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Drug–Protein Interactions: Binding of Chlorpromazine to Calmodulin, Calmodulin Fragments, and Related Calcium Binding Proteins†

Daniel R. Marshak,‡ Thomas J. Lukas, and D. Martin Watterson*

Laboratory of Cellular and Molecular Physiology, Howard Hughes Medical Institute, and Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232
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ABSTRACT: The quantitative binding of a phenothiazine drug to calmodulin, calmodulin fragments, and structurally related calcium binding proteins was measured under conditions of thermodynamic equilibrium by using a gel filtration method. Plant and animal calmodulins, troponin C, S100α, and S100β bind chlorpromazine in a calcium-dependent manner with different stoichiometries and affinities for the drug. The interaction between calmodulin and chlorpromazine appears to be a complex, calcium-dependent phenomenon. Bovine brain calmodulin bound approximately 5 mol of drug per mol of protein with apparent half-maximal binding at 17 μM drug. Large fragments of calmodulin had limited ability to bind chlorpromazine. The largest fragment, containing residues 1–90, retained only 5% of the drug binding activity of the intact protein. A reinvestigation of the chlorpromazine inhibition of calmodulin stimulation of cyclic nucleotide phosphodiesterase further indicated a complex, multiple equilibrium among the reaction components and demonstrated that the order of addition of components to the reaction altered the drug concentration required for half-maximal inhibition of the activity over a 10-fold range. These results (1) confirm previous observations using immobilized phenothiazines [Marshak, D. R., Watterson, D. M., & Van Eldik, L. J. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6793–6797] that indicated a subclass of calcium-modulated proteins bound phenothiazines in a calcium-dependent manner, (2) demonstrate that the interaction between phenothiazines and calmodulin is more complex than previously assumed, and (3) suggest that extended regions of the calmodulin molecule capable of forming the appropriate conformation are required for specific, high-affinity, calcium-dependent drug binding activity.

The calcium-modulated proteins are a class of proteins that bind calcium ions reversibly at physiological ionic strength and pH (Van Eldik et al., 1982). Members of this class include calmodulin, troponin C, parvalbumin, S100α, S100β, intestinal calcium binding protein, and myosin light chains. We previously demonstrated (Marshak et al., 1981) that a subclass of calcium-modulated proteins interacts with immobilized phenothiazines in a calcium-dependent manner. These results suggested that calmodulin, troponin C, S100α, and S100β contain similar structural domains for drug binding. Phenothiazines also inhibit the calcium-dependent activation of enzymes by calmodulin [for a recent review, see Klee & Vanaman (1982)] and therefore are useful probes of the rela-

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‡ Present address: Laboratory of Cell Biology, National Institute of Mental Health, Bethesda, MD 20205.

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tionships between structure and function in calmodulin and other calcium-modulated proteins.

It has been generally assumed that there are two calcium-dependent phenothiazine binding sites on calmodulin. However, this assumption is based on data obtained by several techniques that have specific, inherent limitations. Equilibrium dialysis experiments (Levin & Weiss, 1977, 1979) indicated that there were at least two sites of calcium-dependent phenothiazine binding on calmodulin, but these studies were performed at low ionic strength (<10 mM), a condition known to affect calmodulin's interactions with small ligands (Klee & Vanaman, 1982). Magnetic resonance studies (Klevitt et al., 1981; Krebs & Carafoli, 1982; Thulin et al., 1984) suggested that two sites for trifluoperazine were present on calmodulin but these studies required millimolar protein concentrations and correspondingly high (millimolar) drug concentrations where drug aggregation occurs (Scholtan, 1955; Gariepy & Hodges, 1983). Recently, studies of the adsorption of fragments of calmodulin to immobilized phenothiazines (Brzeska et al., 1983; Vogel et al., 1983; Newton et al., 1984) have been interpreted as indicating at least two phenothiazine binding sites on the protein. However, as discussed previously (Marshak et al., 1985), these studies employed affinity-based adsorption techniques, and quantitative affinity chromatography of calmodulin has not been performed, although such studies have been performed for glutamate dehydrogenase (Veronese et al., 1979). Finally, spectroscopic studies using synthetic peptides from the troponin C sequence (Gariepy & Hodges, 1983) have been the basis of postulations about the atomic structure of the drug binding sites on calmodulin (Reid, 1983). However, peptides from the troponin C sequence may not accurately reflect the drug binding properties of calmodulin. Thus, a quantitative assessment of the interaction between phenothiazines and calcium-modulated proteins is essential to the development of relevant models for drug–protein interactions for calcium-modulated proteins.

