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CALCIUM-DEPENDENT AFFINITY CHROMATOGRAPHY OF CALMODULIN ON AN IMMOBILIZED PHENOTHIAZINE

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SUMMARY: The reversible, calcium-dependent binding of a calmodulin to phenothiazines has been demonstrated using an immobilized chlorpromazine analog. Calmodulin has been purified from crude extracts of bovine brain utilizing calcium-dependent binding to phenothiazine-Sepharose 4B as an initial affinity-based chromatographic procedure. Chromatography of a crude extract of bovine brain, prepared under non-denaturing conditions, yielded calmodulin contaminated with several other minor EGTA-elutable components. These components were removed by calcium-dependent affinity chromatography on calmodulin-Sepharose 4B and ion-exchange chromatography.

INTRODUCTION: Calmodulin is a ubiquitous, small, acidic, calcium binding protein (1,2) which appears to link the intracellular second messengers-calcium and the cyclic nucleotides - through its ability to regulate numerous enzymatic activities (3-14). This protein has been shown[1] (15-17) to be highly conserved functionally and structurally in accordance with its generalized role as a calcium receptor in eukaryotic cells.

One of the activities which has been attributed to calmodulins is their ability to bind a class of neuroleptic drugs - the phenothiazines. This interaction was first demonstrated by Levin and Weiss (18). They found calmodulin to have two high affinity (K_d = 1 x 10^{-6}) and twenty-four low affinity (K_d = 5 x 10^{-3}) sites per molecule for the binding of the phenothiazine trifluoperazine. The binding of these drugs to calmodulin was found to be dependent on the presence of calcium and to block its ability to activate PDE (for review, see 19). This report describes the preparation and use of a 2-chloro-10-(3-aminopropyl)phenothiazine (CAPP)[2]-Sepharose 4B conjugate to demonstrate the interaction of phenothiazines with calmodulin. In addition, we have utilized calcium-dependent affinity chromatography on this phenothiazine-Sepharose 4B conjugate in the purification of calmodulin from bovine brain.

[1] D.M. Watterson, F. Sharief, and T. C. Vanaman; submitted for publication.

[2] Abbreviations: TES - N-Tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid; CAPP - 2-Chloro-10-(3-aminopropyl)phenothiazine; PDE - 3'-5'Cyclic nucleotide phosphodiesterase (EC 3.1.4.17).

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MATERIALS AND METHODS: Ethanolamine was obtained from Eastman Organic Chemicals, acetonitrile from J. T. Baker, cyanogen bromide from Pierce Chemicals and ultra pure guanidine hydrochloride from Schwartz Mann. Electrophoresis reagent grade sodium dodecyl sulfate (SDS), acrylamide and bis-acrylamide were used as supplied by BDH. Sepharose 4B and DEAE Sephadex A-50 were obtained from Pharmacia. 2-Chloro-10-(3-aminopropyl)phenothiazine was the generous gift of Dr. Carl Kaiser of Smith, Kline and French Laboratories. The sources for other chemicals used in these studies have been described previously (1).

Bovine brain was obtained from Pel-Freeze. Bovine brain calmodulin used as a standard and in the preparation of calmodulin-Sepharose 4B was purified as described previously by Watterson et al. (1). "Activator-depleted" 3'-5' cyclic nucleotide phosphodiesterase was prepared and assayed for activation by calmodulin as previously described (1,20) with exact incubation conditions given where appropriate in the text. Polyacrylamide gel electrophoresis and amino acid analyses were performed using previously described procedures (1, 17,21).

