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ISOLATION OF BRAIN Ca^{2+}-CALMODULIN TUBULIN KINASE CONTAINING CALMODULIN BINDING PROTEINS

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Ca^{2+}-calmodulin tubulin kinase activity was isolated from brain cytosol and separated from its substrate protein, tubulin, and Ca^{2+} regulatory protein, calmodulin. Characterization of the Ca^{2+}-tubulin kinase system revealed a Km of 4 μM, 0.5 μM, 60 μM for Ca^{2+}, calmodulin and ATP, respectively. The tubulin kinase system bound to a calmodulin affinity column in the presence of Ca^{2+} and was released from the column by chelation with EGTA. A major 55,000 and a minor 65,000 dalton peptide were identified as the only calmodulin binding proteins in the enzyme fraction, indicating that one or both of these peptides represent the calmodulin binding subunit of the Ca^{2+}-calmodulin tubulin kinase system.

Calmodulin, a major Ca^{2+}-binding protein (1), has been implicated in mediating many of calcium's actions on synaptic function and neuronal excitability (2, 3). Regulation of synaptic protein kinase activity by Ca^{2+} and calmodulin (4) may play a role in modulating release of neurotransmitter substances (5, 6), synthesis of neurotransmitters (7), interactions between synaptic vesicles and membrane (3, 8), and function of the post synaptic density (9). Thus, isolation and characterization of specific Ca^{2+}-calmodulin kinase systems may provide an important insight into the effects of Ca^{2+} on neuronal function.

Tubulin is a major component of the nerve terminal (10, 11), and its interaction with Ca^{2+} and calmodulin (12) may be involved in many dynamic synaptic functions (13). Alpha and beta tubulin are major endogenous substrates for Ca^{2+}-calmodulin kinase systems (13), and endogenous Ca^{2+}-calmodulin dependent tubulin kinase activity has been demonstrated in intact synaptosome (13), synaptic vesicle (14), and synaptic cytoplasm (15) preparations. We now report the separation

Abbreviations: PIPES, 1, 4-piperazinediethane sulfonic acid; EDTA, ethylene diamine tetraacetate; EGTA, ethylene glycol bis(β-aminoethyl ether)-tetraacetate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; MAPs, microtubule associated proteins; PMSF, phenylmethlsulfonylfluoride.

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of the endogenous Ca^{2+}-calmodulin tubulin kinase from its major substrate, tubulin, and its regulatory protein, calmodulin. This Ca^{2+}-calmodulin kinase system contained only two major calmodulin binding proteins.

MATERIALS AND METHODS

[γ-^{32}P] ATP (5-10 Ci/mmol) was purchased from New England Nuclear as the triethylammonium salt. Tubulin preparations were prepared from adult Sprague-Dawley rat brains through two cycles of *in vitro* assembly (16). Calmodulin was prepared from rat brain as described previously (6). Brain cytosol was rapidly prepared from Sprague-Dawley female rats (100-150 g) by established procedures (13). Synaptic membrane was prepared as described previously (17). Proteins from each reaction mixture were separated on 9% sodium-dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE), stained, and quantitated for ^{32}P-phosphate incorporation, as described previously (18, 19). Two-dimensional gel electrophoresis was performed by a modification of the procedure of O'Farrell (20), as performed in our laboratory (13-15).

Cellulose Phosphate Chromatography: Cellulose phosphate (CP, Cellex-P, Bio Rad) resin (1 ml packed bed/rat brain) was equilibrated with 30 mM PIPES, pH 6.9. Brain cytosol was chelated with 10 mM EDTA and 10 mM EGTA and passed over the resin. Following application to the column, the column was washed with a NaCl gradient from 50 mM to 450 mM salt and 1 ml fractions were collected (Fig. 1). Protein in each fraction was monitored by ultraviolet absorbance at 280 nM and confirmed by direct quantitation (21). Aliquots of each fraction were subjected to SDS-PAGE, protein staining, and quantitation of gel proteins by established densitometric procedures (18). Endogenous tubulin and calmodulin in each fraction were identified on the SDS gel by their characteristic molecular weights and comigration with marker tubulin and calmodulin and were quantitated by densitometric scanning of the SDS-gel protein pattern employing standard procedures (19). Ca^{2+}-calmodulin tubulin kinase activity was determined by incubating aliquots of each column fraction with exogenous tubulin and calmodulin under standard conditions. To determine the specific incorporation of ^{32}P-phosphate into alpha and beta tubulin, each reaction mixture was subjected to SDS-PAGE, gel staining, and identification of tubulin, and quantitation of phosphorylation of tubulin by liquid scintillation counting (19).