In order to characterize these drug–protein interactions in solution, we have measured the binding of chlorpromazine by calcium-modulated proteins under conditions of thermodynamic equilibrium. Chlorpromazine is a useful ligand to probe the drug binding domains of calcium-modulated proteins because it is structurally homologous to the phenothiazine derivative [2-chloro-10-(3-aminopropyl)phenothiazine] used in the immbolized drug studies (Marshak et al., 1981), the physical and chemical properties of chlorpromazine in aqueous solutions have been studied in detail (Scholtan, 1955), and the drug is soluble at a wide range of concentrations and ionic strengths. In the present study, we have used the gel permeation technique of Hummel & Dreyer (1962) employing high-performance liquid chromatography. The system may be calibrated by two independent methods, and no experimental corrections are introduced to the data. This technique is amenable to the study of multiple calcium-modulated proteins, chemically modified proteins, and fragments of proteins.

We report here the results of our studies on the binding in solution of phenothiazines by calmodulins, calmodulin fragments, and structurally related calcium-modulated proteins. On the basis of the unexpected results of our binding experiments, we also reexamined the chlorpromazine inhibition of calmodulin activation of cyclic nucleotide phosphodiesterase in attempts to correlate drug binding with drug inhibitory activity. An initial set of studies combining drug binding studies and covalent adducts of calmodulin and drug is described in the following paper (Lukas et al., 1985). Overall, these studies demonstrate that calmodulin's interactions with drugs are more complex than previously assumed and provide a basis for future studies of drug and protein binding sites on calcium-modulated proteins.

Materials and Methods

Isolation of Calcium-Modulated Proteins from Bovine Brain. Calmodulin, S100α, and S100β were isolated from bovine brains by using a modification of procedures previously described (Watterson et al., 1980a,d; Marshak et al., 1981). Conditions for homogenization, ammonium sulfate fractionation, and chromatography on DEAE–Sephadex A-50 were exactly as previously described (Watterson et al., 1980a). Fractions (10 mL) of the effluent from the ion-exchange column were analyzed by polyacrylamide gel electrophoresis. Fractions containing material which comigrated in electrophoresis with either calmodulin or S100 standards were combined in separate pools. These pools were dialyzed against two changes of 4 L of water overnight at 4 °C and against 4 L of buffer F [10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl),[1] pH 8.0, 1 mM 2-mercaptoethanol, 1 mM $MgCl_2$, and 2 mM $CaCl_2$] for 4 h at 4 °C. Each pool was subjected to adsorption chromatography on phenothiazine–Sepharose conjugates (Watterson et al., 1980a) followed by reverse-phase chromatography [(phenylethyl)silanyl]silica as previously described (Marshak et al., 1981). For purification of 10–80 mg of protein by reverse-phase chromatography, a column (0.78 × 30 cm) of Waters μBondapak Phenyl was used. The purified proteins had amino acid compositions, ultraviolet absorption spectra, peptide maps, and partial amino acid sequences which were indistinguishable from those previously reported (Isobe & Okuyama, 1978, 1981; Watterson et al., 1980c).

Equilibrium Binding of Chlorpromazine. The equilibrium for the binding of chlorpromazine by calmodulin was measured by using gel exclusion chromatography as described by Hummel & Dreyer (1962). Binding experiments were performed in buffer HD1 (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1 mM $CaCl_2$) containing various concentrations of 2-chloro-10-[3-(dimethylamino)propyl]phenothiazine (chlorpromazine), a gift of Dr. Harry Green, Smith Kline and French Laboratories. Bovine brain calmodulin was dissolved in buffer HD1 at a concentration of 62.5 μM (1.04 mg/mL) as determined by amino acid analysis. The concentration of chlorpromazine was determined by the absorbance at 254 nm using ϵ^M = 30 000 (Rajeswaran & Kirk, 1962). The calmodulin solution was allowed to equilibrate for at least 30 min at room temperature (22–24 °C). An aliquot (20–80 μL) of the calmodulin solution was applied to a column (0.6 × 30 cm) of Bio-Gel P6 (100–200 mesh, Bid-Rad) which had been equilibrated previously in buffer HD1 containing the same concentration of chlorpromazine as in the calmodulin solution. The column was eluted with buffer HD1 at a flow rate of 0.3 mL/min by using a Waters 6000A pump, and the absorbance of the effluent was monitored at 254 nm by using either a Waters 480 or a Waters 441 detector. Absorbance signals were transmitted to a Hewlett Packard 1000 computer using a Hewlett Packard analog/digital converter. The areas of peaks and troughs in the chromatogram were integrated by using Hewlett Packard 3356 laboratory automation system software.

[1] Abbreviations: AMP, adenosine 5′-monophosphate; cAMP, adenosine cyclic 3′,5′-phosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride.

