Functional Properties of Covalent β-Endorphin Peptide/Calmodulin Complexes. Chlorpromazine Binding and Phosphodiesterase Activation†

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ABSTRACT: The 31-residue neuropeptide porcine β-endorphin was shown to inhibit the Ca^{2+}-dependent calmodulin activation of highly purified bovine brain cyclic nucleotide phosphodiesterase (3′,5′-cyclic AMP 5′-nucleotidohydrolase, EC 3.1.4.17). Using a series of deletion peptides, the minimal inhibitory peptide sequence was found to correspond to β-endorphin residues 14–25, confirming previously reported results for crude enzyme preparations. A correlation was found between the relative inhibitory potency of a particular β-endorphin deletion peptide and the efficacy of cross-linking that peptide to calmodulin with bis(sulfosuccinimidyl) suberate, strongly implicating peptide binding to calmodulin as the mechanism of the observed inhibition. We found that relatively modest concentrations of chlorpromazine significantly reduced the efficiency of cross-linking β-endorphin 14–31 to calmodulin. Chlorpromazine–Sepharose affinity chromatography of peptide/calmodulin adducts showed that a significant portion of the cross-linked β-endorphin 14–31/calmodulin complex (stoichiometry of 1 mol/mol) retained the ability to interact with the immobilized phenothiazine in a Ca^{2+}-dependent and calmodulin-displaceable manner. In contrast, the 2:1 (peptide:protein) product exhibited no affinity for the immobilized phenothiazine. The use of this affinity chromatographic step allowed preparation of homogeneous populations of both 1:1 and 2:1 β-endorphin 13–31/calmodulin complexes and assessment of their functional characteristics. Equilibrium binding studies with chlorpromazine revealed that the covalent attachment of one peptide molecule to calmodulin perturbed all phases of Ca^{2+}-dependent drug binding, but the adduct still bound significant quantities of chlorpromazine. The 2:1 complex, however, showed little detectable binding of the phenothiazine. Thus, β-endorphin-derived peptides and chlorpromazine binding domain(s) on calmodulin possess some commonality. Furthermore, at least a portion of these inhibitory molecule domain(s) seems intimately involved in phosphodiesterase recognition and activation as both the 1:1 and 2:1 complexes exhibited negligible ability to activate or inhibit the calmodulin-dependent activation of the enzyme.

Calmodulin is a ubiquitous acidic, relatively low molecular weight (M_r 16 680) Ca^{2+}-binding protein of known amino acid sequence (Watterson et al., 1980). Extensive sequence analysis of calmodulin from a variety of vertebrate, invertebrate, protozoan, and plant sources has revealed that the molecule is highly conserved throughout evolution (Dedman et al., 1978; Grand & Perry, 1978; Watterson et al., 1980; Jamieson et al., 1980; Takagi et al., 1980; Yazawa et al., 1981), consistent with suggestions that calmodulin plays an important role in intracellular events involving Ca^{2+} (Cheung, 1980; Means & Chafouleas, 1982). Indeed, upon binding Ca^{2+}, calmodulin has been shown to modulate the in vitro activities of a wide variety of enzymes including a form of cyclic nucleotide phosphodiesterase, a form of adenylate cyclase, myosin light chain kinase, and Ca^{2+}–Mg^{2+}-dependent ATPase [cf. review by Klee & Vanaman (1982)]. There is some information available on the two major protein conformations occurring in the presence and absence of saturating Ca^{2+} (Klee, 1977; Wolff et al., 1977). In contrast, the molecular characteristics and structural requirements of the calmodulin/enzyme association and the subsequent modulation process are at this point largely unknown. Since these enzymes are highly complex and do not readily lend themselves to detailed structural studies, we (Giedroc et al., 1983b,c) and others (Malencik & Anderson, 1982, 1983, 1984; Maulet & Cox, 1983; Barnette et al., 1983) have approached the study of these events through an investigation of structures of simpler substances which disrupt calmodulin-dependent processes.

The first reported inhibitors of a calmodulin-stimulated enzymic activity were several agents from the phenothiazine family including trifluoperazine and chlorpromazine (Levin & Weiss, 1977, 1979). Trifluoperazine was shown to inhibit in a concentration-dependent manner the Ca^{2+}, calmodulin-dependent activity of a brain phosphodiesterase preparation with no demonstrable effect on the calmodulin-independent enzymic activity (Weiss et al., 1980). It was suggested that this phenomenon was characterized by Ca^{2+}-dependent binding of trifluoperazine to calmodulin with a stoichiometry of 2 mol of phenothiazine/mol of protein and a K_d in the micromolar range (Levin & Weiss, 1977). In addition, many lower affinity, Ca^{2+}-independent sites on calmodulin for these drugs apparently exist (Levin & Weiss, 1977). More recently, a number of agents with widely diverse structures have been found to exhibit effects similar to the phenothiazines (Hidaka

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et al., 1979; Gietzen et al., 1981). It is now generally accepted that these agents associate with calmodulin primarily on the basis of their hydrophobicity and are not exclusively calmodulin antagonists (Norman & Drummond, 1979; Sharma & Wang, 1981; Moore & Dedman, 1982; Melner et al., 1983). Even so, their use as structural probes of calmodulin is valid given the above functional significance. Indeed, it has been potulated by LaPorte et al. (1980) that a hydrophobic domain is exposed on the surface of calmodulin upon Ca^{2+} binding and that this domain is recognized by these drugs and perhaps functions at the calmodulin/enzyme interface.

β-Endorphin, the basic 31-residue neuropeptide, was the first reported peptide that could mimic the effects of the phenothiazines (Weiss et al., 1980; Sellinger-Barnette & Weiss, 1982). Malencik & Anderson (1982) have demonstrated Ca^{2+}-dependent binding of β-endorphin to a fluorescent derivative of calmodulin with a K_d in the same range as that exhibited by the phenothiazines and have reported a stoichiometry of 1 mol of peptide/mol of protein largely in agreement with equilibrium dialysis experiments (Sellinger-Barnette & Weiss, 1982). No binding was shown in the presence of a Ca^{2+} chelator in either case. We have recently demonstrated by cross-linking experiments with bis(sulfosuccinimidyl) suberate (BS^3)[1] that stable solution complexes of β-endorphin and calmodulin do occur, and stoichiometries of 1 and 2 mol of peptide/mol of protein were detected (Giedroc et al., 1983b). The cross-linking reaction was dependent on exogenous Ca^{2+} and was not influenced by changes in ionic strength (Giedroc et al., 1983b). Furthermore, we suggested that hydrophobic interactions contributed to the β-endorphin binding to calmodulin and that electrostatic interactions were of little importance.

In this paper, we show through the use of deletion peptides that the ability of such peptides to bind to calmodulin is closely coupled to their inhibitory activity in a ternary macromolecular system of peptide, calmodulin and purified brain cyclic nucleotide phosphodiesterase. The abilities of two major cross-linked products (stoichiometries of 1 and 2 mol of peptide/mol of calmodulin) to interact with chlorpromazine–Sepharose in a Ca^{2+}-dependent fashion were found to differ significantly; this served as the basis to prepare homogeneous 1:1 and 2:1 peptide/calmodulin complexes. Assessment of each of these complexes and calmodulin to bind chlorpromazine under equilibrium conditions was compared with their ability to activate phosphodiesterase.