Assay of Phosphorylation: For phosphorylation studies the standard reaction mixture contained 50-100 µg of enzyme fraction protein, 50-60 µM [γ-^{32}R]ATP, 2 mM EDTA and 1 mM EGTA to chelate exogenous divalent cations. $MgCl_2$ was added in sufficient quantities to yield a final concentration of 4 mM free Mg++. Reactions were initiated with addition of 10 µl $CaCl_2$ or 10 µl of deionized distilled water for controls. Tubes were incubated for one minute at 37°C and final reaction volumes were 0.1 ml. Reactions were terminated and samples prepared for electrophoresis by addition of 50 µl of stop solution (360 mM Tris HCl, pH 7.5, 36 mM EDTA, 6% SDS, 3 M sucrose, and Pyronin Y). Samples were heated to 98°C for 3 min and, after cooling, 20 µl of β-mercaptoethanol were added.

Calmodulin Affinity Chromatography: Calmodulin affinity resin (22) was made by linking highly purified calmodulin (5 mg/ml resin) to Biogel 15 (Bio Rad). Linking was accomplished with greater than 90% efficiency. A 8.0 cm x 1.0 cm column was constructed with a total calmodulin content of approximately 40 mg of calmodulin. Fractions collected from the CP column enzyme fraction were diluted to 100 mM NaCl and made 2 mM $MgCl_2$ and 1 mM $CaCl_2$. The fraction was then passed over the calmodulin affinity resin which had been equilibrated with Buffer A (100 mM NaCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 50 mM Tris-HCl, pH 7.1). The column was sequentially washed with Buffer A, Buffer A containing 200 mM NaCl, and Buffer A containing 200 mM NaCl, 1 mM EDTA, and 1 mM EGTA. Peaks from each fraction were pooled, dialyzed against 10 mM Pipes, pH 6.9, and assayed for tubulin kinase activity under standard conditions.

[^{125}I] Calmodulin Gel Overlay: [^{125}I] calmodulin binding to polyacrylamide gels was accomplished using the technique of Carlin et al. (14). Briefly, 50-80 μg of protein were electrophoresed in 9% SDS polyacrylamide gels as above. The gel was then washed with buffer (50 mM Tris HCl, pH 7.1) to remove SDS from the gel. The gel was then overlayed with 0.25 μg of [^{125}I] calmodulin in buffer. After removal of the isotope overlay, the gel was washed, stained, and then dried and subjected to autoradiography as above. [^{125}I] calmodulin (100 Ci/mmol), labelled using Bolton-Hunter reagents, was the gift of Dr. Fred Gorelick (23).

RESULTS AND DISCUSSION

We utilized CP chromatography of chelated cytoplasm in an attempt to separate tubulin kinase activity from its endogenous substrate tubulin (13). 99% of endogenous calmodulin and tubulin passed through the column into the void volume (Figure 1). We assessed kinase activity in each of the fractions eluted from the resin by quantitating ^{32}P-phosphate incorporation into exogenous tubulin added in the presence of calmodulin (Methods). The majority of Ca^{2+}-calmodulin stimulated tubulin kinase activity eluted from the column as a peak between 250 mM and 350 mM NaCl.

The CP isolated tubulin kinase fraction represented only 1.3% of the total cytosol protein and 56.2% of the recovered exogenous tubulin kinase activity (Fig. 1). The protein pattern of the Ca^{2+}-calmodulin tubulin kinase preparation revealed major peptide components with approximate molecular weights of 55,000, 30,000, and 10,000 daltons and minor components of 250,000, 160,000, 90,000, and 70,000 daltons (Fig. 2). This pooled enzyme fraction contained no significant tubulin or calmodulin as assessed by 1 and 2-dimensional gel electrophoresis. A small amount of endogenous tubulin kinase activity remained with tubulin in the void fraction (Fig. 1). Our preparation of substrate tubulin (T_2) also contained high molecular weight microtubule associated proteins (MAPs) (Fig. 2). Figure 2B shows that most of the 50,000 and 60,000 dalton phosphorylation of exogenously added tubulin was actually accounted for by alpha and beta tubulin, as assessed by 2-dimensional gel electorphoresis. The calmodulin dependent tubulin kinase displayed Km's for ATP, Ca^{2+}, and calmodulin of approximately 60 μM, 4 μM, and 0.5 μM, respectively.