2-Chloro-10-(3-aminopropyl)phenothiazine hydrochloride was coupled to Sepharose 4B using essentially the procedure of March et al. (22). Briefly, 30gm (wetcake) of Sepharose 4B was suspended in an equal volume of 2M Na_2CO_3 (pH 11.0) at 0° in a beaker. To the resin slurry was added 3mL of acetonitrile containing 3.95gm of cyanogen bromide. The slurry was stirred upon the dropwise addition of this cyanogen bromide solution and intermittently thereafter. The activation mixture remained at pH 11 without addition of NaOH. After 5 minutes, the slurry was filtered onto a sintered glass funnel, and washed with a total of 1 liter each of cold deionized water and cold coupling buffer (0.2M $NaHCO_3$, pH 9.5). After washing, the slurry was filtered under suction to a moist cake, and then transferred to a 250mL beaker as a 1:1 slurry in coupling buffer. A solution of 61mg of 2-chloro-10(3-aminopropyl)phenothiazine hydrochloride dissolved in 20mL of 5mM HCL was added dropwise to the suspension of activated resin with rapid stirring. It is important to note that phenothiazines are poorly soluble (23,24) at the pH utilized for coupling amino groups to cyanogen bromide activated resins. Therefore, it was necessary to dissolve the phenothiazine to be coupled in a dilute acid solution. Coupling was performed overnight at 4° with gentle stirring. As phenothiazines are light sensitive (23), efforts were made to minimize the exposure of the drug to light during coupling, subsequent treatments, and use of the resin. Accordingly, coupling was carried out in a beaker wrapped in aluminum foil. Following coupling, the resin slurry was filtered and washed on a sintered glass funnel with 1 liter of cold deionized water, followed by 1 resin volume of 2M ethanolamine-HCl (pH 8.0). It was then resuspended in an equal volume of 2M ethanolamine-HCl (pH 8.0) and incubated at 4° with gentle stirring for at least 2 hrs. to block any remaining activated groups. The resulting CAPP-Sepharose 4B conjugate was washed on a sintered glass funnel with 1 liter of cold deionized water, followed by 500mL of 1mM HCl, 1mM 2-mercaptoethanol to ensure the removal of any free phenothiazine, present in a precipitated form in the matrix. Finally, the resin was washed with 5 volumes of storage buffer [100mM sodium acetate (pH 5.5), 1mM 2-mercaptoethanol, and 1mM sodium azide] in which it was suspended as a 2:1 (buffer:resin) slurry and stored at 4° in a light tight container. The coupling of CAPP to the activated Sepharose 4B was judged to be efficient as no alkali precipitable material (23) was recovered from any of the wash fractions described above.

Calmodulin was coupled to Sepharose 4B using the procedure described above for the preparation of CAPP-Sepharose 4B and as previously described (20) except that 5.7gm of cyanogen bromide were used to activate 50gm (wetcake) of Sepharose 4B resin and 53mg of calmodulin were dissolved in deionized water and then added to the coupling mixture dropwise. The final wash sequence also differed. After the final 2 hrs. of incubation at 4° in ethanolamine, the resin was washed with the following buffer: 500mL of 20mM

Tris-HCl (pH 7.8), 1mM 2-mercaptoethanol, 10mM EGTA. Next the resin was washed with 1 liter of storage buffer [20mM Tris-HCl (pH 7.8), 1mM $CaCl_2$, 1mM 2-mercaptoethanol, 1mM sodium azide] and stored as a 2:1 (buffer:resin) slurry in this buffer at 4°. A 50µL aliquot of the slurry was hydrolyzed in 6N HCl and the amount of calmodulin present was determined by amino acid analysis to be 0.68 mg/mL of resin.

RESULTS AND DISCUSSION: 2-Chloro-10-(3-aminopropyl)phenothiazine is an analog of the neuroleptic drug chlorpromazine (see Figure 1a and 1b respectively), differing only in the absence of two methyl groups on its aminopropyl side chain. The free primary amine at the end of the CAPP aliphatic side chain provides an ideal site for the immobilization of this compound on a resin support. This can be accomplished in a straight-forward manner by coupling CAPP by standard methodologies to Sepharose 4B which has been activated with cyanogen bromide as noted in MATERIALS AND METHODS. The structure of the resulting CAPP-Sepharose 4B conjugate, (see Figure 1c) based on the reaction mechanism proposed by March et al.(22), resembles chlorpromazine to a large degree.