Materials and Methods

Chemicals and Reagents. Cyclic [^{3}H]GMP (8.3 Ci/mmol) was purchased from New England Nuclear Corp., Boston, MA, and Na^{125}I (16.9 mCi/μg of iodine) was from Amersham Corp., Arlington Heights, IL. Cyclic AMP, guanosine, glucose oxidase, lactoperoxidase, *Crotalus atrox* venom, and bovine serum albumin were from Sigma Chemical Co., St. Louis, MO. BS^3 was synthesized as described (Staros, 1982) or purchased from Pierce Chemical Co., Rockford, IL. CAPP was kindly supplied by Dr. Stephen Kennedy of the National Institute of Mental Health, Rockville, MD, and chlorpromazine hydrochloride (TLC pure) was supplied by Smith Kline and French Laboratories, Rahway, NJ.

β-Endorphin and Derived Peptides. β-Endorphin and the deletion peptides corresponding to residues 1–17, 14–23, 14–25, 14–27, and 20–31 were based on the sequence of porcine β-endorphin while the peptide corresponding to residues 14–31 was based on the human sequence (cf. Figure 1). These peptides were synthesized by using solid-phase methods, purified, and characterized as described elsewhere (Ling, 1977). [^{14}C-Ile^{23}]-β-Endorphin 13–31, based on the human sequence, was manually synthesized (Giedroc, 1984) by using modifications of the methods outlined by Merrifield et al. (1982). [^{14}C]*t*-BOC-isoleucine was synthesized from [U-^{14}C]isoleucine (Amersham) and di-*tert*-butyl dicarbonate (Aldrich) essentially according to Moroder et al. (1976). The purified peptide, homogeneous by reverse-phase HPLC, gave the expected amino acid composition and exhibited the correct amino-terminal sequence as verified by Edman degradation of the first 14 residues. Sequence analysis was performed by Drs. S. Sinha and K. Brew essentially as described (Shewale & Brew, 1982).

Calmodulin Purification. Calmodulin was purified from either porcine testes or brain by using standard procedures [cf. Klee & Vanaman (1982)]. Briefly, the supernatant following homogenization and low-speed centrifugation (Giedroc et al., 1983c) was batch absorbed to an anion exchanger (Whatman DE-52) and equilibrated with a low ionic strength buffer with subsequent elution of the calmodulin-containing fraction with 0.6 M NaCl. This eluant was carried through ammonium sulfate fractionation followed by CAPP–Sepharose chromatography essentially as described (Giedroc et al., 1983c). This fraction was purified further by either reverse-phase HPLC (Giedroc et al., 1983b) or anion-exchange chromatography (Giedroc et al., 1983a). The resultant calmodulin was judged to be homogeneous by Coomassie blue staining of overloaded 15% Laemmli (1970) gels. The CAPP–Sepharose column was prepared by oxirane coupling of CAPP to Sepharose CL-4B closely following the procedure of Charbonneau & Cormier (1979). Typical capacities were 1.0–1.5 mg of calmodulin/mL of swollen resin.

Phosphodiesterase Purification and Assay. Cyclic nucleotide phosphodiesterase was purified from porcine brain by using a monoclonal antibody coupled to a polyacrylamide gel that recognizes the phosphodiesterase/calmodulin complex (Hansen & Beavo, 1982). Briefly, a homogenate was added to the immobilized antibody in a Ca^{2+}-containing buffer which promotes selective adsorption of the complex. After all other proteins had been washed from the affinity gel, EGTA was added to the buffer, and the effluent was directed to a DEAE-Sephacel column. Calmodulin-free enzyme was eluted from this column. Recovery of enzyme activity from the supernatant was approximately 20–30%.

The enzyme was assayed as described earlier (Keravis et al., 1980), using a Tris-HCl buffer, pH 7.5, containing bovine serum albumin (0.2 mg/mL), 5 mM $MgCl_2$, 1.1 μM cyclic GMP (90 000 cpm), and either $CaCl_2$ or EGTA. The breakdown of [^{3}H]cGMP was measured by converting the nucleotide monophosphate to the nucleoside with snake venom nucleosidase treatment, followed by separation by QAE-Sephadex chromatography.

The concentrations of calmodulin and peptide/calmodulin adducts were determined by amino acid analysis (see below).

Iodination of β-Endorphin Peptide 14–31. The peptide (60 nmol, 122 μg) was iodinated with Na^{125}I using the glucose

[1] Abbreviations: BS^3, bis(sulfosuccinimidyl) suberate; CAPP, 2-chloro-10-(3-aminopropyl)phenothiazine hydrochloride; ED_{50}, effective dose for 50% inhibition; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GMP, guanosine 5′-monophosphate; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; phosphodiesterase, 3′,5′-cyclic adenosine monophosphate 5′-nucleotidohydrolase (EC 3.1.4.17); Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$–PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; TLC, thin-layer chromatography; *t*-BOC, *tert*-butyloxycarbonyl; HPLC, high-pressure liquid chromatography.

oxidase/lactoperoxidase procedure (Graf et al., 1980). The reaction was performed in 0.1 M ammonium acetate, pH 7.0, in a volume of 0.4 mL and an incubation time of 20 min. The reaction was quenched with 4 μL of β-mercaptoethanol and then applied to a 0.9 × 33 cm column of Sephadex G-15 equilibrated and developed in 5% (v/v) acetic acid. The iodinated peptide fraction was lyophilized and stored in 0.5% (v/v) acetic acid. The peptide had a specific radioactivity between 1 and 10 Ci/mmol and comigrated on NaDodSO$_4$–PAGE, in the presence of urea, with standard peptide.

Peptide/Calmodulin Cross-Linking. β-Endorphin peptide 14–31 and calmodulin were cross-linked at room temperature using BS^3 in a 50 mM Hepes buffer, pH 7.5, containing 0.2 M NaCl and 1–10 mM $CaCl_2$ (Giedroc et al., 1983b). The reaction was quenched by the addition of 0.1 volume of 50 mM ethanolamine in 50 mM Hepes, pH 7.5. A 1 mM chlorpromazine stock was prepared in 50 mM Hepes, pH 7.5, and used for cross-linking inhibition studies.

CAPP–Sepharose Chromatography. A preparative cross-linking protocol which allowed appreciable, but submaximal, 2 mol of peptide/mol of protein species to form contained peptide, protein, and cross-linking reagent at respective concentrations of 0.30, 0.12, and 0.25 mM in a 5.0-mL volume. A trace (10^7–10^8 cpm) of ^{125}I-β-endorphin peptide 14–31 was added, and the unbound radiolabeled peptide was subsequently removed by chromatography on a Sephadex G-75 column equilibrated and developed with 0.1 M ammonium bicarbonate. The calmodulin-containing fraction following lyophilization was dissolved in 4.0 mL of 10 mM Tris-HCl, pH 7.5, containing 2 mM $CaCl_2$, and 1-mL aliquots were applied to a 0.7 × 9.5 cm CAPP–Sepharose column (capacity of ca. 4.8 mg of calmodulin) equilibrated and developed with the same Ca^{2+}-containing buffer at a flow rate of 20 mL/h at 22 °C. As indicated in the text and figure legends, NaCl and EGTA were added to the developing buffer. Fractions of 1 mL were collected and monitored both for absorbance at 276 nm and for radioactivity. Aliquots of individual fractions were subjected to NaDodSO$_4$–PAGE [15% gels as described by Laemmli (1970)], and the gels were stained with Coomassie blue and then destained by using standard methods. Between individuals runs, the column was washed with 6 volumes each of 1 M NaCl and 6 M guanidinium chloride containing buffers. Recoveries from all runs were 85 ± 10%.