The tubulin kinase system was further purified by calmodulin affinity chromatography (Table 1). A significant amount of the CP tubulin kinase protein

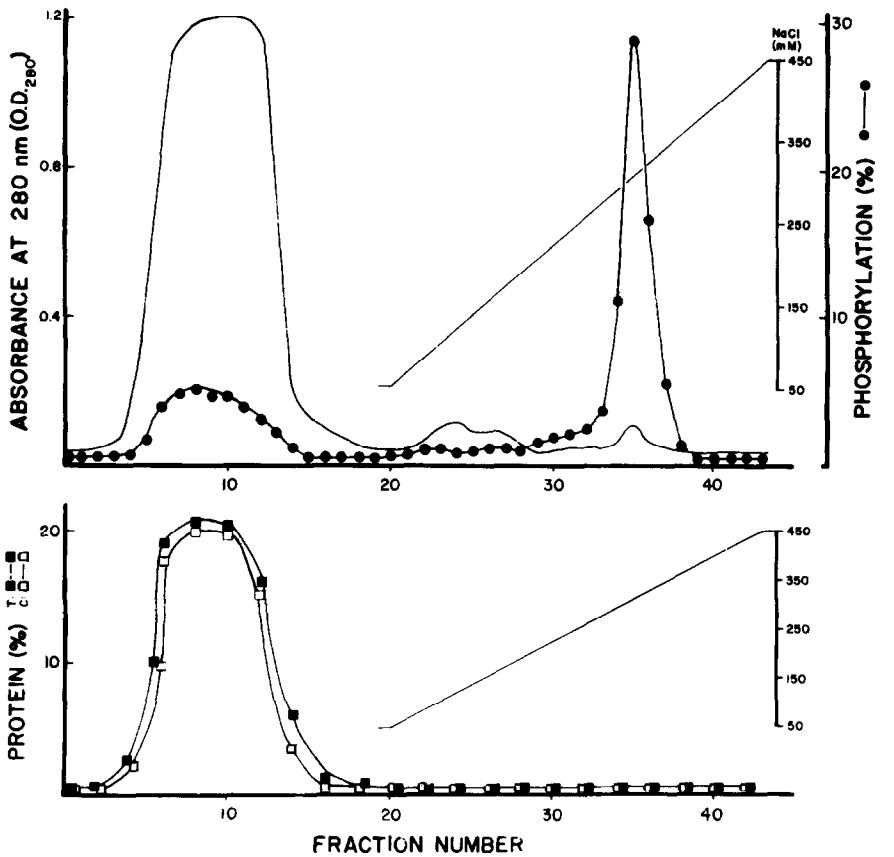

Figure 1. Elution of Ca^{2+}-calmodulin tubulin kinase activity (●-●), total protein (—), tubulin (■-■) and calmodulin (□-□) from cellulose phosphate column chromatography of brain cytosol. Brain cytosol from 10 rat brains was applied to the column and eluted with a NaCl gradient from 50 to 450 mM. Total protein was continuously monitored at 280 nM. Ca^{2+}-calmodulin tubulin kinase activity was assayed in each tube employing tubulin as the substrate (Methods). Calmodulin and tubulin levels were determined for each fraction by SDS-PAGE and quantitation of protein staining (Methods). Tubulin phosphorylation, calmodulin protein and tubulin protein levels are expressed as percent of total value for comparison.

(14.3%) and activity (43.6%) adhered to the calmodulin affinity column in the presence of Ca^{2+}. Washing the affinity column with 50 mM and 200 mM NaCl washes did not release significant bound tubulin kinase activity. However, elution with 1 mM EGTA and EDTA in 200 mM NaCl resulted in the quantitative recovery of the majority of bound tubulin kinase activity (Table 1). The relative specific activity of the calmodulin affinity tubulin kinase preparation was increased 26.7 times over the CP enzyme fraction. Some kinase activity passed through the column in the void, but this fraction had a low relative specific activity (Table 1) and was found to adhere to the affinity column upon

