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Binding of a Synthetic β-Endorphin Peptide to Calmodulin

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SUMMARY

The 31-residue neuropeptide, β-endorphin, inhibits the calmodulin-dependent activity of activatable cyclic nucleotide phosphodiesterase. We have shown that the amino terminal portion of the peptide, which includes the sequence conferring opiate activity, is not required for inhibitory potency and, furthermore, that solution complexes of the peptides and calmodulin render calmodulin functionally inactive in terms of cyclic nucleotide phosphodiesterase activation. An amino terminal deletion peptide of human β-endorphin (β-endorphin 13-31), synthesized using solid phase methods, was shown to interact with calmodulin by cross-linking with bis(sulfosuccinimidyl)suberate and by a gel permeation chromatographic technique. Results from the latter approach, using peptide concentrations of 2–100 μM, demonstrated Ca^{2+}-dependent equilibrium binding with an apparent stoichiometry of approximately 4 mol of peptide/mol of calmodulin and half-maximal binding at 15–20 μM.

INTRODUCTION

Calmodulin is an acidic, low molecular weight Ca^{2+}-binding protein of highly conserved primary structure (1) and known crystallographic structure (2). In view of the diverse group of enzyme activities this protein can modulate *in vitro* in a Ca^{2+}-dependent manner, calmodulin can be regarded as the prototypical member of a class of structurally homologous Ca^{2+}-binding proteins which performs an integral role in transmitting the effects of Ca^{2+} influx on cellular events (for review see Ref. 3). Detailed spectroscopic studies on calmodulin have provided information on the Ca^{2+}-dependent conformational transition that occurs in the protein (3, 4), but the molecular details by which calmodium interacts with enzymes, with subsequent modulation of their activities, have met with relatively slow progress. We (5–7) and others (8–10) have reasoned that structural investigation of simple substances that inhibit calmodulin-dependent events may provide clues concerning the structural requisites of calmodulin-enzyme interactions.

Levin and Weiss (11) initially demonstrated that the phenothiazines, as well as other pharmacologic agents, are inhibitors of the calmodulin-dependent cyclic nucleotide phosphodiesterase activity, presumably by binding to calmodulin at functionally important domains. Weiss and co-workers (8) also reported that a number of small, basic polypeptides, including β-endorphin, dynorphin, and glucagon, could mimic the inhibitory activity of the phenothiazines. These various compounds exhibit K_d and ED_{50} values on the order of micromolar concentrations. Giedroc *et al.* (6) and Malencik and Anderson (9, 12) have assessed general structural characteristics important in the interaction of these and other peptide hormones with calmodulin. Finally, Maulet and Cox (10) have studied the interaction of melittin, a basic peptide derived from bee venom, with calmodulin. Interestingly, this latter peptide and another peptide, mastoparan, exhibit K_d values on the order of nanomolar concentrations for calmodulin (13, 14), while both are potent inhibitors of calmodulin-dependent phosphodiesterase activity (15). Thus, peptide-calmodulin interactions may represent a useful model system which might provide meaningful information on the functional structural requirements associated with the modulatory activity of calmodulin.

Two reports have suggested that intact β-endorphin and calmodulin associate in a Ca^{2+}-dependent fashion with a stoichiometry of 1 mol of peptide/mol of calmodulin and a K_d of 2–4 μM (9, 16). In contrast, we have performed experiments which demonstrated the formation of both 1:1 and 2:1 peptide/protein products as assessed by covalent cross-linking between peptide and protein lysyl residues (5). To address the apparent discrepancy in maximum binding ratio achieved between the two components, it was of interest to reexamine the equilibrium binding properties characteristic of this in-

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teraction. In the present report, we describe binding experiments between calmodulin and the synthetic amino terminal deletion peptide, β-endorphin 13-31, performed with the gel permeation chromatographic technique of Hummel and Dreyer (17).