Determination of CAPP-Sepharose 4B conjugate binding capacity: Calmodulin, purified as previously described (1), was dissolved in a buffer containing 20mM Tris-HCl (pH 7.4), 1mM 2-mercaptoethanol, 1mM $CaCl_2$, 200mM NaCl and was applied to a 10mL bed volume of CAPP-Sepharose 4B which had been equilibrated with the same buffer[3]. This solution of calmodulin (approx. 5mg/ml) was added until it was determined that the column had been saturated by monitoring UV absorbancy at 220 nanometers and later by gel electrophoretic analyses. After washing the column with two column volumes of calcium containing buffer, the calmodulin complexed to the CAPP-Sepharose 4B conjugate was eluted by the addition of 10mM EGTA to the column buffer in place of 1mM $CaCl_2$. The bound material eluted in this manner was detected in fractions by monitoring UV absorbancy at 220nm, these fractions were combined, and $CaCl_2$ in excess of the EGTA present was added immediately. This pooled material was dialyzed once against 1mM $CaCl_2$ and then exhaustively against deionized water. The dialyzed material was freeze-dried, dissolved in deionized water and shown to be pure by polyacrylamide gel electrophoresis. An aliquot was hydrolyzed in 6N HCl and the amount of calmodulin was quantified by amino acid analysis. The CAPP-Sepharose 4B conjugate was determined to bind 2.4mg of calmodulin per mL of resin. Although this is lower than expected assuming that CAPP binding occurred at high affinity, specific sites on calmodulin (18), it is clear that a significant amount of the drug was coupled to Sepharose 4B in an accessible configuration.

[3] CAPP-Sepharose 4B affinity resin was routinely stripped with appropriately buffered solutions containing 1mM 2-mercaptoethanol, 10mM EGTA and 6M guanidine hydrochloride both before and after use.

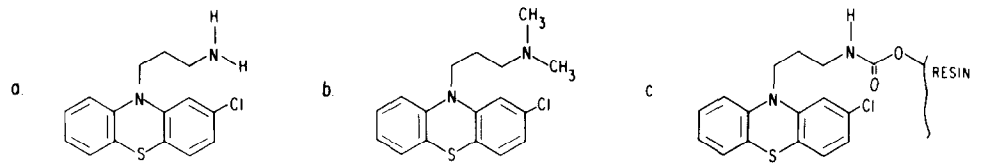

Figure 1. Structures of chemicals referred to in text.

Affinity chromatographic purification of calmodulin: The efficacy of using phenothiazine-Sepharose 4B for the rapid isolation of calmodulin from tissue was tested with bovine brain homogenates for which the purification of calmodulin has been extensively documented in this (1) and other (25) laboratories. All operations were performed at 4°. Ten grams (wet weight) of thawed bovine brain were homogenized in two volumes (20mL) of homogenization buffer [20mM N-Tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid-NaOH (TES-NaOH) pH 7.0, 1mM 2-mercaptoethanol, 1mM EDTA]. The homogenate was centrifuged at 100,000 x g for 1 hr. and the supernate decanted and saved. The pellet was re-homogenized in an equal volume of homogenization buffer and the second homogenate was centrifuged as described above. The supernatant fractions derived from the first and second homogenizations were combined, made 5mM in $CaCl_2$ and 300mM in NaCl and adjusted to pH 7.0 with 1M NaOH or 1M TES. The resulting solution was clarified by an additional centrifugation for 1 hr. at 100,000 x g and applied to a column (15mL resin bed) of CAPP-Sepharose 4B which had been equilibrated with 20mM TES-NaOH (pH 7.0), 1mM 2-mercaptoethanol, 300mM NaCl, 1mM $CaCl_2$ (starting buffer). After the sample was applied, the resin was washed exhaustively with starting buffer until the UV absorbancy at 220 nanometers returned to a baseline value. The material which bound to the CAPP-Sepharose 4B column was eluted by the addition of 10mM EGTA to the starting buffer in place of 1mM $CaCl_2$. Figure 2 shows the elution profile obtained from this separation. As can be seen in the insets above the elution profile, gel electrophoretic analyses of column fractions on 10% polyacrylamide alkaline urea gels showed that calmodulin was quantitatively absorbed by the CAPP-Sepharose 4B conjugate and subsequently eluted with EGTA containing buffer. However, several other components co-eluted with calmodulin from the CAPP-Sepharose 4B conjugate. The contaminating proteins with slower mobilities than that of calmodulin could easily be removed by the passage of the EGTA eluted material from the CAPP-Sepharose 4B column through a column of calmodulin-Sepharose 4B in the presence of excess calcium as described below.