Standard solutions of chlorpromazine in buffer HD1 for calibrations contained 0.01 and 0.1 mM chlorpromazine above the concentration of chlorpromazine in the buffer. Internal calibration of the binding of chlorpromazine to calmodulin was performed as described by Hummel & Dreyer (1962). An aliquot of the calmodulin solution was diluted with various amounts of standard chlorpromazine solutions to give the desired excess amount of drug. This solution was equilibrated for at least 30 min at room temperature and filtered on the Bi-Gel P6 column as described above.

Binding experiments were performed exactly as described above using buffer HD2 containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 4 mM $MgCl_2$, 1 mM EGTA, and various concentrations of chlorpromazine.

Preparation of Chlorpromazine 5-Oxide. Chlorpromazine was dissolved in 97% (v/v) formic acid to a concentration of ~2 mg/mL. The pink solution was placed on ice, and 1 mL of ice-cold 30% (v/v) hydrogen peroxide was added. The solution turned deep purple, then reddish brown, and finally orange on standing on ice. The solution was diluted with 30 mL of ice-cold water which produced a clear solution. The solution was rotary evaporated to dryness and dissolved in water. The absorption spectrum of the product showed the characteristic maxima at 273, 299, and 336 nm (Shine & Mach, 1965) of the phenothiazine 5-oxide group.

Inhibition of Activator Activity. The ability of chlorpromazine to inhibit cyclic nucleotide phosphodiesterase activity was measured by using four protocols. In each protocol, the final concentrations of components of the reaction mixture were as follows: 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 0.4 mM $MnCl_2$, 2 mM cAMP, 24 nM bovine brain calmodulin, 0.06 mg/mL phosphodiesterase preparation (Watterson et al., 1980b), and various amounts of chlorpromazine in a final volume of 6.25 mL. The four protocols varied in the order of addition of the reaction components. In protocol 1, all components except cAMP and chlorpormazine were incubated at room temperature for 10 min. The reaction was initiated by the addition of a mixture of cAMP and chlorpromazine. In protocol 2, all components except cAMP and enzyme were incubated for 10 min at room temperature, and the reaction was initiated by the addition of cAMP and enzyme. In protocol 3, all components except cAMP and calmodulin were incubated for 10 min at room temperature, and the reaction was initiated by the addition of a mixture of cAMP and calmodulin. In protocol 4, all components except cAMP were mixed and allowed to equilibrate for 45 min at room temperature, and the reaction was initiated by the addition of cAMP. Following initiation, all reactions were incubated for 45 min at room temperature (22–24 °C). Duplicate aliquots (0.15 mL) were removed at 1–5-min intervals and placed in a boiling water bath for 10 min. The substrate (cAMP) and product (AMP) in each aliquot were separated by high-performance liquid chromatography on a column (0.46 × 25 cm) of anion-exchange resin (Partisil-10 SAX), and the amount of AMP was quantitated by its absorbance at 254 nm as previously described (Watterson et al., 1980b).

Preparation of Calmodulin Fragments. Fragments of bovine brain calmodulin were prepared by digestion of the protein with protease arg-C from mouse submaxillary gland (Boehringer Mannheim, lot no. 1141403) as previously described (Marshak et al., 1984). Briefly, bovine brain calmodulin was dissolved (1–2 mg/mL) in 0.1 M ammonium bicarbonate containing 1 mM EGTA. The protease was added in two aliquots to give a final ratio of 1:50 (w/w) enzyme to calmodulin. The solution was incubated at 37 °C overnight (14–18 h). The resulting mixture of peptides was fractionated by chromatography on a column (0.94 × 25 cm) of [(octadecyl)silanyl]silica (Whatman, ODS-3) exactly as previously described (Marshak et al., 1984a).

Other Materials. Water was obtained from a Milli-Q (Millipore) or a Darco (Durham, NC) apparatus. Solvents for high-performance liquid chromatography were from Burdick and Jackson or from Fisher. All other reagents were the highest grade available from Mallinckrodt or Fisher. Nucleotide standards were obtained from Boehringer Mannheim. Carp parvalbumin (pI = 3.95) was a gift of Dr. R. H. Kretsinger, Charlottesville, VA. Rabbit skeletal muscle troponin C was a gift of Dr. W. H. Burgess, Nashville, TN.

Results

The binding of chlorpromazine to various calcium-modulated proteins was measured by using the gel permeation technique of Hummel & Dreyer (1962) as described under Materials and Methods. Each protein sample with bound drug eluted as a sharp peak of absorbance in the excluded volume of the column with a retention time of 10 min (Figure 1), and the free drug eluted as a broader peak from the column with a retention time of 49 min. The trough in Figure 1C corresponded to the depletion of drug from the buffer due to drug binding by the protein, and the area of the trough represented the amount of bound drug.