MATERIALS AND METHODS

Chemicals and reagents. Bis(sulfosuccinimidyl)suberate and DCC[2] were purchased from Pierce Chemical Co. (Rockville, MD). BOC-protected L-amino acids were obtained from Peninsula Laboratories (Belmont, CA) and were pure by TLC ($CHCl_3$/isopropyl alcohol/acetic acid, 88:10:2). HOBt was from Aldrich (Milwaukee, WI), and sequenal grade trifluoroacetic acid was obtained from Chemical Dynamics (South Plainfield, NJ). Diisopropylethylamine was obtained from Aldrich, and pyridine was a J. T. Baker (Phillipsburg, NJ) product; both were distilled over ninhydrin (Sigma Chemical Co., St. Louis, MO) before use. Methylene choride was either reagent grade, which was distilled over sodium carbonate, or HPLC grade from Burdick and Jackson (Bodman Chemicals, Doraville, GA). HPLC grade methanol, isopropanol, and dimethylformamide were also Burdick and Jackson products, and HF was from Matheson (Morrow, GA). The solid support was *p*-methyl benzhydrylamine polystyrene (250 μmol/g) obtained from US Biochemicals (Cleveland, OH).

Synthesis, purification, and characterization of [^{14}C] β-endorphin 13-31. Two preliminary chemical syntheses were performed prior to peptide synthesis, [^{14}C]BOC-isoleucine was synthesized according to the method of Moroder *et al.* (18) from [U-^{14}C]isoleucine (Amersham Corp., Arlington Heights, IL) and di-*tert*- butyldicarbonate (Aldrich) with an overall yield of 90%; the product was recrystallized from hexane. This material comigrated with authentic BOC-isoleucine on TLC ($CHCl_3$/isopropyl alcohol/acetic acid, 88:10:2) and was incorporated in the first coupling at the ninth synthetic cycle (i.e., position 23 of β-endorphin). The rationale for incorporating ^{14}C into the synthetic peptide was to facilitate identification, via increased sensitivity, during purification and characterization.

In order to obtain a peptide with an ionizable carboxy terminus upon cleavage from the *p*-methyl benzhydrylamine resin with HF, the carboxy terminal α-carbonyl protected BOC-amino acid, 4-BOC-(glutamyl(*O*-benzyl)-oxymethyl)phenylacetic acid, was synthesized from 5 mmol of 4-bromomethylphenylacetic acid phenylacyl ester (Chemical Dynamics) and 5.5 mmol of BOC-glutamic acid γ-benzyl ester (Vega-Fox Biochemicals, Tucson, AZ) according to the method of Tam *et al.* (19). The product was recrystallized from ethyl acetate and petroleum ether with an overall yield of 50% (2.49 mmol), based on the BOC-amino acid starting material, and gave one UV-positive spot on TLC ($CHCl_3$/acetic acid, 95:5).

Peptide synthesis was performed manually in a 14 × 200 mm Pyrex culture tube modified (Ace Glass, Vineland, NJ) with a fritted bottom and a single stopcock valve for removal of solvents via aspiration and a Teflon-lined cap for introduction of solvents. Shaking was performed on a Chipco shaker (San Francisco, CA) which took the vessel through a 180° arc motion.

The coupling protocols employed followed that of Merrifield *et al.* (20) except that DCC-mediated coupling was effected *in situ* at room temperature. In the case of Asn, preformed HOBt esters were used to reduce nitrile formation (21). *p*-Methyl benzhydrylamine resin (0.83 mmol) was used as starting material. For all couplings, 3 eq (2.5 mmol) each of the protected amino acid and DCC were added for a final concentration of reactants of 0.125 M.

[2] The abbreviations used are: DCC, *N,N'*-dicyclohexylcarbodiimide; BOC, N$^{\alpha}$-*tert*-butyloxycarbonyl; EGTA, ethylene glycol-bis-(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; HOBt, 1-hydroxy-benzotriazole; HPLC, high performance liquid chromatography; HF, hydrogen fluoride; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; TLC, thin layer chromatography.

