Drug–Protein Interactions: Isolation and Characterization of Covalent Adducts of Phenoxybenzamine and Calmodulin†

Thomas J. Lukas, Daniel R. Marshak,‡ 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: Phenoxybenzamine, an α-adrenergic antagonist containing a (chloroethyl)amine group, labels calmodulin in the presence of calcium. The covalent interaction is inhibited by chlorpromazine in a concentration-dependent manner. Adducts of calmodulin and phenoxybenzamine were separated by high-performance liquid chromatography into four major fractions: two containing 0.6 and 1.2 mol of drug per mol of protein and two different fractions each containing 2.0 mol/mol. Each adduct had a reduced ability to activate cyclic nucleotide phosphodiesterase and myosin light chain kinase, and the chlorpromazine binding capacities of the phenoxybenzamine–calmodulin adducts were diminished to the extent of phenoxybenzamine incorporation into each adduct. Isolation and characterization of labeled peptides from phenoxybenzamine-modified calmodulins indicated that peptides encompassing residues 38–75, 107–126, and 127–148 contained phenoxybenzamine label. These studies directly demonstrate the relatedness between the binding activities of two structurally dissimilar calmodulin antagonists, demonstrate that covalent adducts of calmodulin and drugs with equal stoichiometries of labeling can have quantitative differences in activity and sites of modification, and provide direct evidence of distinct drug binding regions in calmodulin located in the amphipathic α-helical regions of the second and fourth domains.

Calmodulin is a calcium-modulated protein that stimulates a variety of enzymes and appears to be involved in the intracellular transduction of signals generated by calcium ion fluxes [for a review, see Klee & Vanaman (1982)]. Several classes of drugs bind to calmodulin in a calcium-dependent manner (Weiss et al., 1983) and inhibit the calmodulin activation of enzymes (Levin & Weiss, 1979; Hidaka et al., 1981). In the presence of calcium, calmodulin undergoes conformational changes that form or expose sites which facilitate the interaction with drugs and target proteins. These sites have been proposed to be hydrophobic in nature (Tanaka & Hidaka, 1980; LaPorte et al., 1980). However, many of the drugs and dyes (Weiss et al., 1983) and peptides (Malencik & Anderson, 1982) that bind to calmodulin are amphiphilic, containing both basic regions and hydrophobic regions, and information correlating calmodulin's known structural domains with drug binding sites is limited.

As described in the preceding paper, a phenothiazine drug (chlorpromazine) shows complex calcium-dependent binding to calmodulin (Marshak et al., 1985). The solution binding studies, however, provide only the thermodynamic parameters of the interaction such as dissociation constants and stoichiometry. Covalent labeling is necessary for the elucidation of binding sites. One reagent for such labeling is phenoxybenzamine, an α-adrenergic antagonist that inhibits the calmodulin stimulation of cyclic nucleotide phosphodiesterase in a dose-dependent, calcium-dependent manner (Watanabe & West, 1982; Watterson et al., 1984) and that forms covalent adducts with calmodulin (Lukas et al., 1983; Weiss et al., 1983). Thus, phenoxybenzamine is a covalent probe that is itself an inhibitor of calmodulin. We report here results of our studies on the purification and characterization of products formed in the calcium-dependent reaction of phenoxybenzamine with calmodulin, including data on the quantitative relationship between phenoxybenzamine–calmodulin adducts and chlorpromazine binding activity.

Materials and Methods

[^{3}H]Phenoxybenzamine was from New England Nuclear (lot 1414–209, 32 Ci/mmol) and was diluted with unlabeled phenoxybenzamine in methanol to obtain the desired specific activity and stored at −20 °C. Unlabeled chlorpromazine and phenoxybenzamine were gifts from Dr. Harry Green of Smith Kline and French Laboratories. High-performance liquid chromatography (HPLC)[1] grade acetonitrile was from Burdick and Jackson, TPCK–trypsin from Worthington, and Bio-Gel P6 resin from Bio-Rad.

General Methods. Chlorpromazine binding measurements were performed as described in the preceding paper (Marshak et al., 1985). Protein and peptide separations were done with a Hewlett-Packard 1084B liquid chromatograph. Wavelength chromatography was done on-line with a Hewlett-Packard 8450B spectrometer equipped with a 10-mm path-flow cell (Hellma). Spectra were acquired and stored in a host HP3357 laboratory automation system interfaced to the 8450B spectrometer. Software for data acquisition and surface plotting were developed in collaboration with the Department of

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

[1] Abbreviations: cAMP, adenosine cyclic 3′,5′-phosphate; AMP, adenosine 5′-monophosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; 1,5-IAEDANS, 5-[[[(iodoacetyl)-amino]ethyl]amino]naphthalene-1-sulfonic acid; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin; Me_3Lys, N^{ϵ},N^{ϵ},N^{ϵ}-trimethyllysine; TPCK, tosylphenylalanine chloromethyl ketone; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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Biomedical Engineering at Vanderbilt University. Automated Edman degradations were done with an Applied Biosystems 470A protein sequencer as described (Hunkapiller et al., 1981), with the following modifications. Glass fiber filters were treated with polybrene (1.8 mg) and precycled for 6 cycles. Peptides, dissolved in water, were applied in 30-μL aliquots. Phenylthiohydantoin derivations were analyzed by reverse-phase HPLC as described previously (Zimmerman et al., 1977). Repetitive yield on sequence analyses averaged 90% or greater. Amino acid analyses were done by using an LKB 4400 instrument and a standard sodium citrate buffer system. Performic acid oxidation was done as described previously (Hirs, 1967).