The trough area was measured by using two independent methods of calibration, external and internal, as described by Hummel & Dreyer (1962). An example of external calibration is shown in Figure 1A. The amount of drug corresponding to a particular trough area was calculated by linear regression analysis, and at each chlorpromazine concentration, the response of the detector was linear with the amount of drug applied in standard samples. An example of internal calibration is shown in Figure 1B and in Figure 2 of the supplementary material (see paragraph at end of paper regarding supplementary material). By addition of excess drug to the sample of protein, the area of the trough was reduced or converted to a peak (Figure 2 of the supplementary material). The relationship between the areas of these peaks or troughs and the amount of excess drug added to the sample was linear within the range of values used for this study, as shown Figure 1B. The amount of drug bound to the protein is equal to the amount of excess drug required to bring the area of the trough to zero, shown by the dashed line in Figure 1B. The values for the amount of chlorpromazine bound to bovine brain calmodulin calculated by external and internal calibrations differed by <5% below 20 μM drug and <20% at higher drug concentrations.

Chlorpromazine Binding by Calcium-Modulated Proteins. The binding of chlorpromazine to bovine brain calmodulin is shown in Figure 2 for a range (0.5–100 μM) of drug concentrations. In the presence of calcium, bovine brain calmodulin bound up to 5–6 mol of drug per mol of protein with apparent saturation above 20 μM chlorpromazine. In the presence of 1 mM EGTA and 4 mM $MgCl_2$, calmodulin bound less than 0.6 mol of drug per mol of protein (Figure 2). Chlorpromazine 5-oxide is a pharmacologically less active metabolite of chlorpromazine (Levin & Weiss, 1976, 1979). Bovine brain calmodulin bound 0.59 mol of chlorpromazine 5-oxide at 49 μM drug concentration in the presence of calcium.

We compared the results for bovine brain calmodulin with those for other four-domain calcium-modulated proteins. Spinach leaf calmodulin has a selected number of amino acid sequence differences and has quantitative activator activity