The EGTA eluted material was pooled as indicated in Figure 2, $CaCl_2$ in excess of the EGTA present added as quickly as possible, and the material dialyzed two times against four liters of calmodulin-Sepharose 4B column

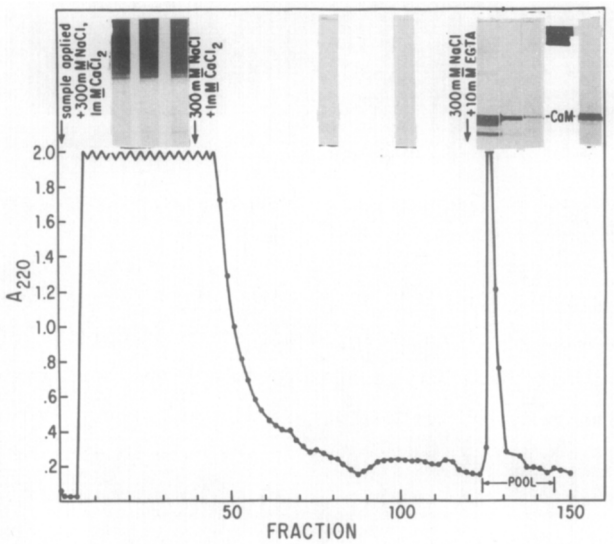

Figure 2. CAPP-Sepharose 4B affinity chromatography. The separation shown was performed and monitored as described in the text. Two milliliter fractions were collected. Sample was applied commencing at fraction 1. The column was washed with calcium containing buffer commencing at fraction 40. Elution was accomplished, beginning at fraction 120, by the replacement of calcium with 10mM EGTA in the column buffer. The column was operated at a flow rate of 30 mL/hr. The results of 10% polyacrylamide alkaline urea gel electrophoretic analyses of column fractions are inserted above corresponding fractions (50µl of fractions 10,25,40,80,100,125,130 and 135) of the elution profile. The insertion at the far right represents 5µg of bovine brain calmodulin purified by the method of Watterson et al. (1), and analyzed on the same gel. Fractions 124-145 were pooled.

buffer [75mM Tris-HCl (pH 8.0), 15mM 2-mercaptoethanol, 1mM $CaCl_2$]. The resultant solution was applied to a 15mL column (1 cm x 12.5 cm) of calmodulin-Sepharose 4B in the same buffer. The unbound material from this column was pooled, dialyzed once against 10mM ammonium bicarbonate and then exhaustively against deionized water, freeze-dried and analyzed by alkaline urea 10% polyacrylamide gel electrophoresis. As can be seen in Figure 3 (left panel, slot B) this material contained the calmodulin with none of the slower migrating contaminants described above. However, the more rapidly migrating contaminants were still present. Homogeneous calmodulin was separated from these contaminating proteins by ion-exchange chromatography on DEAE Sephadex A-50 (vide infra). The material which was bound to the calmodulin-Sepharose 4B conjugate was eluted with buffer containing 10mM EGTA in place of $CaCl_2$, dialyzed against 1mM Tris-HCl (pH 8.0) and freeze dried. It was anticipated that this fraction contained PDE and/or possibly other calmodulin regulated enzymatic activities. Our initial analyses (data not shown) of this material were unable to detect PDE activity in this fraction. However, we have repeatedly been able to remove these proteins by calmodulin-Sepharose 4B chromatography from preparations of