Preparation of Phenoxybenzamine-Labeled Calmodulin. Bovine brain calmodulin (540 nmol) was purified as described (Marshak et al., 1981) and dissolved in 9 mL of 50 mM Tris-HCl buffer containing 1.0 mM $CaCl_2$ and 150 mM NaCl, pH 7.4. To this solution were added three aliquots (27 μL) each of 0.02 M [^{3}H]phenoxybenzamine solution (13 336 cpm/nmol). After each addition of drug, the solution was mixed and allowed to stand at room temperature (22–24 °C) for 60 min. After the reaction period, the solution was applied to a column (2.0 × 30 cm) of Bio-gel P6 (Bio-Rad) and was eluted with 0.1 M ammonium bicarbonate and 0.1 mM EGTA, pH 7.9, at a flow rate of 2.0 mL/min. Fractions containing protein were identified by the absorbance at 280 nm and by the radioactivity. These fractions were concentrated on a Speed Vac evaporator (Savant, Hicksville, NY) to about 1 mL, and each was injected on a semipreparative (10 mm × 250 mm) RP-P column (Synchrom, Linden, IN) equilibrated in 10 mM sodium phosphate, 0.5 mM EGTA, pH 6.10, and 20% (v/v) acetonitrile. Drug-modified protein fractions were separated by using a program of linear gradients and isocratic steps as shown in Figure 1. Protein fractions were pooled from successive 1-mL injections and concentrated to remove acetonitrile. Each fraction was then subjected to gel filtration as described above using 0.1 M ammonium bicarbonate and 0.1 mM EGTA adjusted to pH 7.0 with acetic acid. Fractions containing protein were evaporated to dryness and then redissolved in 0.1 M ammonium bicarbonate to a concentration of approximately 2 mg/mL. The exact concentration of protein was determined by amino acid analysis. Portions of each fraction were used for all subsequent assays and trypsin digestion.

Trypsin Digestion. Calmodulin–phenoxybenzamine adducts (2 mg/mL) were digested with trypsin in 0.1 M ammonium bicarbonate and approximately 1.0 mM EGTA. Aliquots of enzyme, 1:100 (w/w), were added and the solutions incubated at 37 °C for 2 h. An additional aliquot of enzyme was added, and the digests were incubated at 37 °C for an additional 6 h. The digest was immediately frozen and stored at −20 °C until thawed for chromatography. Control samples were incubated without enzyme for the same periods of time, and a sample of unmodified calmodulin was also treated in the same manner. Peptides were separated on a Whatman ODS-3 column (0.94 × 25 cm) using 0.01 N HCl and acetonitrile as described previously (Lukas et al., 1984). The gradient program used for elution is given in Figure 2.

Filter Assay for Drug-Modified Calmodulin. Calmodulin was dissolved in 50 mM Tris-HCl, 150 mM NaCl, and 1.0 mM $CaCl_2$, pH 7.4, to make a stock solution of 0.17 mg/mL by amino acid analysis. [^{3}H]Phenoxybenzamine was dissolved in methanol to a concentration of 0.082 mM at a specific activity of 1.17×10^6 cpm/nmol. Twenty microliters of calmodulin stock solution and 68 μL of buffer (50 mM Tris-HCl, pH 7.4, and 150 mM NaCl), containing either $CaCl_2$ or EGTA (1.0 mM), were mixed, and the reaction was initiated by addition of 12 μL of phenoxybenzamine solution. Experiments with competing chlorpromazine were done similarly except that 2, 4, or 8 μL of a 0.5 mM chlorpromazine solution in water was added and the amount of diluting buffer reduced by the same amount. The solution was incubated at room temperature for 20 min and then the reaction initiated by addition of phenoxybenzamine. After 20 min of reaction at room temperature, aliquots (10 μL) were removed and spotted onto 25-mm circles of Whatman 3A filter paper. The filters were air-dried and then immersed into ice-cold 10% (w/v) trichloroacetic acid for 4 min. The filters were washed successively with 95% (v/v) ethanol at 0 °C (2 × 4 min) and room temperature (2 × 4 min). The filters were air-dried and transferred to glass scintillation vials, and the amount of radioactivity was measured in 12 mL of Liquiscint (National Diagnostics) in a Beckman 7500 scintillation counter. Controls with calcium or EGTA buffer and phenoxybenzamine but without calmodulin were also measured. Radioactivity in these controls was substracted from the reactions containing protein. No correction was made for quenching, and all experiments were done in duplicate.

Table I: Inhibition of Phenoxybenzamine Modification of Calmodulin

additive	method 1[a] cpm	method 1[a] mol/mol[c]	method 2,[b] % calmodulin modified
none	7000 ± 1400	0.36 ± 0.07	96
10 μM chlorpromazine	6800 ± 200	0.34 ± 0.01	
20 μM chlorpromazine	4700 ± 400	0.23 ± 0.02	50
40 μM chlorpromazine	2300 ± 600	0.11 ± 0.03	30
1.0 mM EGTA instead of $CaCl_2$	1800 ± 300	0.09 ± 0.02	4

[a] Conditions were the following: 1.7 μM calmodulin, 10 μM [^{3}H]-phenoxybenzamine, 50 mM Tris-HCl, 150 mM NaCl, and 1.0 mM $CaCl_2$, pH 7.4. Control values from reactions in the absence of protein were 1200 cpm in the presence of calcium and 1600 cpm in the presence of EGTA. These were subtracted from the observed cpm in reactions containing protein. In protein recovery experiments, 80% of added calmodulin was retained on the filters after the washing steps. [b] Conditions were the following: 4.5 μM calmodulin, 45 μM [^{3}H]-phenoxybenzamine, 50 mM Tris-HCl, 150 mM NaCl, and 1.0 mM $CaCl_2$, pH 7.4. The percent calmodulin modified was determined from integrated areas of calmodulin and modified calmodulin found by HPLC and UV detection at 220 nm. The predominant protein product migrates as adduct 2 (Figure 1A). [c] The stoichiometry is based upon the specific activity of [^{3}H]phenoxybenzamine used in these experiments.