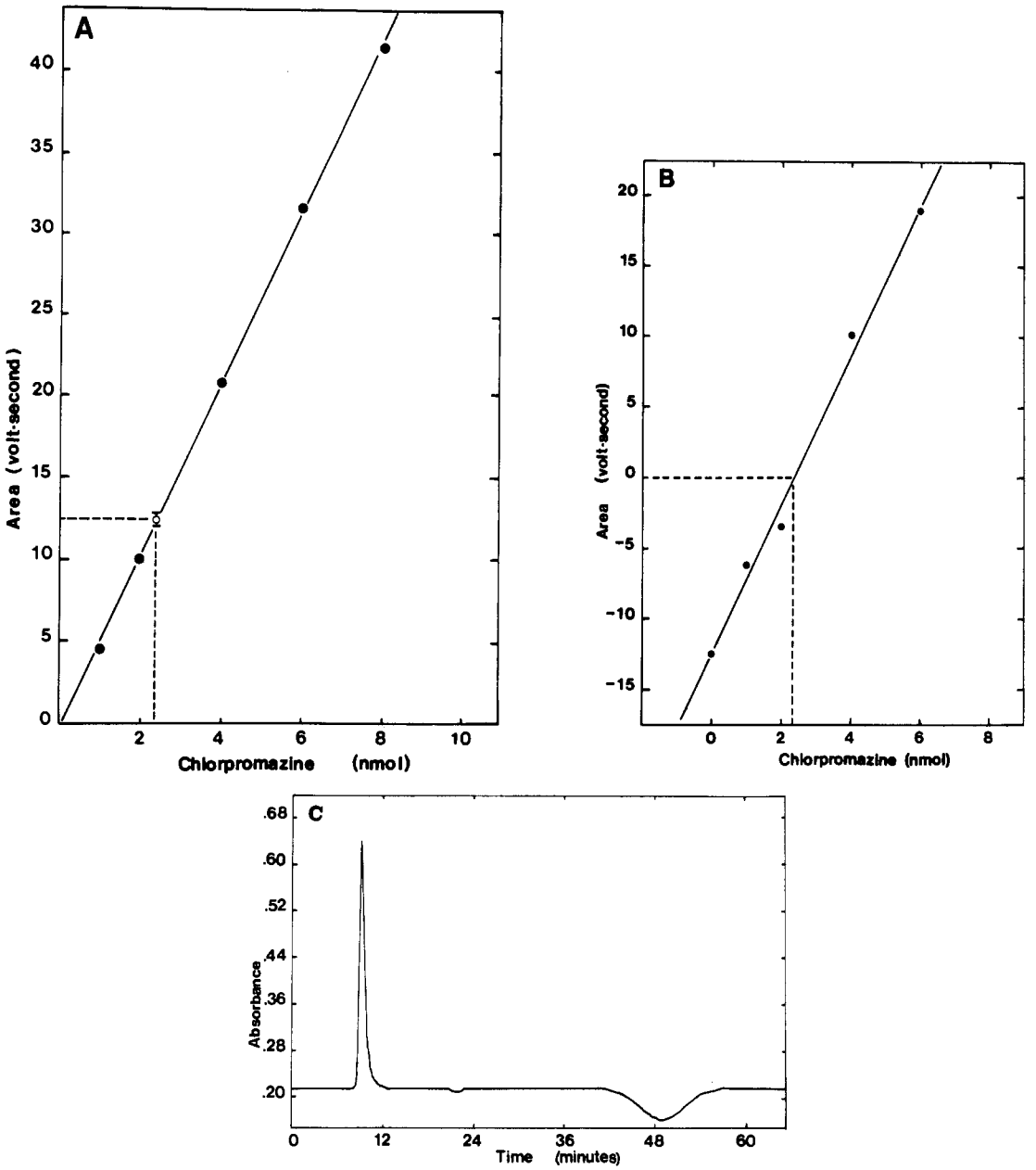

FIGURE 1: Binding of chlorpromazine to bovine brain calmodulin by high-performance gel filtration liquid chromatography. Binding measurements were performed by the method of Hummel & Dreyer (1962), modified as described in the text. The samples were applied to a column (0.6 × 30 cm) of acrylamide resin (Bio-Gel P6) equilibrated in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1mM $CaCl_2$, and 12.5 μM chlorpromazine at 0.3 mL/min. (A) External calibration of the binding measurements. Various amounts of chlorpromazine were applied to the column, and the areas of the peaks that eluted with a retention time of 49 min are shown (●). The solid line was calculated by linear regression ($r > 0.99$). An aliquot of bovine brain calmodulin (1.25 nmol) equilibrated in the column buffer was applied to the column. (B) Internal calibration of the binding measurements. Various amounts of excess chlorpromazine (0, 1, 2, 4, or 6 nmol) were added to samples containing 1.25 nmol of bovine brain calmodulin dissolved in the column buffer. The samples were applied to the column, and the area of the trough (negative) or the peak (positive) eluting at 49 min was plotted (●). The solid line was calculated by linear regression ($r > 0.99$). The chromatographic analysis of each sample is shown in Figure 2 of the supplementary material. (C) Absorbance at 254 nm as a function of the retention time. The area of the trough shown is plotted on the calibration curve [see (○) in panel A] and represents the mean of duplicate determinations with the range of values shown by the bars.

differences from vertebrate calmodulin (Lukas et al., 1984; Roberts et al., 1984). Therefore, we measured chlorpromazine binding to spinach calmodulin, as shown in Figure 3. Spinach calmodulin bound about 1 mol of drug per mol of protein at 10 μM chlorpromazine and 4–5 mol of drug per mol of protein at 47–100 μM drug. Spinach calmodulin bound 0.34 mol of chlorpromazine per mol of protein in the presence of EGTA, Mg^{2+}, and 47 μM drug, while the protein bound 0.61 mol of chlorpromazine 5-oxide per mol of protein in the presence of calcium and 49 μM drug. Thus, differences between bovine brain and spinach leaf calmodulins were not readily detected under the conditions of this study.

Rabbit skeletal muscle troponin C is a structural and functional analogue of calmodulin (Watterson et al., 1980c). Chlorpromazine binding to troponin C is shown in Figure 3. Troponin C bound only 0.3 mol of drug per mol of protein at 10 μM drug and maximally bound 2.2–2.5 mol of drug at 47–100 μM chlorpromazine. In the presence of EGTA, Mg^{2+}, and 47 μM chlorpromazine, troponin C bound 0.48 mol of drug per mol of protein. In addition, at 49 μM chlorpromazine 5-oxide in the presence of calcium, troponin C bound 0.28 mol of drug per mol of protein. Thus, troponin C has specific, calcium-dependent drug binding activity, but its total chlorpromazine binding capacity is less than that of calmodulin.