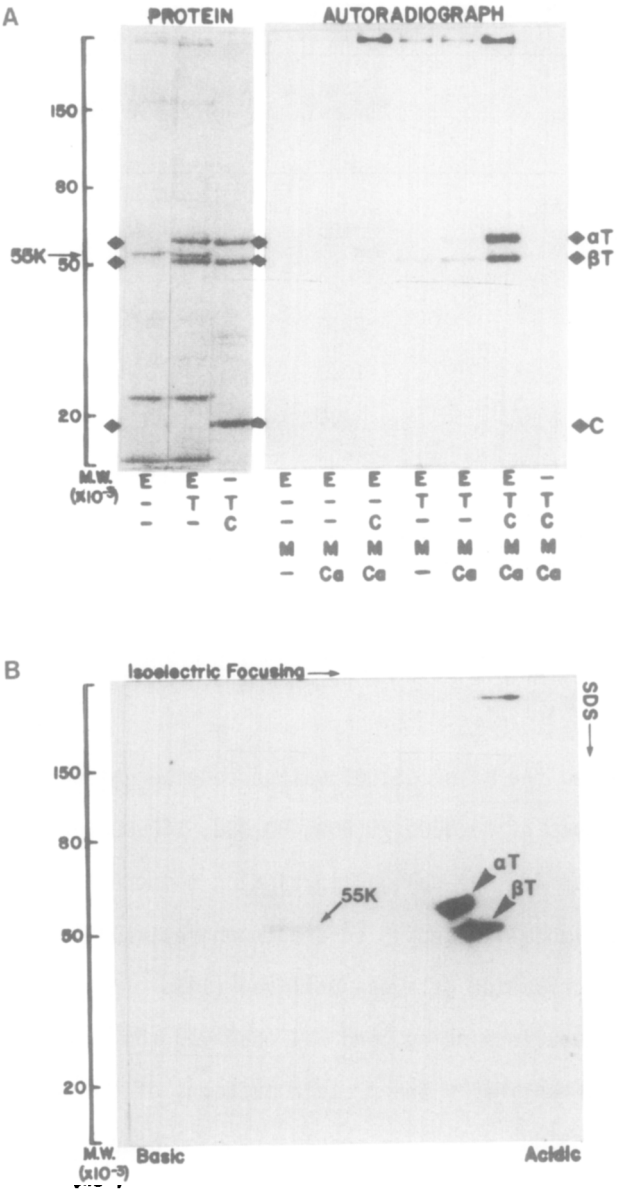

Figure 2. Protein pattern and phosphorylation autoradiography for the Ca^{2+}-calmodulin tubulin kinase system. A. Protein pattern of the tubulin kinase system (E) on SDS-PAGE with marker tubulin (T) and calmodulin (C) and autoradiograph of protein phosphorylation under various conditions: Magnesium (M), calcium (Ca). The positions of α and β tubulin and the 55,000 dalton (55 k) calmodulin binding protein are designated. B. Autoradiograph of the two-dimensional phosphorylation pattern of tubulin by the tubulin kinase fraction in the presence of Ca^{2+}, Mg^{2+}, and calmodulin. The positions of α and β tubulin and the 55 k enzyme component are designated.

reapplication. These results demonstrate that the calmodulin-depleted tubulin kinase system binds to calmodulin in the presence of Ca^{2+} and could be released from its Ca^{2+} receptor protein by chelation. The calmodulin affinity kinase

Table 1. Isolation of Tubulin Kinase Activity by Calmodulin Affinity Chromatography

Fraction	Total Protein %	Tubulin Kinase Activity %	Relative Specific Activity
Cellulose Phosphate Enzyme	100	100	1.00
Calmodulin Affinity Column			
Void	73.0	39.4	0.54
50 mM NaCl	10.3	3.0	0.29
200 mM NaCl	2.5	0.6	0.24
200 mM NaCl, 1 mM EGTA, 1 mM EDTA	1.5	40.0	26.70

Total protein and tubulin kinase activity recovered from the calmodulin affinity column are expressed as percent of total protein and kinase activity of the CP enzyme fraction applied to the column (Fig. 1). Relative specific activity is expressed as percent kinase activity/percent protein recovered from the column. The percent recovered of protein and kinase activity from the affinity column were 87.3 and 83.0 percent, respectively.

preparation contained the major 55,000 dalton peptide, as well as minor bands with molecular weights of 30,000, 70,000, 80,000, 160,000, and 250,000 daltons.

In order to identify the calmodulin binding component in the enzyme fraction, [^{125}I] calmodulin binding to electrophoretically resolved proteins in denaturing polyacrylamide gels was utilized (14). In the presence of $CaCl_2$, a single major calmodulin binding band was observed that corresponded to the major 55,000 dalton peptide in the protein patterns of the enzyme fractions (Fig. 3). A minor 65,000 dalton calmodulin binding doublet was also consistently visualized (Fig. 3). [^{125}I] calmodulin failed to bind to any of the other protein components in the enzyme fractions. The specificity of calmodulin binding was indicated by the total elimination of [^{125}I] calmodulin labelling in the presence of either chelator (2 mM EGTA and 2 mM EDTA) or preincubations with 1000 fold excess of non-radioactive calmodulin (Fig. 3).