Enzyme Activation Assays. Calmodulin activation of cyclic nucleotide phosphodiesterase was done as described (Watterson et al., 1980a). Activation of chicken gizzard myosin light chain kinase was measured with 100 μM bovine cardiac light chains as substrate as described (Roberts et al., 1984).

Results

Phenoxybenzamine Binding to Calmodulin and Inhibition by Chlorpromazine. On the basis of the thermodynamic binding data presented in the previous paper (Marshak et al., 1985), two different methods were used to examine the inhibition by chlorpromazine of the calcium-dependent reaction of phenoxybenzamine and calmodulin. Various concentrations of chlorpromazine were preincubated with calmodulin, and the reaction was initiated by the addition of phenoxybenzamine. The buffer and conditions used for this reaction were the same as those used in the equilibrium binding of chlor-

Table II: Amino Acid Analysis of Phenoxybenzamine-Modified Calmodulins after Performic Acid Oxidation

amino acid	residues by sequence[a]	adduct 1	adduct 2	adduct 3	adduct 4
Asp	23	24.0[b]	23.0	22.9	22.8
Thr	12	11.2	11.8	10.8	10.8
Ser	4	4.7	4.7	5.9	6.2
Glu	27	27.5	27.0	27.4	27.2
Pro	2	2.2	2.1	2.2	2.4
Gly	11	12.1	13.7	13.8	14.6
Ala	11	11.0	11.0	11.0	11.0
Val	7	6.7	6.7	6.7	6.8
$Met(O_2)$[c]		6.8	6.4	6.0	6.2
HSe[d]		0.4	0.5	0.8	0.6
Met	9	<0.1	<0.1	0.4	<0.1
Ile	8	7.2	7.0	7.3	7.3
Leu	9	8.4	8.3	8.8	8.6
Tyr	2	1.8	1.7	2.0	1.8
Phe	8	7.5	7.2	7.1	7.3
His	1	1.1	1.2	1.3	1.3
Me_3Lys	1	0.9	1.3	1.3	1.3
Lys	7	6.6	6.8	6.7	6.7
Arg	6	5.8	5.9	6.1	6.4
recovery of protein (nmol)[e]		36	120	59	84
mol of drug/mol of protein		0.6	1.2	2.0	2.0

[a] Residues in bovine calmodulin from Watterson et al. (1980b). [b] Adduct compositions are normalized to Ala = 11. [c] Methionine in the adducts was determined as the sulfone. [d] Homoserine. Homoserine lactone was not quantitated. [e] 540 nmol of calmodulin was in the phenoxybenzamine reaction as described under Materials and Methods.

promazine (Marshak et al., 1985). These results are summarized in Table I. In method 1, the extent of reaction was followed by precipitation of labeled protein on Whatman 3A filters as described under Materials and Methods. In method 2, the amount of unreacted calmodulin was determined by quantitative HPLC separation of the modified and unmodified protein. Regardless of the method of analysis, the incorporation of phenoxybenzamine was calcium dependent and was inhibited by chlorpromazine.

Isolation and Characterization of Covalent Adducts of Phenoxybenzamine and Calmodulin. Phenoxybenzamine–calmodulin covalent products were prepared by using a calmodulin concentration of 60 μM and initial drug concentrations of less than 100 μM. In the preparative reaction, the methanol concentration was less than 1%. In other experiments, phenoxybenzamine was found to precipitate at concentrations greater than 100 μM in the absence of added organic solvent.

Separation of the products from the phenoxybenzamine reaction was achieved by reversed-phase HPLC (Figure 1). Four major products, designated adducts 1–4, were obtained from the reaction (Figure 1A). As illustrated in Figure 1B, the elution of the adducts can be observed from spectra obtained with scans of the effluent. Fine structure of the spectra in the 260-nm region imparted by the drug is evident during the elution of a modified protein. The apparent stoichiometry of the modified calmodulin fractions was calculated from the specific activity of the tritiated drug incorporated into the protein.

The yields of modified calmodulins and the stoichiometries of drug incorporation are given in Table II. Adduct 1 (Figure 1A) contains 0.6 mol of drug per mol of protein, adduct 2 contains an overall stoichiometry of 1.2 mol of phenoxybenzamine per mol of protein, and adducts 3 and 4 contain 2 mol of drug per mol of protein. After purification, adduct 1 was found to contain a mixture of drug–protein adduct and some modified protein that, based upon the incorporation of isotopic label, lacked drug. Analysis of the reaction mixture prior to gel filtration and HPLC indicated that no native calmodulin could be detected (Figure 1C of supplementary material; see paragraph at end of paper regarding supplementary material). Therefore, a loss of drug occurred during the purification of the adducts. This loss of drug could be due to either chemical decomposition or dissociation of tightly bound drug. Additional purification of adduct 1 was not done as part of these studies.