Purification of [^{14}C]-β-Endorphin 13–31/Calmodulin Cross-Linked Complexes. A preparative cross-linking protocol, modeled after the procedure above, was carried out in a total volume of 15 mL. After removal of free ^{14}C-peptide, a portion of the calmodulin-containing fraction was dissolved in 2 mL of Tris/Ca^{2+} buffer and applied directly to a 0.9 × 20 cm CAPP–Sepharose column equilibrated as above. After elution of the void or weakly bound components, a 0.5 M NaCl wash, followed by subsequent elution with EGTA, was performed. Fractions of 1 mL were collected and monitored at 277 nm and for ^{14}C content. Aliquots of pooled fractions were subjected to NaDodSO$_4$–PAGE as above. Homogeneous 1:1 and 2:1 β-endorphin 13–31/calmodulin complexes obtained from this column, developed as described in the text, were used for subsequent experiments. CAPP–Sepharose reapplication experiments were carried out under identical chromatographic conditions.

Chlorpromazine Binding by Calmodulin and β-Endorphin 13–31/Calmodulin Complexes. The gel permeation binding technique of Hummel & Dreyer (1962) was used to assess chlorpromazine binding properties of peptide/calmodulin complexes. A Bio-Gel P-10 (Bio-Rad) column, packed into a 4.6 × 250 mm HPLC column support, was equilibrated at constant pressure and 0.1 mL/min flow rate with a Perkin-Elmer Series 10 liquid chromatograph pump in 50 mM Hepes, pH 7.5, 0.2 M NaCl, and 1 mM $CaCl_2$ or 2 mM EGTA containing various concentrations of chlorpromazine. The column effluent was directed to a Perkin-Elmer LC 75 UV–vis detector, and absorbance was monitored at 305 nm. After exhaustive equilibration of the column, lyophilized samples (typically 1.5–2 nmol) of calmodulin and 1:1 or 2:1 β-endorphin 13–31/calmodulin complexes were dissolved in the column buffer containing chlorpromazine and injected (20 μL) onto the column. The amount of protein injected onto the column was quantitated by amino acid analysis as described below; values were normalized to 11, 13, and 15 nmol of Ala per nmol of calmodulin, 1:1 complex, and 2:1 complex, respectively. Quantitation with other amino acids gave comparable results. Bound chlorpromazine was quantitated by cutting and weighing the trough and comparing it with known quantities of chlorpromazine chromatographed on the column alone.

Amino Acid Analysis. Peptides and proteins were hydrolyzed in 6N HCl with phenol present at 110–115 °C for 24 or 72 h in evacuated tubes. Compositions were determined with an automated postcolumn *o*-phthalaldehyde derivatization package supplied with a Perkin-Elmer amino acid analyzer module. Proline is not detected under these conditions.

Results

Inhibition of Calmodulin-Stimulated Activity of Purified Phosphodiesterase. The cyclic nucleotide phosphodiesterase preparation was essentially free of contaminating protein bands as judged by NaDodSO$_4$–PAGE and migrated with an apparent subunit molecular weight of 61 000 [data not shown; cf. Hansen & Beavo (1982)]. The effects of β-endorphin and selected amino-terminal and carboxy-terminal deletion peptides on the Ca^{2+}/calmodulin-dependent phosphodiesterase activity are shown in Figure 1. As previously shown for crude enzyme preparations, the dodecapeptide corresponding to β-endorphin residues 14–25 represented the minimal inhibitory sequence under these conditions; removal of two addition carboxy-terminal residues (resulting in the decapeptide defined by β-endorphin residues 14–23) abolished the inhibition under these conditions. It is also interesting to note that the amino-terminal 13 residues, ending with Pro-13 and containing the opiate portion of β-endorphin (i.e., residues 1–5), appear to contribute little to the peptide-mediated inhibition since peptide 14–31 is nearly as effective as β-endorphin. Thus, in this purified ternary macromolecular system constiting of Ca^{2+}/calmodulin, phosphodiesterase, and peptide, the rank order of potency of inhibition as determined by the ED_{50} was found to be β-endorphin 1–31 > 14–31 > 14–27 > 14–25, while peptides 1–17 and 14–23 and the basic peptide 20–31 were not effective inhibitors. Interestingly, β-endorphin appears to increase the basal activity of phosphodiesterase slightly in a peptide concentration dependent manner.

Correlation of Inhibitory Potency with Efficiency of Cross-Linking to Calmodulin. Solution complexes of β-endorphin and calmodulin were previously demonstrated by cross-linking the two components with BS^3 in the presence of exogenous Ca^{2+} (Giedroc et al., 1983b). We have utilized this cross-linking reaction between calmodulin and β-endorphin-derived peptides to estimate the extent of binding between the two species. This approach offers at least an approximation of binding efficacy as all of the peptides contain potentially reactive nucleophilic residues (i.e., lysine). The time course of cross-linking various peptides to calmodulin is shown in

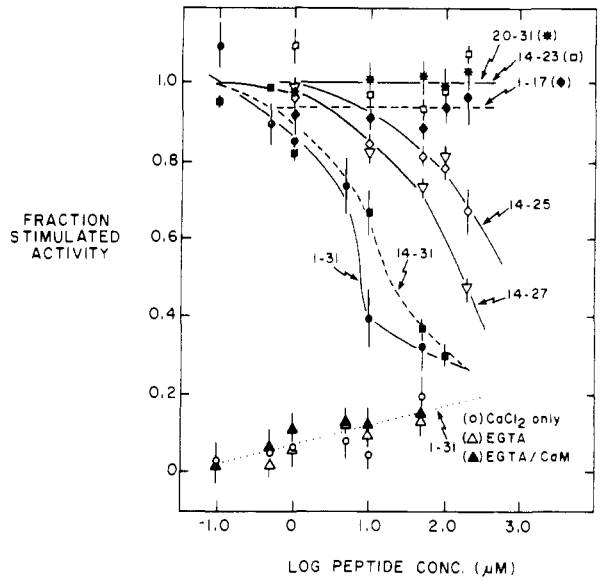

FIGURE 1: Inhibition of calmodulin-stimulated phosphodiesterase activity by β-endorphin and deletion peptides. The results, presented as mean ± SEM from triplicate determinations, have been normalized to a scale of 0–1.0. Here, zero represents the basal enzymic activity (14 nmol min^{-1} μg^{-1}), and 1.0 denotes the activity in the presence of 30 nM calmodulin plus 0.1 mM Ca^{2+} (66 nmol min^{-1} μg^{-1}; data point not shown). This 4.5–5-fold activation was standard under the conditions used where the concentration of calmodulin was such that peptide inhibition could be readily monitored [cf. Puett et al. (1983)]. Basal activities were determined in the presence of 0.1 mM $CaCl_2$ but no calmodulin (○), in 1 mM EGTA (△), and in 1 mM EGTA plus 30 nM calmodulin (▲). Stimulated activities were measured in the presence of 30 nm calmodulin plus 0.1 mM Ca^{2+}. The effects of β-endorphin 1–31 on basal activity (○, △, ▲) and stimulated activity (●) are shown. Also, the effects of various β-endorphin deletion peptides on the stimulated activity are presented. With the exception of β-endorphin peptide 14–31, all peptide sequences are based on that of porcine β-endorphin [cf. Li (1978)]: NH_2-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Val-Lys-Asn-Ala-His-Lys-Lys-Gly-Gln-COOH. β-Endorphin 14–31 is based on the human sequence which differs from the porcine sequence in three positions (Li, 1978): Ile^{23}, Tyr^{27}, and Glu^{31}.