The ninhydrin monitoring of the synthesis, as well as determination of capacity, followed the method of Sarin *et al.* (22). After coupling the first amino acid, deprotection, and neutralization, the capacity of sites available for growing peptide chains was approximately 160 μmol/g, or 60% of theoretical capacity. Typical values obtained upon ninhydrin analysis after the second coupling were in the range of 0.2–0.4 μmol/g (or 0.12–0.25% unblocked sites), which is near the background values of the assay. A third coupling was employed if necessary.

To effect cleavage, the fully protected peptide-resin (0.79 g) was swollen in 30 ml of CH_2Cl_2, deprotected, and dried *in vacuo* overnight. This resin was transferred to a cleavage vessel and 1.5 ml of *p*-cresol were added. Approximately 10 ml of anhydrous HF were distilled into the vessel through a commercial HF cleavage apparatus and mixed for 60 min at 0°. The HF was then removed by water aspiration and finally under vacuum. Crude peptide was extracted from the mixture of peptide and resin with 10% acetic acid and filtration. After addition of 0.6 volumes of ethyl acetate to the peptide extract to remove the *p*-cresol, the aqueous layer was rotoevaporated to remove traces of ethyl acetate, lyophilized, and relyophilized in H_2O.

A portion of this material was then applied directly to a Whatman CM-32 cation exchange column (2.6 × 45 cm) equilibrated in 20 mM ammonium acetate, pH 6.0, and developed with a linear gradient of 20–500 mM ammonium acetate, pH 6.0, with fractions monitored for ^{14}C content. Analytical reverse phase HPLC analysis of individual fractions was performed on a Synchropak (SynChrom, Inc., Linden, IN) C-18 colunm (250 × 46 mm, 300 Å, 10 μm) equilibrated in 0.1% trifluoroacetic acid and developed with a 30-min 0–40% linear acetonitrile gradient initiated 5 min after injection. This solvent composition was held until completion of the chromatogram. For compositional determination, the purified peptide was hydrolyzed *in vacuo* for 72 hr at 110° in 6 N HCl with phenol present. Amino acid analysis was performed with an automated post-column *o*-phthalaldehyde derivatization package supplied with a Perkin-Elmer amino acid analyzer module (proline is not detected under these conditions). Automated sequence analysis of the amino-terminal-14 residues was kindly performed by Drs. S. K. Sinha and K. Brew (University of Miami, Miami, FL).

Iodination of β-endorphin 13-31. Since the ^{14}C incorporated into the peptide was of insufficient specific activity to perform binding measurements, the peptide (60 nmol) was iodinated with Na^{125}I (New England Nuclear Corp., Boston, MA) using the glucose oxidase/lactoperoxidase procedure (7). The reaction was conducted in 0.4 ml of 0.1 M ammonium acetate, pH 7.0, for 20 min at room temperature and then quenched with 4 μl of 2-mercaptoethanol. The entire reaction mixture was loaded directly onto a Sephadex G-15 (Pharmacia Fine Chemicals, Piscataway, NJ) column (1 × 20 cm) equilibrated and developed with 5% acetic acid. The iodinated peptide peak was pooled, lyophilized, and stored in 0.5% acetic acid at a specific activity of 1–10 Ci/mmol. [^{125}I]β-Endorphin 13–31 comigrated with unlabeled peptide on SDS-PAGE in the presence of urea. If the iodinated peptide was stored longer than 1 month (−20°), undiluted solutions were subjected to HPLC (see above), and the iodinated peptide fraction (>80% of the applied radioactivity) was pooled and used for binding studies.

Calmodulin preparation and cross-linking methods. Porcine testis calmodulin was prepared according to standard procedures essentially as described (7). Cross-linking experiments were performed and the products analyzed by Coomassie blue staining of 15% SDS-PAGE gels (5). Briefly, the reactions were carried out for 60 min at room temperature with 24 μM calmodulin and various concentrations of β-endorphin 13–31 and of bis(sulfosuccinimidyl)suberate in 50 mM HEPES, pH 7.5, containing 0.2 M NaCl and 1 mM $CaCl_2$. The concentrations of all species were based on dry weights.