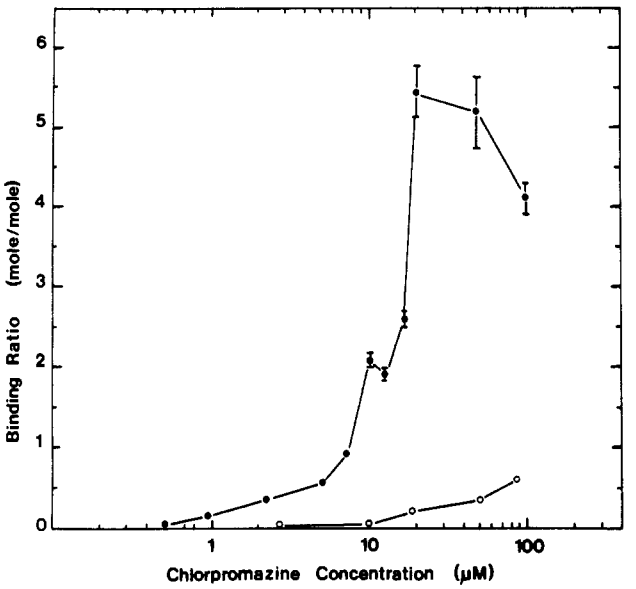

FIGURE 2: Equilibrium binding of chlorpromazine by bovine brain calmodulin as a function of chlorpromazine concentration. The binding of chlorpromazine to calmodulin was measured by using the method of Hummel & Dreyer (1962) in buffer containing calcium (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1 mM $CaCl_2$) (●) or in buffer containing magnesium (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 4 mM $MgCl_2$, and 1 mM EGTA) (○). The ordinate expresses the number of moles of chlorpromazine bound per mole of protein. The abscissa shows the concentration of chlorpromazine in the buffer. Results shown are the means of values calculated from external and internal calibrations. The range of values is shown when >5%. For experiments in which the binding ratio was less than 0.4 mol of chlorpromazine per mol of protein, the results shown are the means of duplicate determinations calculated from external calibration, and the range of values was <0.02 mol/mol.

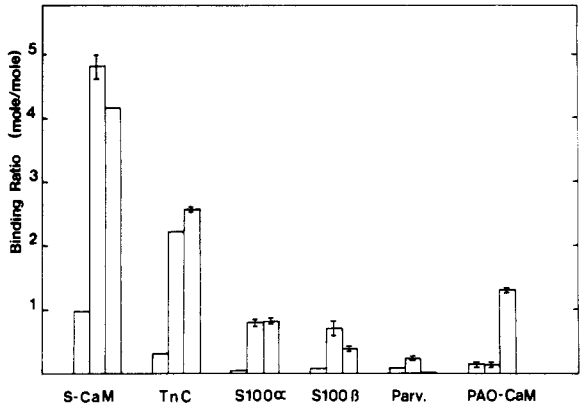

FIGURE 3: Binding of chlorpromazine by calcium-modulated proteins. Equilibrium binding experiments were performed by using the method of Hummel & Dreyer (1962) in buffer containing calcium (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1 mM $CaCl_2$) as described in the text. The number of moles of chlorpromazine bound per mole of protein are shown for spinach leaf calmodulin (S-CaM), rabbit skeletal muscle troponin C(TnC), bovine brain S100β(S100β), bovine brain S100α (S100α), carp paravalbumin (Parv.) and performic acid oxidized bovine brain calmodulin (PAO-CaM). For each protein, results are shown for three chlorpromazine concentrations: 9–10 μM (left bars), 45–50 μM (center bars), and 100 μM (right bars). The results shown are the means of duplicate determinations with the range of values as shown when >0.02 mol of drug per mol of protein.

Bovine brain S100α and S100β and carp parvalbumin were also tested for their ability to bind chlorpromazine because they represent two- and three-domain calcium-modulated proteins. In addition, parvalbumin does not interact with immobilized phenothiazines, as shown previously (Marshak et al., 1981). As shown in Figure 3, S100α and S100β maximally bound up to 0.82 and 0.68 mol of drug per mol of protein, respectively, in the presence of calcium, and 0.17 and 0.20 mol/mol, respectively, in the presence of EGTA and Mg^{2+}. Parvalbumin bound less than 0.5 mol of chlorpromazine per mol of protein at all drug concentrations tested in the presence of either Ca^{2+} or EGTA and Mg^{2+} (Figure 3).

Table I: Chlorpromazine Binding[a] to Fragments of Bovine Brain Calmodulin

peptide[b]	mol of drug bound/mol of peptide at 10 μM chlorpromazine	mol of drug bound/mol of peptide at 100 μM chlorpromazine
calmodulin (1–148)	2.02	4.10
residues 1–90	0.014	0.201
residues 1–37	0.009	0.067
residues 107–148	0.000	0.049

[a] Binding measurements were performed by using the method of Hummal & Dreyer (1962), modified as described in the text, in buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, and either 10 or 100 μM chlorpromazine. [b] The peptides are numbered according to the amino acid residues contained, using the numbering system for bovine brain calmodulin (Watterson et al., 1980c). The amino acid compositions of the peptides are shown in Table II of the supplementary material.