Figure 2. The cross-linking efficiency, as monitored by complex formation, yields the following rank order: β-endorphin 1–31 > 14–31 > 14–27 > 14–25; peptides 1–17 and 20–31 do not form detectable complexes with calmodulin. Note that this trend in cross-linking efficiency followed that observed in Figure 1 for inhibitory potency. Thus, an effective inhibitor exhibited good cross-linking efficacy to calmodulin while an ineffective inhibitor appeared to show little, if any, association with the protein.

Inhibition of β-Endorphin 14–31/Calmodulin Cross-Linking by Chlorpromazine. The data presented in Figure 3 demonstrate that chlorpromazine dramatically inhibits the formation of both the 1:1 and 2:1 β-endorphin 14–31/calmodulin complexes in a concentration-dependent manner. Cross-linking conditions were chosen to allow formation of the 2:1 complex (Figure 3A). At 120 μM chlorpromazine, corresponding to a 10- and 4-fold molar excess of chlorpromazine over calmodulin and peptide, respectively, the extent of cross-linked products was significantly reduced. At 240 μM chlorpromazine, the yield was essentially nil. In another experiment, identical molar ratios of reactants were incubated, but concentrations of all components were reduced by half (Figure 3B). Significant inhibition occurred in the range of 42–60 μM drug, corresponding, as above, to 7–10-fold molar excesses of chlorpromazine over calmodulin. Similar trends were observed in each case as shown in Figure 3C where the

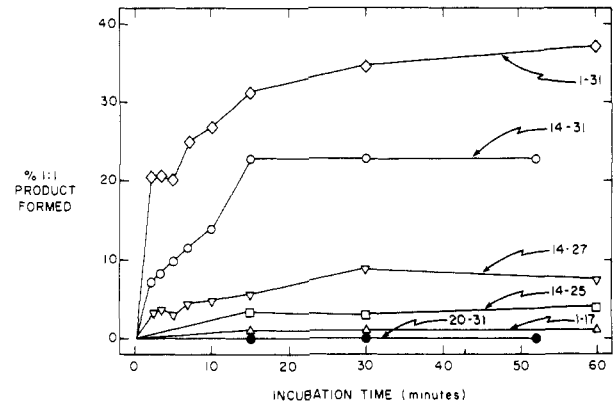

FIGURE 2: Time course of cross-linking β-endorphin and deletion peptides to calmodulin. Each peptide, calmodulin, and BS^3 were present at 24, 24, and 100 μM, respectively, in the Hepes buffer described under Materials and Methods. At the indicated times, the reactions were terminated, and the samples were processed and then analyzed by $NaDodSO_4$–PAGE. The amounts of complex (1 mol of peptide/mol of calmodulin) and calmodulin were quantitatively determined by scanning gel denistometry and expressed as the percent of 1:1 complex formed (i.e., percent of total, 1:1 plus calmodulin). Under these conditions, no complex containing 2 mol of peptide/mol of calmodulin was observed.

percent inhibition of formation of the 1:1 complex is plotted as a function of the molar ratio of drug over that of calmodulin and peptide. The curves essentially overlap. Furthermore, when reactions were carried out at an identical calmodulin concentration (6 μM) but at 30 μM peptide, approximately twice as much chlorpromazine was required to effect the same amount of inhibition observed at 15 μM peptide (i.e., conditions of Figure 3B), strongly suggesting a competitive phenomenon (data not shown). Control experiments with rabbit muscle aldolase [cf. Staros (1982)] showed that the cross-linking reagent functioned in an indistinguishable fashion with or without the phenothiazine present under comparable conditions, thus ruling out micelle partitioning or nucleophilic scavenging of BS^3 by chlorpromazine (data not shown).

CAPP–Sepharose Chromatography of Peptide/Calmodulin Complexes. Given the above result, it was of interest to assess the ability of covalent 1:1 and 2:1 β-endorphin 14–31/calmodulin complexes to interact with immobilized chlorpromazine (CAPP–Sepharose) in the presence of Ca^{2+}. This drug matrix binds calmodulin and structurally related Ca^{2+}-binding proteins in the presence of Ca^{2+}; chelators such as EGTA promote release of the bound proteins (Marshak et al., 1981). Initial experiments showed that when a mixture of calmodulin and 1:1 ^{125}I-peptide/calmodulin complex was applied to a small CAPP–Sepharose column and subsequently eluted with EGTA, greater than 80% of the ^{125}I applied to the column was retained by the drug matrix with Ca^{2+} present, but was displaced with EGTA (data not shown). However, 0.5 M NaCl, without chelator present, caused the elution of an appreciable amount of this bound iodinated 1:1 complex, conditions under which free calmodulin remained bound (data not shown). It was found that ^{125}I-β-endorphin 14–31 possessed no affinity for the drug matrix (data not shown).

In another cross-linking protocol (cf. Materials and Methods), appreciable formation of the 2:1 complex was allowed to occur. After removal of free ^{125}I-β-endorphin 14–31, this preparation was applied to the CAPP–Sepharose column in the presence of Ca^{2+} and subsequently eluted with EGTA (Figure 4A). Note that all of the 2:1 complex applied to the column eluted in the void volume (peak I); in contrast, 50% of the 1:1 complex was retained by the drug matrix with Ca^{2+} present and was eluted upon the addition of EGTA. Once