These results indicate that the major 55,000 and minor 65,000 dalton peptides in the calmodulin kinase represent the only calmodulin binding sites for this enzyme system. The presence of only two significant calmodulin binding polypeptides in the tubulin kinase fraction indicates that one or both of these

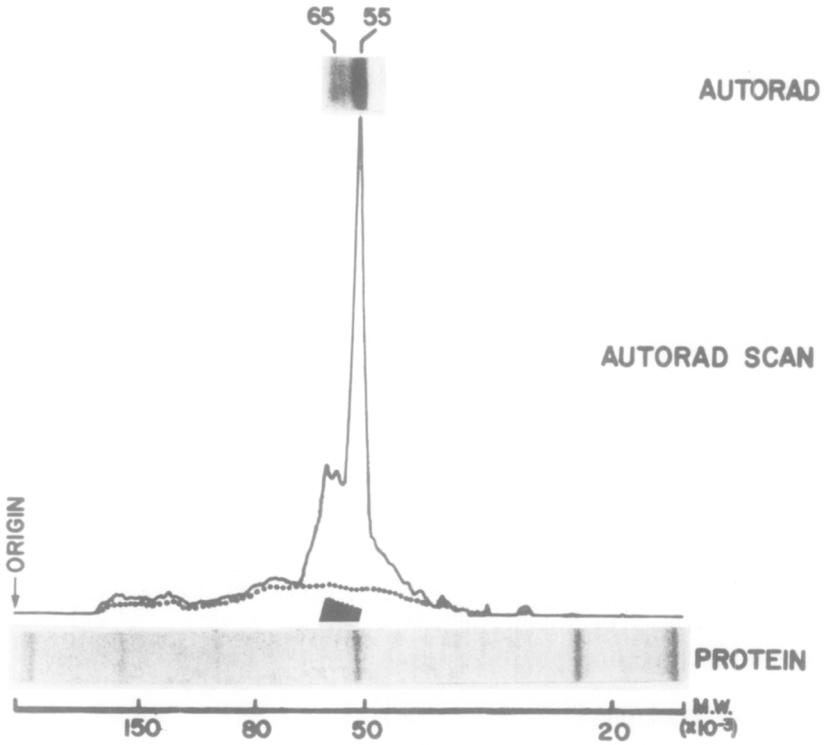

Figure 3. [^{125}I] Calmodulin binding to the Ca^{2+}-calmodulin tubulin kinase fraction in denaturing gels (Methods). The kinase protein pattern (40 µg) reveals major peptides with approximate molecular weights of 55,000, 30,000, and 10,000 daltons and minor peptides of 250,000, 160,000, 90,000, and 70,000 daltons (confirmed by PAGE of a 120 µg sample). The densitometric scan of autoradiograph of [^{125}I] calmodulin binding reveals a major 55,000 and minor 65,000 dalton bands (——) that were eliminated in the presence of EGTA or excess cold calmodulin (·····). The data shown represent the CP kinase fraction and essentially identical results were obtained with the calmodulin affinity column preparations.

proteins is the calmodulin binding subunit of this Ca^{2+}-calmodulin kinase system. The 55,000 and 65,000 dalton calmodulin binding peptides in the enzyme fraction also manifested some endogenous phosphorylation which could be easily distinguished from tubulin on 2-dimensional PAGE (Fig. 2).

Membrane bound endogenous tubulin kinase activity has been identified in brain with essentially identical characteristics to the soluble brain tubulin kinase system (3, 14). The presence of the tubulin in kinase system in brain membrane suggests that membrane fractions may contain the same calmodulin binding proteins as in the isolated cytosolic kinase fraction. Carlin et al. (24) have described several calmodulin binding proteins in both post synaptic densities (PSDs) and synaptic membrane, including a major band of 51,000 daltons and a

minor band of 60,000 daltons. These two calmodulin binding membrane proteins, like the calmodulin binding proteins in the cytosolic tubulin kinase system (Fig. 2), were also endogenous substrates for protein kinase activity (25). We have replicated the calmodulin binding studies of Carlin et al. (22) for membrane and found that the 51,000 and 60,000 dalton calmodulin binding membrane proteins comigrated on the same SDS-PAGE system with the two calmodulin binding peptides observed in the brain cytosolic Ca^{2+}-calmodulin tubulin kinase system. These results support the hypothesis that both membrane bound and unbound Ca^{2+}-calmodulin dependent tubulin kinase share the same calmodulin binding subunit.

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