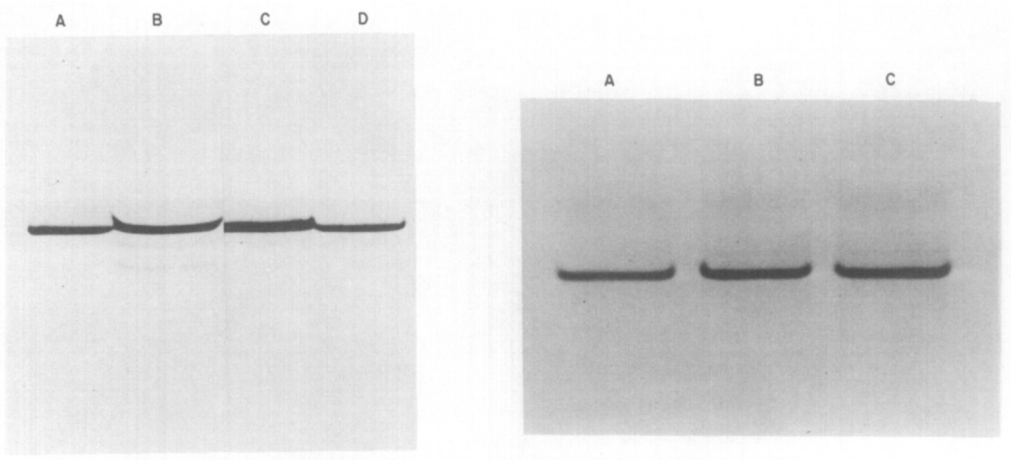

Figure 3. Gel electrophoretic analyses of purified bovine brain calmodulin. Left panel. Electrophoresis was performed on a 10% polyacrylamide slab gel containing 6.9M urea with a continuous alkaline buffer system as previously described (26). Labelled slots contained: A and D - 5µg of bovine brain calmodulin; B - a 25µL aliquot of a 2.0mL solution of the unretarded material from chromatography on calmodulin-Sepharose 4B; C - 5µg of the calmodulin obtained from DEAE-Sephadex A-50 chromatography as described in the text. Right panel. Electrophoresis was performed on a 12.5% polyacrylamide slab gel with discontinuous buffers (1) with the following samples: Slot A - 10µg Bovine brain calmodulin (standard purification procedure). Slot B - 5µg Bovine brain calmodulin (standard purification procedure) plus 5µg Bovine brain calmodulin (affinity purification procedure) and Slot C - 10µg Bovine brain calmodulin (affinity purification procedure). Protein concentrations were determined by amino acid analysis.

calmodulin from bovine brain, and other sources. Studies are in progress to determine if one or more of the known calcium-calmodulin activated enzyme activities can be detected in this fraction.

The unretarded material from the calmodulin-Sepharose 4B affinity column, containing the calmodulin, was applied in 20mM Tris-HCl (pH 7.5), 2mM 2-mercaptoethanol, 200mM NaCl to a DEAE-Sephadex A-50 column (1 cm x 10 cm) which had been equilibrated in the same buffer. The column was washed with one column volume of buffer containing 300mM NaCl and eluted with a 300mM to 600mM NaCl gradient (100mL/100mL) in the same buffer. Fractions identified by polyacrylamide gel electrophoresis and UV absorbancy at 280 nanometers as containing calmodulin, free of contaminating proteins, were pooled and dialyzed against 0.5mM 2-mercaptoethanol and finally against deionized water before being freeze-dried. This final product was demonstrated to be homogeneous, migrating as a single Coomassie blue staining band on alkaline urea 10% and discontinuous 12.5% polyacrylamide gels (see Figure 3- slot C, left and right panels). It comigrated with bovine brain calmodulin purified by the method of Watterson et al. (1) in both gel electrophoretic systems. The amino acid composition of the purified material agreed with that determined by amino acid