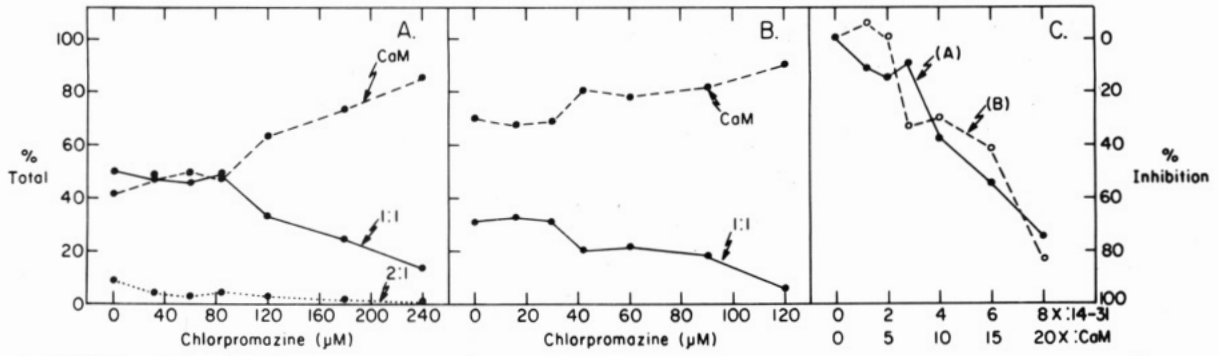

FIGURE 3: Inhibition of β-endorphin 14–31 cross-linking to calmodulin by chlorpromazine. (A) Peptide, calmodulin, and BS^3 were present at 30, 12, and 100 μM, respectively. Chlorpromazine was included in the incubations at the indicated concentrations. The reaction was terminated, and the mixture was processed as described under Materials and Methods. Following $NaDodSO_4$–PAGE and staining with Coomassie blue, the amounts of 2:1 complex (2:1), 1:1 complex (1:1), and free calmodulin (CaM) were quantified by scanning gel densitometry and normalized to 100%; the percent contribution of each species is given at each chlorpromazine concentration. (B) An identical experiment was conducted as above, but the peptide, protein, and cross-linking reagent were present at 15, 6, and 50 μM, respectively. The chlorpromazine concentrations were also reduced by half to yield identical ratios of drug to peptide and calmodulin as in panel A. (C) The percent inhibition of formation of the 1:1 complex obtained under the conditions described in panels A (●) and B (○) is presented as a function of the molar ratios of reactants.

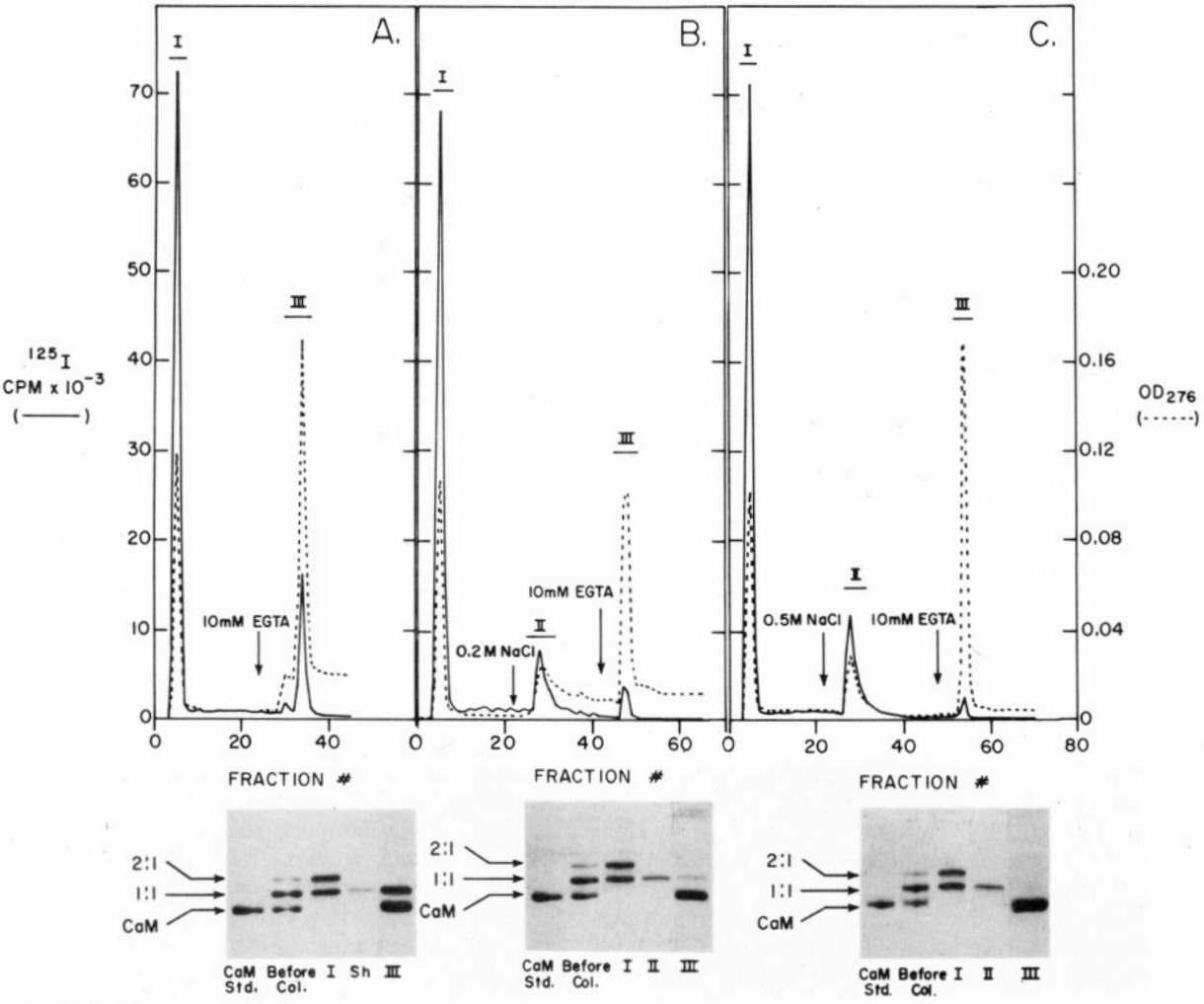

FIGURE 4: CAPP–Sepharose affinity chromatography of β-endorphin peptide 14–31/calmodulin complexes. Cross-linking was based on a protocol which yields formation of 1:1 and 2:1 peptide/protein complexes. (A) The mixture of complexes and calmodulin was added to a CAPP–Sepharose column. At the position indicated by the arrow, 10 mM EGTA was added to the developing buffer. The major peaks were pooled as indicated, and $NaDodSO_4$–PAGE profiles are shown for the calmodulin standard (CaM Std.), the mixture before the column (Before Col.), peak I (I), the small shoulder preceding peak III (Sh), and peak III (III). (B) Same as in panel A except that 0.2 M NaCl was added to the developing buffer as indicated; 10 mM EGTA was then added at the indicated position. $NaDodSO_4$–PAGE profiles are shown. (C) Same as panel B except that 0.5 M NaCl was used instead of 0.2 M NaCl; the $NaDodSO_4$–PAGE profiles are presented.

again, however, if the column was washed with 0.2 M NaCl (Figure 4B) or 0.5 M NaCl (Figure 4C) prior to elution with EGTA, nearly all of the iodinated 1:1 complex was removed from the column.