Binding of β-endorphin 13-31 to calmodulin. Peptide binding to calmodulin was measured by the gel permeation binding technique of Hummel and Dreyer (17) essentially as described by Ackers (23). The specific activity of equilibrating [^{125}I]β-endorphin 13-31 solutions was based either on dry weight, UV spectroscopy ($\epsilon_{276\ nm}$ = 1420 $M^{-1}cm^{-1}$,

assuming only the contribution of Tyr), or amino acid analysis; quantitation by each of these methods gave comparable results. Briefly, a column (1 × 22 cm) of Bio-Gel P-30 (Bio-Rad Laboratories, Richmond, CA) was equilibrated with 50 mM HEPES, pH 7.5, 0.2 M NaCl, and either 1 mM $CaCl_2$ or 2 mM EGTA with the desired concentration of β-endorphin 13-31 containing [^{125}I]β-endorphin 13-31 in trace amounts (at least 0.1% by weight). Lyophilized aliquots of calmodulin, quantified by amino acid analysis, were dissolved in 200 μl of equilibration buffer containing the peptide. An aliquot of this solution (1.3–2.6 nmol of calmodulin in 50–200 μl) was loaded directly onto the bed of the column after which the reservoir was reattached. The amount of calmodulin recovered in the peak fraction was estimated to be 88 ± 4% based on chromatography of trace acetylated [^{14}C]calmodulin in peptide-containing equilibration buffer. Some measurements were also made using a Sephadex G-75SF (Pharmacia) column (0.4 × 68 cm) exactly as described for the Bio-Gel support. Constant flow rates were maintained with a peristaltic pump at 1–2 ml/hr to ensure adequate resolution between the peak and trough. Fractions (0.25–0.30 ml) were collected and counted by gamma spectrometry. The amount of bound ligand was quantitated by averaging the areas of the peak and the trough, and these generally agreed to within ±10%. Two or three independent column runs were made for each peptide concentration.

RESULTS

Peptide purification and characterization. The purification of [^{14}C]β-endorphin 13-31 by cation exchange chromatography is shown in Fig. 1, and a reverse phase HPLC chromatogram of the pooled fraction, A-I (Fig. 1, *inset*), suggests >98% homogeneity. The amino acid sequence of the peptide is given in Fig. 2, and the composition of the purified peptide was as follows (expected residues are in parentheses): Asp (2), 2.03; Thr (1), 0.93; Glu (1), 1.00; Gly (1), 0.95; Ala (2), 2.00; Val (1), 0.85; Ile (2), 1.75; Leu (2), 1.99; Tyr (1), 0.96; Phe (1), 0.96; Lys (4), 4.01. Proline is not detected by the *o*-phthalaldehyde system used, but its presence at the amino terminus was confirmed by sequence analysis. Indeed, 14 cycles on the sequenator gave the expected amino acid sequence.

Chemical cross-linking of β-endorphin 13-31 and calmodulin. We have previously shown that stable solution complexes of intact β-endorphin and calmodulin occur in the presence of Ca^{2+} as indicated by the formation of both 1:1 and 2:1 peptide/protein products mediated by the hydrophilic protein cross-linker bis(sulfosuccinimidyl)suberate (5). Fig. 3 shows that purified β-endorphin 13-31 can be cross-linked to calmodulin with similar yields of both the 1:1 and 2:1 products as achieved with intact β-endorphin, thus demonstrating the importance of the carboxy terminal region of β-endorphin in this reaction (cf. Ref. 6). Thus, covalent cross-linking would implicate a 2:1 stoichiometry between peptide and protein; however, conditions were not found in which all of the 1:1 complex was quantitatively converted to the 2:1 complex (data not shown).