Table III: Peptides Recovered from Trypsin Digests of [^{3}H]Phenoxybenzamine–Calmodulin Adduct[a]

adduct[a]	stoichiometry[b]	% modified 38–71	% modified 107–126	% modified 127–148
1	0.6	0	0	64.0
2	1.2	31.8	19.3	47.0
3	2.0	50.6	49.6	85.4
4	2.0	80.2	10.7	43.0

[a] The percent labeled peptide is based on the recovery of labeled and unlabeled peptide from trypsin digestion of the appropriate fraction. Data are from Tables IV and VI (supplementary material) and Figure 2. Adducts are numbered with respect to their elution as in Figure 1A. [b] From Table II; moles of drug per mole of protein.

Table IV: Compositions of Phenoxybenzamine-Labeled Peptides from a Trypsin Digest of Adduct 3[a]

amino acid	peptide[b] 1L (107–126)	2L (127–148)	3L (38–71)
Asp	3.2 (3)	4.3 (4)	7.1 (6)
Thr	1.7 (2)	1.0 (1)	2.5 (3)
Ser	0.7 (0)	0.2 (0)	1.3 (1)
Glu	3.9 (4)	5.2 (5)	6.5 (6)
Pro			2.0 (2)
Gly	1.9 (1)	2.2 (2)	3.6 (3)
Ala	0.4 (0)	2.1 (2)	2.4 (2)
Val	1.9 (2)	2.0 (2)	1.2 (1)
Met	0.9 (2)	0.8 (2)	0.8 (2)
Hse[d]	0.2	0.2	<0.1
Ile	1.0 (1)	1.0 (1)	2.0 (2)
Leu	2.0 (2)		2.8 (3)
Tyr		1.0 (1)	
Phe		1.0 (1)	1.8 (2)
His	0.8 (1)		
Me_3Lys	1.0 (1)		
Lys		1.0 (1)	
Arg	1.0 (1)		
nmol recovered[c]	11.7	13.5	9.0
cpm recovered	70894	58005	65588

[a] Compositions of peptides from other adducts are in the supplementary material (Table VI). [b] Peptides are labeled with respect to their elution as shown in Figure 2A. Numbers in parentheses are number of residues calculated from the sequence of bovine calmodulin as determined previously (Watterson et al., 1980b). [c] From fractionation of 36 nmol of phenoxybenzamine–calmodulin adduct 3 trypsin digest. [d] Homoserine. Homoserine lactone was not quantitated.

Identification of Phenoxybenzamine-Labeled Sites. Each of the labeled adducts was subjected to digestion with trypsin. An example of the separation of the peptides from trypsin digestion of adduct 3 and of unmodified calmodulin is given in Figure 2A,B. Similar chromatograms of adducts 1, 2, and 4 are in the supplementary material (Figure 2C–E). All calmodulin-derived peptides containing covalently attached drug were found to migrate signficantly later in the chromatogram compared to the unmodified peptides. Thus, the extent of modification of each peptide was determined from the recoveries of both labeled and unlabeled peptide.

Three tryptic peptides were found to contain radioactivity (see Figure 2A). A summary of the labeling pattern is given

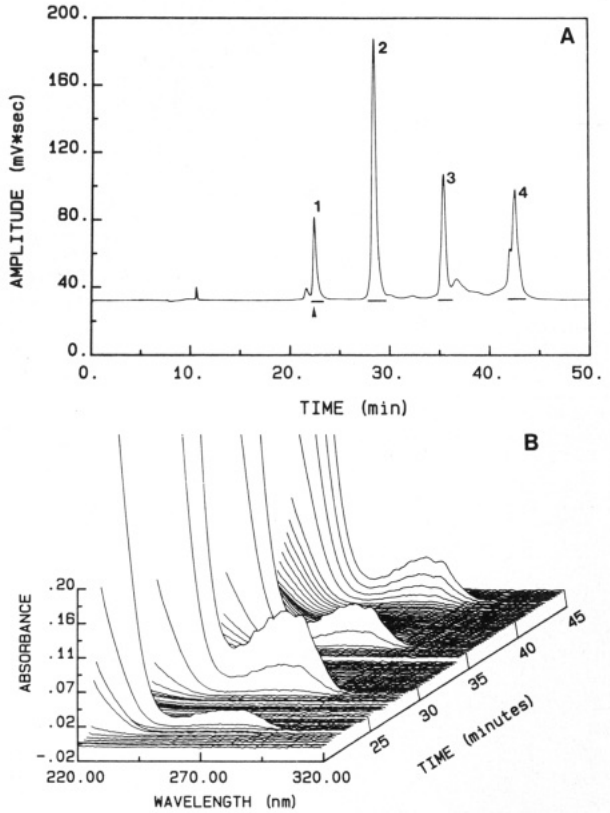

FIGURE 1: (A) Separation of [^{3}H]phenoxybenzamine–calmodulin adducts by HPLC monitored at 220 nm. The flow rate was 2.0 mL/min. Protein was loaded on the column initially equilibrated in 20% (v/v) acetonitrile (solvent B) and sodium phosphate buffer (solvent A) as described under Materials and Methods. The gradient program was the following: 6.0 min, % B = 20; 12.0 min, % B = 20; 18 min, % B = 30; 19 min, % B = 32; 25 min, % B = 32; 26 min, % B = 34; 32 min, % B = 34; 33 min, % B = 40; 40 min, % B = 40; 42 min, % B = 45. Adducts are numbered as they were eluted. Native calmodulin elutes as indicated by the arrow. The ordinate is the signal output from the detector. (B) Three-dimensional representation of UV spectra obtained in 1-s scans of the 20–45-min effluent from the chromatogram in (A). Scans begin at 20 min and continue in 15-s intervals.