To show that this binding of the 1:1 complex is nonrandom, the EGTA-eluted material (i.e., peak III in Figure 4A) was reapplied to the column, resulting in 80% of the iodinated 1:1 complex again being bound by the column (data not shown). In addition, when aother preparation of ^{125}I-labeled 1:1 complex, previously shown to bind to CAPP–Sepharose, is reap-

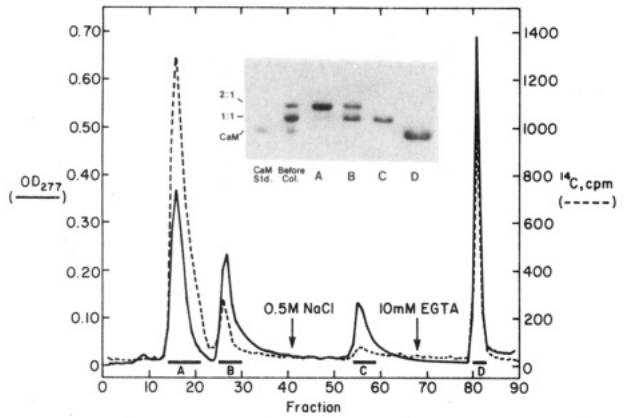

FIGURE 5: Preparative CAPP–Sepharose chromatography of [^{14}C]-β-endorphin 13–31/calmodulin complexes. Both 1:1 and 2:1 peptide/calmodulin complexes and free calmodulin were obtained and applied to a CAPP–Sepharose column and chromatographed as outlined under Materials and Methods. 0.5 M NaCl and 10 mM EGTA were added to the developing buffer at the indicated positions. ^{14}C content (---) and absorbance at 277 nm (—) are shown. Fractions A–D were pooled, and protein composition was analyzed by NaDodSO$_4$–PAGE as shown in the inset. Lane designations are as follows: calmodulin standard (CaM Std.); mixture of complexes before chromatography (Before Col.); peak A (A); peak B (B); peak C (C); and peak D (D).

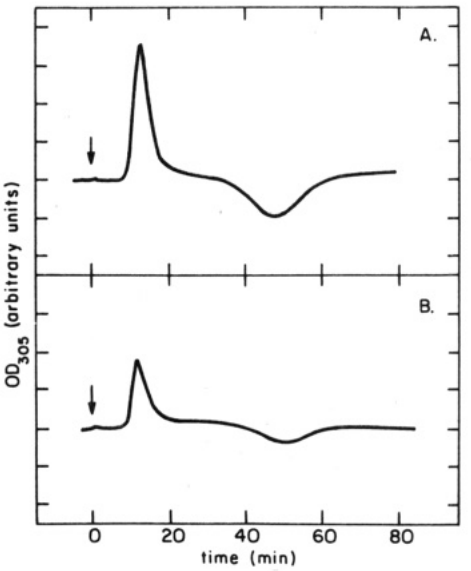

FIGURE 6: Binding of chlorpromazine by calmodulin and the 1:1 β-endorphin 13–31/calmodulin complex. A Bio-Gel P-10 column was equilibrated as described under Materials and Methods with 20 μM chlorpromazine. (A) 1.1 nmol of calmodulin, dissolved in equilibration buffer containing chlorpromazine, was injected into the column at the position designated by the arrow. The effluent was monitored at 305 nm. (B) Same as in panel A except that 1.7 nmol of 1:1 peptide/calmodulin complex was applied to the column.

plied to the column, essentially 100% of this complex was reabsorbed. More importantly, this bound 1:1 complex was quantitatively displaced from the drug matrix by free calmodulin (data not shown). In contrast, reapplication of a mixture of unretained 1:1 and 2:1 complexes lacking free calmodulin (cf. peak I, Figure 4A) resulted in the 1:1 complex being largely absorbed with a portion being eluted with 0.5 M NaCl and the remainder with chelator. On the other hand, the majority of the 2:1 complex again passed through the column with the rest elutable with a high ionic strength wash. Thus, the ability of 1:1 and 2:1 peptide/calmodulin complexes to interact with CAPP–Sepharose appears markedly influenced by whether or not calmodulin is present in the mixture of components, i.e., the overall capacity of available interaction sites. It is presumed, then, that there is no fundamental difference between interacting and noninteracting populations of 1:1 complex from the CAPP–Sepharose column.

Regardless of these uncertainties, as an initial means of resolving similar amounts of various peptide/calmodulin complexes from one another and from free calmodulin, the utilization of CAPP–Sepharose chromatography under the conditions established suggested that this could serve as a suitable means for purifying these complexes. Here, [^{14}C]-β-endorphin 13–31 was used in place of ^{125}I-β-endorphin 14–31 to monitor the fate of covalent peptide/calmodulin complexes through various manipulations. A mixture of cross-linked ^{14}C-peptide/calmodulin products, after removal of free ^{14}C-peptide, was chromatographed on a semipreparative CAPP–Sepharose column under identical conditions as in Figure 4C (Figure 5). NaDodSO$_4$–PAGE analysis of each of the four peaks is shown in the inset of the chromatogram. Note that the void fraction (A) contained a significant amount of the 2:1 complex; a slightly retained fraction (B) contained the remainder of the 2:1 product as well as a major portion of the 1:1 complex. Addition of 0.5 M NaCl gave another fraction (C) which is composed of a homogeneous 1:1 complex while subsequent elution with EGTA gave calmodulin (D) with only a trace of the 1:1 complex.

To remove residual 1:1 complex from fraction A, this pool, after exhaustive dialysis and lyophilization, was reapplied to the same column, and the void fraction was collected. This fraction represents highly purified 2:1 [^{14}C]-β-endorphin 13–31/calmodulin complex (Figure 7A). Homogeneous 1:1 [^{14}C]-β-endorphin 13–31/calmodulin complex was obtained directly from the preparative CAPP–Sepharose column as fraction C (Figure 7A).

Chlorpromazine Binding and Phosphodiesterase Activation by β-Endorphin 13–31/Calmodulin Complexes. To further probe the presence of overlapping chlorpromazine and β-endorphin-derived peptide binding domains on calmodulin, equilibrium binding studies with chlorpromazine, using the method of Hummel & Dreyer (1962), were conducted with calmodulin and 1:1 and 2:1 β-endorphin 13–31/calmodulin complexes purified as described above.

Typical chromatograms obtained upon loading calmodulin (Figure 6A) or 1:1 complex (Figure 6B) onto the column, equilibrated in 20 μM chlorpromazine and 1 mM Ca^{2+}, are shown. Quantitation of the trough provides a determination of the nanomoles of chlorpromazine bound. Data were collected at ligand concentrations ranging from 5 to 100 μM in the presence of Ca^{2+} or EGTA for both calmodulin and the 1:1 peptide/calmodulin complex and are summarized in Figure 7B where the binding ratio is plotted as a function of the free chlorpromazine concentration on a logarithmic scale. The presence of a minor UV-absorbing contaminant in the 2:1 complex preparation precluded a thorough analysis of these data; however, the area of the trough was unobstructed, and the binding ratios estimated were 0, 0.19, and 1.48 at chlorpromazine concentrations of 10, 20, and 100 μM, respectively (i.e., similar to the values determined for calmodulin and the 1:1 complex in the presence of EGTA). Figure 7B (inset) shows a Scatchard (1949) plot of the Ca^{2+}-dependent binding of chlorpromazine by calmodulin and the 1:1 peptide/calmodulin complex.