Equilibrium binding between [^{125}I] β-endorphin 13-31 and calmodulin. [^{125}I]β-Endorphin 13-31 was indistinguishable from the unmodified peptide in its efficacy in cross-linking to calmodulin (data not shown), and the binding results given in Table 1 are consistent with similar, if not identical, affinities of the native and iodinated peptide to calmodulin. In these experiments the column was equilibrated with unlabeled peptide with varying amounts of radioiodinated peptide present in trace quantities. The results show that the binding ratio r (i.e., moles of peptide bound/mole of calmodulin) is, within experimental error, independent of the amount of tracer used to ascertain equilibrium binding. Fig. 4A illustrates a typical chromatogram obtained with 10 μM [^{125}I]β-endorphin 13-31 in a Ca^{2+}-containing buffer following the application of calmodulin. Fig. 4B shows that the binding of the peptide to calmodulin appears Ca^{2+}-dependent since EGTA precludes the formation of the peptide-calmodulin complex.

All binding measurements were determined under buffer conditions directly comparable to the cross-linking experiment described in Fig. 3. Binding experiments were performed in the presence of Ca^{2+} and of EGTA at peptide concentrations of 2–100 μM and 10–50 μM, respectively. The results are summarized in Fig. 5 where the binding ratio r is expressed as a function of the logarithm of the equilibrating peptide concentration. At the higher concentration of β-endorphin 13-31, approximately 4 mol of peptide are bound per mol of calmodulin in the presence of Ca^{2+}, whereas no binding was detected in the presence of EGTA. The peptide concentration corresponding to 50% binding saturation (based on an end-point stoichiometry of $r = 4$) is between 15 and 20 μM. This can only be considered an approximation to the

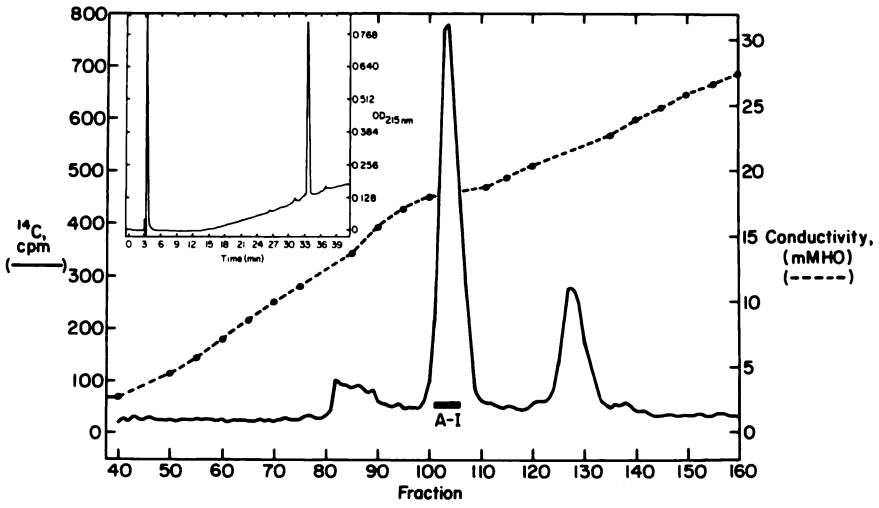

FIG. 1. *CM-32 chromatography of crude [^{14}C] β-endorphin 13-31*

The crude peptide extract resulting from HF cleavage was chromatographed, and fractions were monitored for ^{14}C content (—) and conductivity (- - -). Homogeneous peptide peak A-I was pooled as indicated. *Inset*, reverse phase HPLC analysis of purified β-endorphin 13-31.

$$NH_2\text{-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile}^{*}\text{-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu-COOH}$$

(15, 20, 25, 30)

FIG. 2. *Amino acid sequence of human β-endorphin 13-31*
The *asterisk* designates the position where [^{14}C]isoluecine was incorporated in the synthesis.

K_d since the binding isotherm cannot be completely described in terms of noncooperative binding. For example, a Scatchard plot of these data exhibited some curvature reminiscent of positive cooperativity, and a Hill plot yielded an apparent Hill coefficient of 1.5.