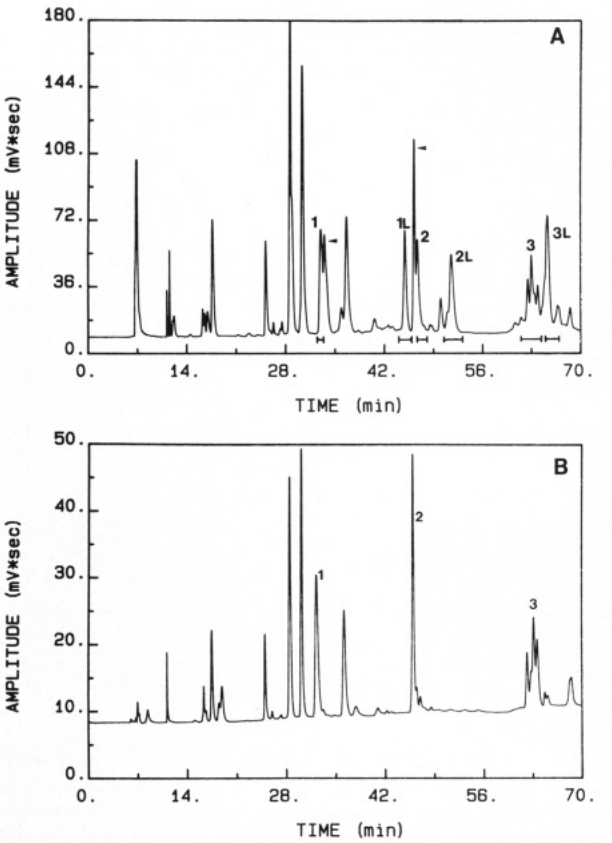

FIGURE 2: (A) Separation of labeled and unlabeled peptides from 30 nmol of trypsin-digested adduct 3. The peptides were loaded onto an ODS-3 column initially equilibrated in 5% (v/v) acetonitrile (solvent B) and 0.01 N HCl (solvent A). The peptides were eluted with the following programmed changes in solvent B: 5 min, % B = 5; 5.5 min, % B = 10; 10 min, % B = 10; 20 min, % B = 23; 30 min, % B = 25; 40 min, % B = 34; 50 min, % B = 34; 60 min, % B = 44; 65min, % B = 45. The ordinate is the detector signal output at 215 nm. Fractions 1, 2, and 3 are the unlabeled 107–126, 127–148, and 38–71 peptides, respectively. Fractions 1L, 2L, and 3L are the phenoxybenzamine-labeled peptides. The bars under the peaks indicate that portion of the effluent collected as a fraction. Arrows indicate the elution positions of uncharacterized phenoxybenzamine by-products. (B) Separation of peptides from an unmodified bovine calmodulin trypsin digest with the same conditions as (A).

in Table III. The chromatogram from the elution of peptides and the amino acid compositional and recovery data for labeled peptides from adduct 3 are given in Figure 2A and Table IV as an example. Similar data for adducts 1 ,2, and 4 and additional data for adduct 3 are in Tables VI–VIII of the supplementary material. Because the modified peptides were resolved from the corresponding unmodified peptide, a percent modification could be calculated (Table III) from the data in Table IV and Table VI of the supplementary material. This allows a direct comparison of the quantitative distribution of label in each adduct without making any assumptions about relative recovery or stability. Because there appears to be a loss of drug during purification and characterization, the data summarized in Table III may represent a low estimate of the amount of modification.

Adduct 1, which had 0.6 mol of drug per mol of protein, has only one labeled peptide (Table III). This peptide was identified by composition and amino-terminal sequence analysis to be residues 127–148 of calmodulin (Table VI of the supplementary material). Although the extent of modification varied among the adducts, peptide 127–148 was modified in all of the adducts. Adduct 2, which contained at least 1.2 mol of drug per mol of protein, has two additional modified peptides which were also identified by amino acid composition and amino-terminal sequence analysis (Table VI–VIII of the supplementary material). These additional peptides corresponded to residues 38–71 or 38–75 and residues 107–126; methionines are found at residues 51, 71, 72, 109, and 125 in the protein sequence, and the cleavage between Met-71 and Met-72 by tryspin has been noted previously (Watterson et al., 1980b).

Adducts 3 and 4 have 2 mol of drug per mol of protein and contain more label in peptide 38–71 than adduct 2 (Table III). Adduct 3 contains the greatest labeling of peptides 127–148 and 107–126. Adduct 4 is distinguished from adducts 2 and 3 by the increased amount of label recovered in peptide 38–71 (Table III).

When the purified phenoxybenzamine-modified calmodulins were subjected to acid hydrolysis and amino acid analysis, a loss of methionine residues and the presence of homoserine were observed. This suggested that phenoxybenzamine reacted with certain methionine residues presumably through the initial formation of the drug aziridinium ion and alkylation to give a sulfonium ion. Sulfonium ions may decompose under hydrolytic conditions to yield homoserine and methionine (Walsh & Stevens, 1977). Peptides containing the label were also found to give some homoserine after acid hydrolysis and amino acid analysis (Table IV and Table VI of the supplementary material).