Finally, it was of interest to compare the ability of these peptide/calmodulin complexes to activate and/or inhibit phosphodiesterase with their chlorpromazine binding capacity. Figure 8A illustrates that the 1:1 complex is greatly reduced in its ability to activate phosphodiesterase; in fact, the apparent

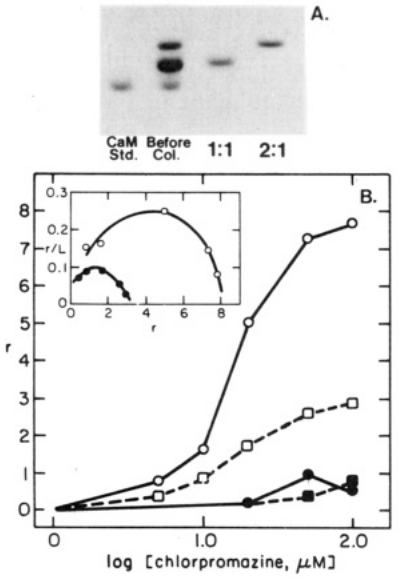

FIGURE 7: Summary of chlorpromazine binding by calmodulin and the 1:1 β-endorphin 13–31/calmodulin complex in the presence and absence of exogenous Ca^{2+}. (A) $NaDodSO_4$–PAGE analysis of homogeneous 1:1 and 2:1 β-endorphin 13–31/calmodulin complexes used in the present experiment as well as those detailed in Figure 8. Designations are as follows: calmodulin standard (CaM Std.); mixture of products before preparative CAPP–Sepharose chromatography (Before Col.); homogeneous 1:1 complex (1:1); and homogeneous 2:1 complex (2:1). (B) Reduced data collected as in Figure 6 and quantitated as described under Materials and Methods are presented as the binding ratio (r = moles of chlorpromazine bound per mole of protein) as a function of log chlorpromazine concentration for calmodulin plus $CaCl_2$ (○), calmodulin plus EGTA (●), 1:1 complex plus $CaCl_2$ (□), and 1:1 complex plus EGTA (■). (Inset) Scatchard (1949) plot of the data in panel A from calmodulin plus $CaCl_2$ (○) and from 1:1 complex plus $CaCl_2$ (●) presented as r/L [L = equilibrating (free) peptide concentration] vs. r.

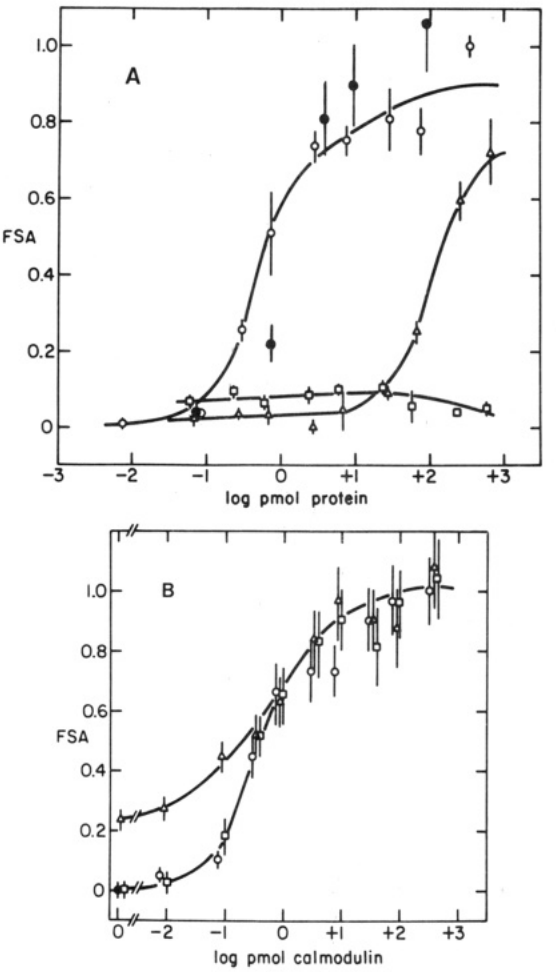

FIGURE 8: Determination of the ability of homogeneous 1:1 and 2:1 β-endorphin 13–31/calmodulin complexes to activate and inhibit phosphodiesterase. The assay of purified phosphodiesterase was carried out as described under Materials and Methods. As in Figure 1, the data are normalized to a scale of 0–1.0 where 0 represents basal activity with 0.1 mM $CaCl_2$ while 1.0 represents maximally stimulated activity with 1.38 μM calmodulin and 0.1 mM $CaCl_2$. (A) Activation of phosphodiesterase by calmodulin (○), calmodulin exposed to cross-linker but with no peptide bound (●), 1:1 complex (△), and 2:1 complex (□). The concentrations are based on molar units using molecular weights of 16 680, 18 810, and 20 940 for calmodulin and 1:1 and 2:1 complexes, respectively. (B) Activation by calmodulin (○), calmodulin plus 0.3 μM 1:1 complex (△), and calmodulin plus 0.3 μM 2:1 complex (□). The concentration of calmodulin which results in 50% maximal stimulation is approximately 1.6 nM. Basal enzymic activity in the presence of 0.1 mM $CaCl_2$ is also indicated (●).

potency of activation found with this species can be explained by a level of calmodulin contamination of less than 0.5%, which certainly cannot be precluded. The 2:1 peptide/calmodulin complex also shows negligible ability to activate the enzyme relative to calmodulin. Note that free calmodulin isolated from the cross-linking mixture is indistinguishable from untreated calmodulin in its ability to activate phosphodiesterase; thus, inactivation of the calmodulin molecule is directly attributed to the covalent attachment of the peptide. Figure 8B shows that neither covalent peptide/calmodulin complex is capable of inhibiting the calmodulin-dependent activation of phosphodiesterase.

Discussion

The data presented herein attempt to address the functional significance of β-endorphin interaction domain(s) on calmodulin in terms of phenothiazine binding and phosphodiesterase recognition and/or activation. Initial experiments demonstrated that the Ca^{2+}-dependent calmodulin activation of purified phosphodiesterase is inhibited in a dose-dependent manner by β-endorphin with an ED_{50} largely constitent with results previously obtained by using crude enzyme preparations (Giedroc et al., 1983c). These studies using purified components were essential to eliminate any possible contribution of another component, e.g., a contaminant in the crude enzyme preparation, in describing the inhibition results. Further, we have shown that peptide 14–25 corresponded to the minimal inhibitory sequence under the conditions employed. It has been suggested that this region of β-endorphin fully encompasses the domain predicted to form an amphipathic α-helix based both on CD studies in helix-promoting solvents and on studies with model synthetic peptides correlating β-endorphin bioactivity with this proposed secondary structure (Yang et al., 1977; Wu et al., 1979; Hammonds et al., 1982; Taylor et al., 1982, 1983). Interestingly, calmodulin/peptide mixtures are characterized by induced α-helical structures concomitant with complex formation (Giedroc et al., 1983c; Puett et al., 1983). Furthermore, other inhibitory peptides of substantial affinity in interacting with calmodulin are known or predicted amphipathic α-helices, including mastoparan (Malencik & Anderson, 1984) and melittin (Maulet & Cox, 1983). Thus, a similar structural unit may occur in the calmodulin binding domain on calmodulin-regulated enzymes.