DISCUSSION

The interaction of calmodulin with inhibitory peptides and phenothiazines may provide some information of the minimal structural requisites involved in calmodulin-enzyme interactions. Indeed, β-endorphin and amino terminal deletion peptides (cf. Ref. 6), in addition to other inhibitory peptides including melittin and mastoparan (15), are all characterized by a postulated or known amphipathic α-helix connected to a carboxy terminal basic sequence of two to four residues (13, 24, 25). This secondary structural unit may be important in interacting with calmodulin since we have shown that β-endorphin and β-endorphin 14-31, when complexed with calmodulin, results in induced helicity associated with the interaction (6, 26); a similar phenomenon was reported for melittin (10). The phenothiazines are also amphiphilic in that they consist of a hydrophobic ring system coupled to a positively charged aliphatic chain, both of which appear important in the calmodulin interaction (27). It is generally believed that the interaction of these substances with calmodulin competitively inhibits its ability to activate phosphodiesterase; however, a detailed assessment of the relationship between binding and inhibition of peptides and phenothiazines is required to further define the details of this model system (cf. Refs. 7 and 28).

The results described herein show that the inhibitory peptide β-endorphin 13-31 interacts with calmodulin in a multisite, Ca^{2+}-dependent manner. The maximum binding ratio achieved is approximately 4 mol of peptide bound/mol of calmodulin under the concentration range