Chlorpromazine Binding by Modified Calmodulin. Performic acid oxidation of calmodulin quantitatively converts methionines to methionine sulfone (Watterson et al., 1984). Performic acid oxidized calmodulin does not retain enzyme activator activity but does adsorb to immobilized phenothiazine. We tested performic acid oxidized bovine brain calmodulin for chlorpromazine binding in solution, as shown in Figure 3. The oxidized protein bound less than 0.2 mol of drug per mol of protein below 100 μM and bound 1.3 mol of drug per mol of protein at 100 μM chlorpromazine. These results are consistent with the low level of covalent labeling of performic acid oxidized calmodulin described in the following paper (Lukas et al., 1985). Thus, the affinity and stoichiometry of calmodulin for chlorpromazine appear to be reduced by performic acid oxidation.

Calmodulin contains four structural domains that are homologous to each other (Watterson et al., 1980c). Domains 1 and 3 and domains 2 and 4 share higher structural homologies than other pairs of domains, suggesting the evolution of the protein by gene duplication. Because the two-domain, calcium-modulated proteins S100α and S100β had chlorpromazine binding activity, it was possible that large fragments of calmodulin containing one or two structural domains might have significant drug binding activity. In order to test this possibility, we investigated the binding of chlorpromazine to fragments of calmodulin in solution (Table I). Complete digestion of calmodulin with trypsin or cyanogen bromide destroys phenothiazine binding activity (Watterson et al., 1984). We prepared large fragments of bovine brain calmodulin using an arginine-specific protease from mouse submaxillary gland, as previously described (Marshak et al., 1984). The amino acid compositions of the fragments are shown in Table II of the supplementary material. The binding of chlorpromazine by these fragments was measured at 10 and 100 μM drug concentrations in the presence of calcium. As shown in Table I, none of these fragments bound the drug stoichiometrically. At 100 μM chlorpromazine, the fragment containing residues 1–90 retained only approximately 5% of the drug binding activity of the native protein. Thus, even a fragment of calmodulin which contains 60% of the molecule has very little chlorpromazine binding activity.

Chlorpromazine Inhibition of Activator Activity. The complexity of chlorpromazine binding to calmodulin led us

Table II: Inhibition of Calmodulin-Stimulated Cyclic Nucleotide Phosphodiesterase by Chlorpromazine

protocol[a]	IC_{50} (μM)[b]	activity[c]
1	>190	206
2	19	230
3	20	183
4	42	153

[a] The protocol refers to those described under Materials and Methods. [b] IC_{50} is the concentration of chlorpromazine necessary for 50% inhibition of activator activity. [c] Activity refers to the rate of production of AMP in the absence of chlorpromazine and is given in nanomoles of AMP per minute per milligram of enzyme preparation.

to reinvestigate the inhibition of calmodulin's activator activity by chlorpromazine. The chlorpromazine inhibition of calmodulin-stimulated cyclic nucleotide phosphodiesterase was investigated by using four assay protocols. These protocols were designed to examine the possibility that the order of addition of reagents and drug might affect the apparent IC_{50}, the concentration of chlorpromazine necessary for half-maximal inhibition of activator activity. In protocol 1, calmodulin and enzyme were preincubated in buffer containing calcium, and the reaction was initiated by the addition of a mixture of cAMP and drug. In protocol 2, calmodulin was preincubated with drug in buffer containing calcium, cAMP was added, and the reaction was initiated by the addition of enzyme. In protocol 3, the enzyme preparation was preincubated with drug, and the reaction was initiated by the addition of a mixture of cAMP and calmodulin. In protocol 4, all components except cAMP were preincubated, and the reaction was initiated by the addition of substrate. As shown in Figure 5 of the supplementary material, the rates of reaction, as measured by the product (AMP) formed, were nonlinear for protocols 1–3. The curves using method 1 are convex upward, those using protocols 2 or 3 are concave upward, and those using method 4 are approximately linear.

The relative rates of phosphodiesterase reaction and the concentration of chlorpromazine necessary for 50% inhibition (IC_{50}) are shown in Table II. The rates for protocols 1–3 were calculated from the initial slope of the linear portion (0–10 min) of the curves in Figure 5 of the supplementary material. With 1, the apparent IC_{50} for chlorpromazine was >190 μM while with protocols 2,3, and 4 the apparent IC_{50}'s for chlorpromazine were 19, 20, and 42 μM, respectively. These experiments were carried out at 24 nM calmodulin, which gives 80–90% activation of phosphodiesterase in the absence of drug. In the absence of calmodulin, the drug had little effect on the basal activity of the enzyme under these conditions. These data indicate that the order of addition of reagents to the phosphodiesterase assay can result in a 10–fold difference in the apparent IC_{50} for the drug. The overall nonlinearity of the rates of the reaction may be a result of the multiple, complex interactions of calmodulin, the drug, and the enzyme. No further investigation of the mechanism of inhibition was done as part of this study.