The positive correlation that was found between the binding of a particular β-endorphin-derived peptide to calmodulin, as estimated by the efficacy of cross-linking the two species, and the inhibitory potency of that peptide strongly suggests that the observed inhibition results from the formation of a pep-

tide/calmodulin complex. Indeed, β-endorphin peptides 20–31 and 1–17, which are ineffective inhibitors, showed only weak, if any, apparent association with calmodulin in solution. Moreover, inhibitory peptides exhibited only minor effects on basal enzymic activity (Giedroc et al., 1983c). Consistent with this suggestion is the finding that the homogeneous 1:1 β-endorphin 13–31/calmodulin complex, as well as the 2:1 complex, exhibited negligible ability to activate phosphodiesterase. Furthermore, neither of these covalent peptide/calmodulin complexes was able to inhibit the calmodulin-dependent activation of phosphodiesterase at concentrations 2–3 orders of magnitude greater than that of calmodulin. Thus, the covalent attachment of β-endorphin and derived peptides to calmodulin would appear to prevent the activation of phosphodiesterase by rendering calmodulin unable to interact with the enzyme. These findings are in apparent contrast to results reported for another covalent calmodulin adduct, CAPP/calmodulin, by Newton et al. (1983). They found that the phenothiazine/calmodulin adduct was unable to activate phosphodiesterase but was an inhibitor of the calmodulin-dependent activation of the enzyme. It was reasoned that CAPP/calmodulin still possessed a functional enzyme interaction domain while attachment of the phenothiazine perturbed only the activation ability of calmodulin. Our results suggest that attachment of one β-endorphin-derived peptide to calmodulin seems to prevent the interaction of the complex with phosphodiesterase.

We have previously reported that trifluoperazine greatly reduced the degree of cross-linking between intact β-endorphin and calmodulin (Giedroc et al., 1983b). Herein, we show that a similar phenomenon occurred with another phenothiazine, chlorpromazine, and the amino-terminal deletion peptide β-endorphin 14–31. Given this result, it was of interest to probe the peptide binding sites on calmodulin by assessing the ability of both the 1:1 and the 2:1 β-endorphin peptide 14–31/calmodulin complexes to associate with immobilized chlorpromazine in the presence of Ca^{2+}. We have previously reported that the binding of β-endorphin does not appear to grossly alter the Ca^{2+}-saturated conformation of calmodulin (Giedroc et al., 1983c); in addition, immobilized chlorpromazine does not interact with the peptides. The data presented herein demonstrate that a significant population of the species containing 1 mol of peptide/mol of calmodulin interacted in a Ca^{2+}-dependent fashion with the immobilized drug, albeit with reduced affinity relative to calmodulin, in a Ca^{2+}-dependent and calmodulin-displaceable manner. In contrast, 100% of the complex containing 2 mol of peptide/mol of calmodulin was not retained by the matrix. Although reapplication experiments revealed that the observed fractionation was influenced by the presence of free calmodulin, the data indicated that 1:1 and 2:1 complexes, relative to each other and to calmodulin, possessed qualitatively different chlorpromazine binding properties. Thus, an initial suggestion is that peptide binding and phenothiazine binding to calmodulin are influenced by one another. These suggestions were considerably strengthened by analyzing the chlorpromazine binding properties under equilibrium conditions of β-endorphin peptide/calmodulin complexes. As can be clearly seen, the covalent attachment of one β-endorphin 13–31 molecule significantly affects the Ca^{2+}-dependent phenothiazine binding at all concentrations tested. Furthermore, the maximum binding was reduced by ca. 65% from $n = 8$ with calmodulin to $n = 3$ with the 1:1 complex. The 2:1 complex expressed very little capacity to bind the drug. Interestingly, the distinction between specific "high-affinity" and nonspecific "low-affinity" chlorpromazine binding sites on calmodulin, as originally reported (Levin & Weiss, 1979), is not readily recognizable in the present data. Indeed, at relatively modest concentrations of chlorpromazine, e.g., 20 μM, 1 mol of calmodulin is able to bind perhaps as many as 5 mol of the drug; a level of 7–8 mol of drug/mol of calmodulin seems to represent a maximum value. The binding of one to two drug molecules only occurs in the range of 5–10 μM, and this binding is clearly not saturated at these concentrations. A qualitatively similar chlorpromazine/calmodulin binding curve was reported by Lukas et al. (1983).

The Scatchard plot for chlorpromazine association with calmodulin suggests positive cooperativity. Interestingly, the binding of chlorpromazine to the 1:1 complex, although significantly reduced in the maximum binding ratio achieved, also appears to bind in a positively cooperative fashion among the remaining sites. When fitted to a Hill plot, Hill coefficients of 1.9 and 1.7 for the calmodulin ($n = 8$) and 1:1 ($n = 3$) curves, respectively, were obtained. Thus, the covalent attachment of 1 mol of peptide/mol of calmodulin significantly perturbs the amount of chlorpromazine binding, and, if high- and low-affinity sites actually exist, these sites, while overlapping with peptide binding sites, are still apparently interacting with one another to an appreciable extent.

Alternatively, chlorpromazine may self-associate to form a complex that may interact with both calmodulin and, to a lesser extent, the 1:1 complex under the present conditions of ionic strength and pH. This is plausible, given a recent report by Cann et al. (1981) of a critical micelle concentration (CMC) for chlorpromazine of 200 μM in 1 M sucrose and 125 mM NaCl, pH 6.8. They found that the positively cooperative binding of eight to nine chlorpromazine molecules to brain tubulin previously observed (Hinman & Cann, 1976) could be explained by the interaction of a chlorpromazine micelle with a single site on tubulin. Even though concentrations less than 200 μM were used in the present study, calmodulin may provide a nucleation site which, after binding one or two phenothiazines, promotes the formation of a protein-bound "micelle-like" particle at concentrations far less than the CMC. Nonetheless, whatever the mechanism of chlorpromazine binding to calmodulin, covalent attachment of β-endorphin 13–31 to calmodulin significantly affects this equilibrium.

It is intriguing that the 1:1 peptide/calmodulin complex, although significantly reduced in its ability to bind chlorpromazine, still possesses available drug interaction sites. Indeed, two covalently bound peptide molecules are sufficient to essentially prohibit the binding of the phenothiazine. However, the 1:1 and 2:1 peptide/calmodulin complexes appear unable to interact with and activate phosphodiesterase; thus, all available chlorpromazine binding sites need not be occupied for perturbation of the stimulated enzymic activity. Functionally then, the 1:1 and 2:1 complexes are distinguished by their phenothiazine binding properties, but not in terms of phosphodiesterase activation properties. More importantly, the present study, while illustrating the commonality of phenothiazine and inhibitory peptide interaction domains on calmodulin, provides compelling evidence for the direct involvement of peptide binding domain(s) on calmodulin in its modulatory effect on activatable phosphodiesterase.

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phosphodiesterase assays and Dr. Arthur E. Jackson for helpful discussions.

Registry No. Porcine β-endorphin, 60149-45-3; cAMP phosphodiesterase, 9040-59-9; chlorpromazine, 50-53-3; human β-endorphin (14–31), 77761-24-1; porcine β-endorphin (1–17), 60893-02-9; porcine β-endorphin (14–23), 84741-72-0; porcine β-endorphin (14–25), 87281-35-4; porcine β-endorphin (14–27), 87281-36-5; porcine β-endorphin (20–31), 81539-48-2.

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