To further investigate the possibility of methionine as the alkylation target, the modified calmodulins were subjected to performic acid oxidation, followed by acid hydrolysis and amino acid analysis to quantitate the methionine sulfone content. Because sulfonium ions are resistant to oxidation, the oxidation of unmodified methionines to methionine sulfone allows quantitation of the number of methionine residues unmodified (Walsh et al., 1979). As shown in Table II, the number of methionine residues recovered as methionine sulfone after hydrolysis of the phenoxybenzamine-modified proteins decreases with increased incorporation of drug. All of the adducts give some homoserine after hydrolysis, indicating that one mode of decomposition of the modified residue is to homoserine. Performic acid oxidation is a quantitative procedure for converting methionine to methionine sulfone. Therefore, even small amounts of methionine detected in acid hydrolysates of an oxidized sample could be significant. We detected less than 0.1 mol of methionine in adducts 1, 2, and 4, while adduct 3 gave 0.4 mol of methionine per mol of oxidized protein. Thus, another mode of decomposition of the modified residue appears to regenerate methionine. If the decomposition of the adducts leads to a methionine derivative or to methionine and the loss of label, then the actual number of modified methionine residues may be higher than that found by incorporation of radioactivity. Because performic acid oxidized calmodulin retains some affinity for chlorpromazine, we quantitated the labeling of performic acid oxidized calmodulin with phenoxybenzamine under the same conditions used for unoxidized calmodulin. As would be expected if all methionines were oxidized prior to treatment with phenoxybenzamine, less than 0.1 mol of phenoxybenzamine per mol of protein could be incorporated into performic acid oxidized calmodulin.

As indicated in Table II, the lysine, arginine, and histidine contents of the adducts are unchanged. The modification of the side-chain carboxyl groups of glutamic and aspartic acids would not normally be detected after acid hydrolysis. Because esters hydrolyze under the basic conditions used in the reaction and purification steps, transient ester formation cannot be ruled out in a phenybenzamine labeling experiment.

It attempts to determine the exact sites of phenoxybenzamine labeling, peptides were subjected to automated Edman degradation as described under Materials and Methods. Unfortunately, the drug–peptide bonds are apparently unstable under the conditions of Edman degradation chemistry and appear to decompose during sequence analysis. Overall, less than 50% of the applied radioactivity was recovered. Position assignments of the phenoxybenzamine label to particular methionines, therefore, must be considered tentative. In sequence analysis of the labeled 107–126 peptides, the recovery of PTH-methionine in cycle 3 was indistinguishable from that of an unlabeled peptide, and only 2.4% of the recovered label was found in this cycle (Table VII of the supplementary material). This result is consistent with Met-109 not being the major site of labeling and suggests Met-124 may be the phenoxybenzamine reactive residue in this peptide. Sequence analysis of peptides 38–71 implicated Met-51 as an alkylation site, because 17–25% of the total radioactivity recovered during Edman degradation appeared in the sequencer cycle corresponding to this residue (data for adduct 3 are in Table VIII of the supplementary material). A labeled peptide encompassing residues 38–75 also was isolated from one of the adducts (Table VI of the supplementary material). Therefore, it is possible that label is distributed among Met-51, Met-71, and Met-72. Analysis of the labeled peptides 127–148 indicated that 92% of the radiolabel was lost from the peptide by

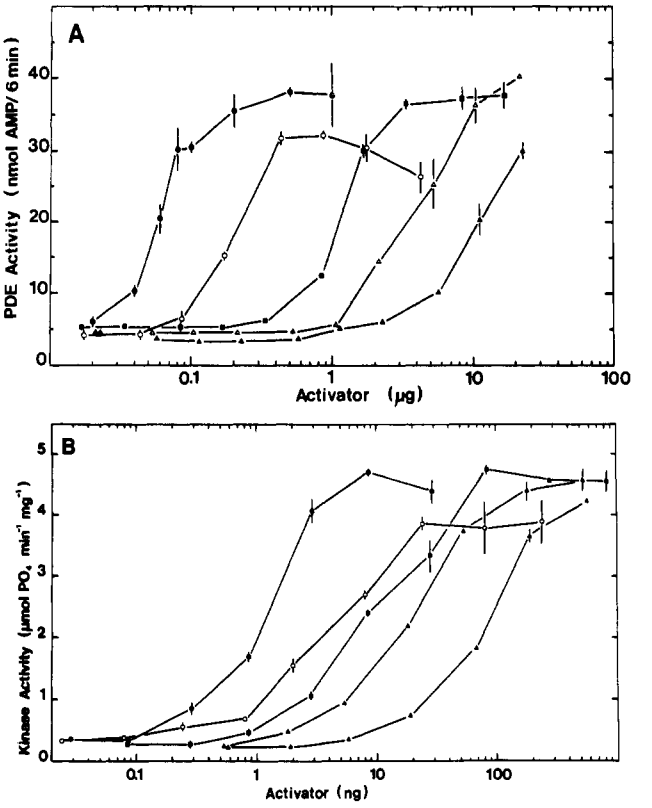

FIGURE 3: (A) Activation of 3′,5′-cyclic-AMP phosphodiesterase by bovine brain calmodulin and phenoxybenzamine–calmodulin adducts. Adduct fractions 1 (○), 2 (■), 3 (△), and 4 (▲), and unmodified calmodulin (●) were incubated with a calmodulin-depleted fraction of bovine brain phosphodiesterase, and the reaction was initiated by addition of cAMP (Marshak et al., 1985). AMP produced was measured by quantitative HPLC as described (Watterson et al., 1980a). (B) Activation of chicken gizzard myosin light chain kinase by bovine brain calmodulin and phenoxybenzamine–calmodulin adducts. Adduct fractions 1 (○), 2 (■), 3 (△), and 4 (▲) and unmodified calmodulin (●) were tested for myosin light chain kinase activator activity as described (Roberts et al., 1984). Incorporation of $^{32}PO_4$ into bovine cardiac light chain in 20 min was measured by precipitation onto phosphocellulose papers and counting in Betafluor (National Diagnostics). Points in both graphs are means of duplicate determinations.

cycle 15, which is before the Met–144 and 145 cycles. Thus, assignment of the alkylation sites to either Met-144 or Met-145 was not possible, although these are the only methionine residues in this peptide.