TABLE I

Activity of Bovine Brain 3'-5' Cyclic Nucleotide Phosphodiesterase in the Presence of:

Assay mixture	nmol cAMP hydrolyzed/mg enzyme/min
1. Standard[a] without additions	4.8
2. Standard - Plus 0.2µg control[b] bovine brain calmodulin	57.2
3. Standard - Plus 0.2µg affinity purified bovine brain calmodulin	51.2
4. Same as 2 but containing 2mM EGTA	1.6
5. Same as 3 but containing 2mM EGTA	1.6
6. Same as 4 but containing an additional 4mM $CaCl_2$	32.8
7. Same as 5 but containing an additional 4mM $CaCl_2$	27.6

[a]Assay conditions: 2mM cAMP, 20mM Tris-HCl (pH 8.0), 1mM Ca^{2+}, 0.4mM Mn^{2+}, 50µg of enzyme plus calmodulin, EGTA and Ca^{2+} as indicated. Inorganic phosphate production resulting from a two-stage assay system as previously described (1) was monitored according to Sanui (30).

[b]Purified as per Watterson et al. (1).

sequence of the bovine brain protein[1] (14). In addition, the protein obtained by the procedure described here stimulated "activator-depleted" 3'-5'cyclic nucleotide phosphodiesterase (PDE) in a calcium-dependent manner identical to the activation obtained with bovine brain calmodulin purified by standard methodologies (1), as shown in Table I. The yield of pure calmodulin obtained as final product was determined by amino acid analysis to be 87.5mg per kg of tissue. This represents a substantial improvement in yield over previously reported procedures. (For example, Watterson et al. (1) reported a yield of 41mg per kg of bovine brain). It should be noted that this yield was obtained without the use of denaturants (26) or thermal denaturation steps (25).

CONCLUSIONS: The reversible formation of a calcium-dependent complex between a phenothiazine and calmodulin has been directly demonstrated in this study. We have also presented here a non-denaturing, two-step, calcium-dependent, affinity chromatographic procedure which when coupled to a final DEAE ion-exchange chromatographic step yields homogeneous calmodulin from brain. This method appears to be of greatest value when a small amount of starting material is available, but may be utilized as the final step in large scale calmodulin purifications. These procedures with minor modifications appear to be generally applicable to the purification of calmodulins, as we have

recently used them to isolate calmodulins from such divergent sources as the macrophage-like murine cell line $P388D_1$[4], and Tetrahymena pyriformis[5]. Charbonneau et al. (27,28) have made similar observations concerning the applicability of phenothiazine-Sepharose 4B conjugates to the purification of plant, animal, and fungal calmodulins. Therefore it would appear that the ability to bind phenothiazines in a calcium-dependent manner has been a property of calmodulins since their appearance in eukaryotes.

In addition to the obvious use of phenothiazine-Sepharose conjugate in purifying calmodulins, these immobilized drugs undoubtedly will be a valuable tool for studying the mechanism by which they block other calmodulin activities and for attempting to identify other phenothiazine binding components in tissues.

Studies are currently in progress to determine if any of the components reported here as eluting with calmodulin from CAPP-Sepharose 4B (see RESULTS) consist of (1) calmodulin binding proteins whose complexes with calmodulin are not phenothiazine dissociable and/or (2) brain-specific phenothiazine binding proteins which might comprise a pharmacologically important site for phenothiazine action.

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