Discussion

The studies presented in this report demonstrate the following: (1) The interaction between calmodulin and chlorpromazine is a complex, calcium-dependent phenomenon with a stoichiometry of approximately 5 mol of drug bound per mol of protein over a range of drug concentrations used in biological studies; (2) extended regions of calmodulin encompassing most, if not all, of the protein are required for specific, high-affinity, calcium-dependent drug binding; (3) the inhibition of calmodulin's activator activity by phenothiazines is complex, and the quantitative effect of phenothiazines on activator activity can be altered by the order of addition of reactants to enzyme assays; (4) several structurally related calcium-modulated proteins bind chlorpromazine in a calcium-dependent manner with various stoichiometries and affinities for the drug.

The interaction between chlorpromazine and calmodulin appears to be a multisite, calcium-dependent phenomenon. Under the experimental conditions reported here, bovine brain calmodulin maximally binds approximately 5 mol of drug per mol of protein with overall, apparent half-maximal binding at 17 μM chlorpromazine. Several explanations are consistent with the binding phenomenon found in these studies. Multiple classes of nonidentical binding sites for chlorpromazine on calmodulin could exist, each class being calcium dependent and within the range of drug concentrations considered here. There could be cooperativity among some of the sites. Combinations of nonidentical, cooperative sites are also plausible. Finally, there could be alterations in calmodulin structure as a result of binding 2 mol of drug such that additional drug binding sites are exposed. It is not possible from any studies currently available to distinguish among these possibilities. The availability of accurate stoichiometries from the studies reported here will allow the possible mechanism to be addressed in future studies.

In order to examine the drug-binding domains of calmodulin in more detail, we measured binding of chlorpromazine to fragments of the molecule. To our knowledge, these are the only quantitative measurements of phenothiazine binding to fragments of calmodulin in solution. The results indicate that large fragments of calmodulin retain very little drug binding activity, consistent with reports (Newton et al., 1984) on activator activity of calmodulin fragments. Our results indicate that extended regions of the calmodulin molecule are required for specific, high-affinity, calcium-dependent drug binding. Several studies (Marshak et al., 1980; Head et al., 1982; Brzeska et al., 1983; Vogel et al., 1983; Newton et al., 1984) have shown that large fragments of calmodulin adsorb to phenothiazine–Sepharose conjugates. However, these studies are subject to the limitations of the methods as discussed in the introduction. The results reported here are direct measurements of the drug binding capacity of fragments of calmodulin in solution. More detailed characterization of the drug binding sites requires covalent modification of calmodulin with drugs that bind to calmodulin in a calcium-dependent manner. An initial study utilizing phenoxybenzamine, a calmodulin antagonist that covalently labels the protein, is presented in the following paper (Lukas et al., 1985).

The kinetics of chlorpromazine inhibition of calmodulin activation of cyclic nucleotide phosphodiesterase indicate that there may be a time-dependent equilibrium among the drug, calmodulin, and the enzyme. The concentration of chlorpromazine required for half-maximal inhibition was similar to the concentration required for half-maximal binding to calmodulin only under conditions where a calmodulin–enzyme complex is not formed prior to the initiation of the reaction. When all components were preincubated prior to the addition of substrate, the IC_{50} for the drug was 42 μM, in agreement with values measured previously (Levin & Weiss, 1976; Hidaka et al., 1980). These data indicate that the order of addition of the enzyme, calmodulin, and drug may alter the apparent IC_{50} for the drug by as much as 10-fold. These observations suggest that if calmodulin is bound to its target enzymes prior to the introduction of the drug, then the effective inhibitory concentration of the drug may be much higher than the actual affinity of the drug for calmodulin.

Our results for chlorpromazine binding to various calcium-modulated proteins support the hypothesis (Marshak et al., 1981) that these proteins contain related drug binding domains. However, there appear to be quantitative differences in the stoichiometry and affinity of the drug–protein interaction. These differences may be due to the specific three-dimensional structures of the drug binding sites of these different calcium-modulated proteins. Selected amino acid sequence differences among these structurally related proteins may result in altered chemical complementarities between atoms on the drug and amino acid side chains of the proteins. The details of the drug–protein interaction for phenothiazines and calcium-modulated proteins may provide insights for the future design of new drugs that are selective for particular calcium-dependent processes.

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SUPPLEMENTARY MATERIAL AVAILABLE

Table II giving the amino acid composition of the bovine brain calmodulin peptides, Figure 2 showing the chromatographic results of the internal calibration for the drug binding measurements described in Figure 1, and Figure 5 showing the cyclic nucleotide phosphodiesterase activity as a function of time for the four assay protocols and various concentrations of chlorpromazine (7 pages). Ordering information is given on any current masthead page.

Registry No. Chlorpromazine, 50-53-3; cyclic nucleotide phosphodiesterase, 9040-59-9.

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