On the basis of our data, the major phenoxybenzamine reactive sites appear to be at selected methionines found within residues 38–75, 107–126, and 127–148. These regions include methionines at residues 51, 71, 72, 109, 124, 144, and 145. However, no evidence for alkylation of Met-36 or -109 was found. A major labeled peptide containing Met-76 was not identified, indicating that this methionine either is not labeled or is labeled but rapidly decomposes under the conditions used.

Functional Properties of Drug-Modified Calmodulins. Activation profiles for phosphodiesterase by the phenoxybenzamine–calmodulin adducts are given in Figure 3A. The drug-modified calmodulins activate phosphodiesterase to the same extent as unmodified calmodulin. However, the amount of protein required for half-maximal activation is displaced 5–180-fold compared to native bovine calmodulin. The same general profile of activity was observed with myosin light chain kinase, as shown in Figure 3B.

Each of the protein adducts was tested for its ability to bind to chlorpromazine, by using the method described in the preceding paper (Marshak et al., 1985), at a chlorpromazine

Table V: Chlorpromazine Binding by Calmodulin and Phenoxybenzamine-Modified Calmodulins

sample	phenoxybenzamine incorporated[a]	chlorpromazine bound[b]	sum of chlorpromazine + phenoxybenzamine
unmodified calmodulin	0	5.28	5.28
adduct 1	0.6	5.05	5.65
adduct 2	1.2	3.90	5.10
adduct 3	2.0	2.98	4.98
adduct 4	2.0	2.53	4.53

[a] From Table II; moles of drug per mole of protein. [b] Binding was measured at a chlorpromazine concentration of 35 μM as described under Materials and Methods. Buffer was 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1 mM $CaCl_2$. Results are given as moles of drug per mole of protein.

concentration of 35 μM. The results are shown in Table V. Unmodified bovine calmodulin bound 5.3 mol of drug/mol of protein. Except for adduct 1 which contains only 0.6 mol of phenoxybenzamine per mol of calmodulin, reduced capacity for chlorpromazine binding could be detected for the adducts. Adduct 2, which has 1.2 mol of phenoxybenzamine per mol of protein, bound only 3.9 mol of chlorpromazine. Similarly, adducts 3 and 4, which have approximately 2 mol of phenoxybenzamine per mol of protein, bound 2.5–3 mol of chlorpromazine. The total drug binding capacity calculated from the sum of chlorpromazine bound and phenoxybenzamine incorporated gave 4.5–5.6 mol of drug per mol of protein. Thus, the chlorpromazine binding properties of the adducts are consistent with the stoichiometry determined for chlorpromazine and native calmodulin (Marshak et al., 1985).

Discussion

Many covalent labeling studies of proteins require that a ligand be modified to introduce a reactive moiety at a position in the molecule that is not directly involved in protein binding. Phenoxybenzamine is a drug that inhibits calmodulin activity and is able to covalently label calmodulin without prior derivatization. Thus, the modification of calmodulin with phenoxybenzamine provides a logical starting point in attempts to map drug binding domains and potentially allows labeling of residues directly involved in drug binding. The studies summarized in this report (1) demonstrate that in the presence of calcium, calmodulin is covalently labeled by phenoxybenzamine, resulting in at least four chromatographically distinct products; (2) suggest that selected methionines in proposed amphipathic α-helices may be the amino acid residues modified, and these sites are distinct from those readily modified in studies not utilizing affinity-based labeling; and (3) demonstrate that the total chlorpromazine binding activity, phosphodiesterase activator activity, and myosin light chain kinase activator activity of the adducts are diminished to varying extents.

The stoichiometry of alkylation of the four adducts ranged from 0.6 to 2 mol of phenoxybenzamine per mol of protein. Even though fractions of modified proteins were separated by HPLC, homogeneity with respect to modification site(s) within each adduct was not assumed. If phenoxybenzamine, like chlorpromazine (Marshak et al., 1985), binds to as many as five or six sites on calmodulin, then a number of sites may be labeled and multiple products would be expected. Further characterization of the phenoxybenzamine–calmodulin adducts indicated at least three sites were labeled. These sites are found within residues 38–75, 107–126, and 127–148. Circumstantial evidence for methionine modification was provided by amino acid analysis of phenoxybenzamine-modified calmodulins and labeled peptides. This suggests that phenoxybenzamine reacts with certain methionine residues in calmodulin, presumably through the formation of an aziridinium ion (Henkel et al., 1976) and subsequent alkylation. A proposed mechanism of the reaction is shown in Figure 4. Although phenoxybenzamine has been used to label or inhibit various receptor proteins (Weiner, 1982), a specific amino acid residue has not been implicated previously.

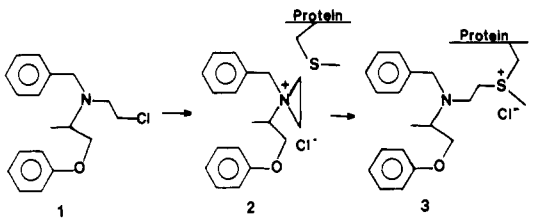

FIGURE 4: Scheme of a proposed reaction of a protein methionine group and the aziridinium ion (**2**) from phenoxybenzamine (**1**).

Chemical modification studies of calmodulin have shown that methionines are modified by reagents such as iodoacetate (Walsh & Stevens, 1977), 1,5-IAEDANS (Olwin et al., 1983), benzyl bromide (Walsh et al., 1979), and *N*-chlorosuccinimide (Walsh & Stevens, 1978). These modifications are not affinity based, and the distributions of modification sites are distinct from those modified by phenoxybenzamine. For example, Walsh & Stevens (1978) reported that in the presence of calcium, three to four methionines in calmodulin may be specifically oxidized by *N*-chlorosuccinimide, resulting in a loss of phosphodiesterase activator activity. Tanaka et al. (1983) found that this oxidized calmodulin also had substantially lower affinity for W-7 and chlorpromazine. Walsh & Stevens (1978) identified the modified residues as Met-71, -72, and -76, and possibly Met-109, and concluded that these residues are likely to be exposed on the surface of calmodulin in the presence of calcium. As shown here, Met-76 and -109 do not appear to be major alkylation sites for phenoxybenzamine.

Two of the phenoxybenzamine-modified peptides surround the proposed fourth calcium binding loop of calmodulin (Kretsinger, 1980). Another peptide encompasses the helices of the second domain. Thus, domains 2 and 4, which share structural homology (Watterson, et al., 1980b), also contain functional homology with respect to phenoxybenzamine alkylation sites. Because phenoxybenzamine appears to be a methionine-selective alkylating agent with respect to calmodulin, regions lacking methionines may not be labeled. Calmodulin, however, has methionines in most of the amphipathic α-helical regions (Watterson et al., 1980b). The only regions lacking a methionine are the proposed E helices of the first domain (residues 9–19) and third domain (residues 85–92).

Chlorpromazine inhibits the reaction of calmodulin with phenoxybenzamine at total chlorpromazine concentrations consistent with the observed binding parameters (Marshak et al., 1985). It should not be assumed that chlorpromazine binding is necessarily singularly competitive with phenoxybenzamine such that the binding of drug molecules is mutually exclusive on a site basis. On the basis of our results, however, the binding of chlorpromazine to sites unaffected by phen-

oxybenzamine does not appear to be impaired since the total drug binding capacity as measured by chlorpromazine binding in solution plus the phenoxybenzamine–calmodulin stoichiometry is not dramatically changed. Thus, the modification of calmodulin by phenoxybenzamine results in a stoichiometric loss of drug binding activity.

A relationship between phenoxybenzamine reactive sites and protein functional activity was demonstrated by the decreased ability of the modified proteins to activate two calmodulin-stimulated enzymes, phosphodiesterase and myosin light chain kinase. Adduct 1, which is modified at the peptide 127–148 site, displays a 4-fold decrease in activity. This result is consistent with that obtained with alkylation by 1,5-IAEDANS (Olwin et al., 1983). This modification, possibly in the peptide 127–148 site, resulted in about 5-fold decrease in the apparent association of 1,5-IAEDANS-labeled calmodulin with phosphodiesterase. Phenoxybenzamine modification of additional sites as in adducts 3 and 4 appears to futher weaken the interaction of calmodulin with phosphodiesterase and myosin light chain kinase. The activity displayed by these fractions represents an upper limit since the presence of less than 1% native calmodulin could account for some of the observed activity.

The greatest loss of activity is found with adduct 4 which has increased modification of the second domain peptide. Marshak et al. (1985) found significant drug binding activity in a fragment of calmodulin that includes residues 1–90. Together these results implicate regions of the highly conserved second domain of calmodulin in the binding of drugs and, indirectly, in the binding of phosphodiesterase and myosin light chain kinase. However, because calmodulin modified only in the second domain was not one of the major adducts isolated, an assessment of the second domain modification cannot be made from these data alone.

Clearly, the interaction of calmodulin with drugs and target proteins is a complex process that involves conformational changes and multiple binding sites. Phenoxybenzamine modification of some of these sites is only an initial step in mapping functional domains. To map further the functional domains on calmodulin, other specific reagents are required. These reagents could be other calmodulin inhibitors with inherent covalent reactivity similar to phenoxybenzamine, derivatives of drugs (Newton et al., 1983), or peptides synthesized to contain reactive groups such that specific regions of calmodulin can be modified and identified. However, a limitation of most covalent labeling studies is that the reactive residue may be spacially proximal but not necessarily identical with the residues directly involved in ligand interaction. Therefore, detailed quantitative analyses, such as those described by Marshak et al. (1985), of the drug binding properties of calmodulins with selected amino acid changes in drug binding sites are also required. Together, these investigations might provide further insight into the molecular and atomic requirements for specific drug–calmodulin interactions.

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SUPPLEMENTARY MATERIAL AVAILABLE

Tables VI–VIII containing amino acid composition and amino acid sequence data and Figures 1C and 2C–E showing chromatograms of the separation of adducts and labeled peptides (9 pages). Ordering information is given on any current masthead page.

Registry No. Chlorpromazine, 50-53-3; cyclic nucleotide phosphodiesterase, 9040-59-9; myosin light chain kinase, 51845-53-5; Ca, 7440-70-2.

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