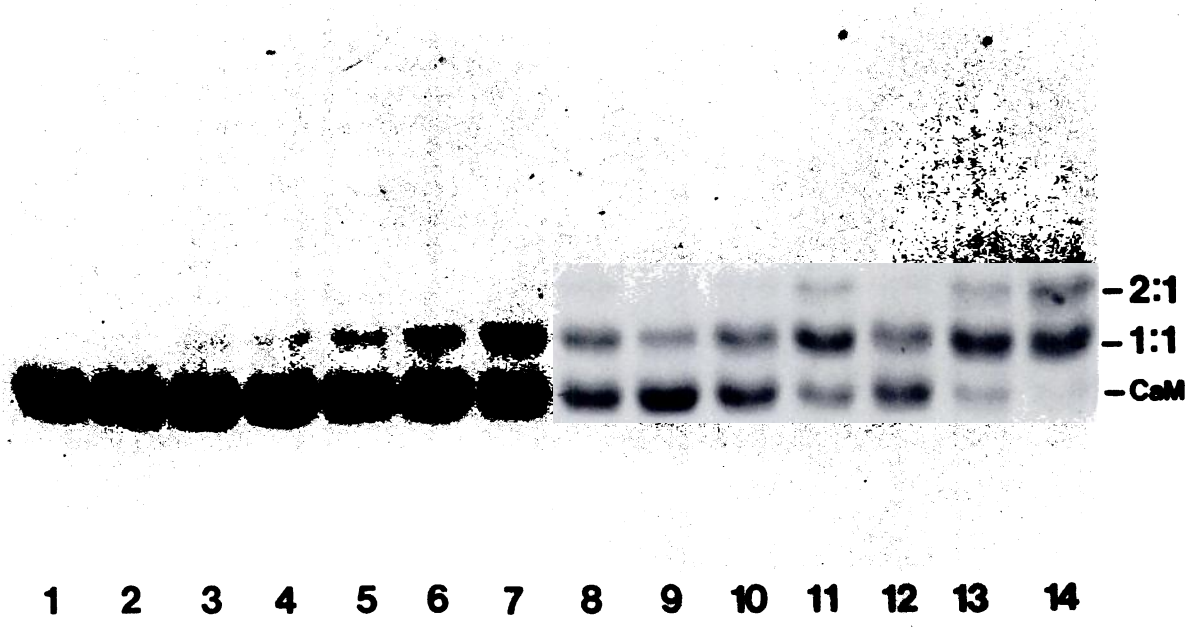

FIG. 3. *Cross-linking between β-endorphin 13-31 and calmodulin at various concentrations of bis(sulfosuccinimidyl)suberate and peptide/protein molar ratios*
Cross-linking was carried out with 24 μM calmodulin and 1 mM $CaCl_2$ with the following: *lanes 1–2*, unmodified and cross-linked calmodulin; *lanes 3–5*, 24 μM (*lane 3*), 60 μM (*lane 4*), and 120 μM (*lane 5*) peptide each at 24 μM cross-linker. This triplet of peptide/protein molar ratios is then repeated using increasing concentrations of cross-linker as follows: *lanes 6–8*, 50 μM cross-linker; *lanes 9–11*, 100 μM cross-linker; *lanes 12–14*, 200 μM cross-linker. The numbers to the right of the gel image designate the 2:1 and 1:1 peptide/calmodulin complexes, and *CaM* designates calmodulin according to relative migrations with molecular weight standards (cf. Ref. 5).

TABLE 1

Evidence that the binding of iodinated and non-iodinated β-endorphin 13-31 to calmodulin is similar[a]

Peptide concentration[b]	Peptide specific radioactivity[c] / calmodulin ((cpm/nmol)/nmol)	r[d]
μM		mol/mol
15	947	2.1[e]
15	5296	1.3
20	1016	2.8
20	2637	3.0[f]
20	4516	2.2
50	1564	3.9
50	1635	4.6
50	3242	3.4

[a] These experiments are based on the Hummel-Dreyer gel permeation system (17, 23) described in the text.

[b] These values represent the peptide concentrations used to equilibrate the column.

[c] Trace amounts of [^{125}I]β-endorphin 13-31 were added to the equilibrating peptide solutions to achieve varying levels of specific radioactivity, i.e., cpm/nmol of total peptide, and calmodulin was then added (either 1.35, 2.0, or 2.5 nmol) to initiate the binding experiment. The results in this column give the peptide specific radioactivity divided by the amount of calmodulin loaded. Bound radioactivity based on total counts in the peak or trough fractions ranged from 15,000–75,000, with an estimated error of 5–10%.

[d] As defined in the text, r represent the moles of peptide bound per mole of calmodulin. The calculation of r using either the peak or the trough agreed to within ±10% unless indicated otherwise.

[e] The range for peak and trough determinations of r was 1.4–2.8.

[f] The range for peak and trough determinations of r was 2.0–4.0.

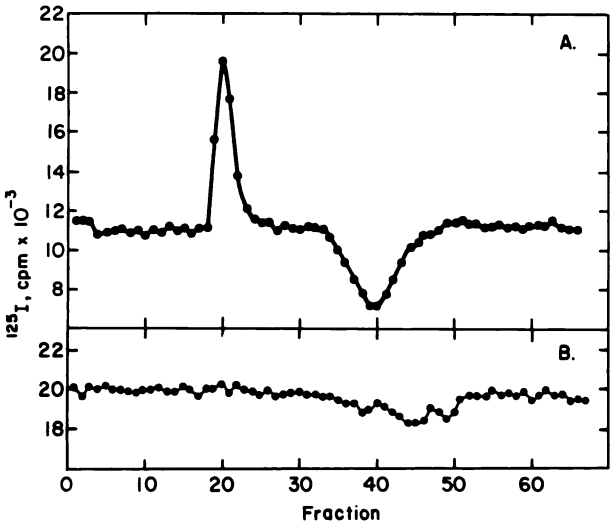

FIG. 4. *Binding of [^{125}I]β-endorphin 13-31 to calmodulin*

A, Hummel-Dreyer binding experiment (17, 23) obtained with 10 μM [^{125}I]β-endorphin 13-31 in the presence of calmodulin and $CaCl_2$. Quantitation of peptide bound gave an r value of 2.2; a similar experiment with Sephadex G-75SF gave r = 1.6. B, as in A except that EGTA replaced $CaCl_2$ in the equilibrating buffer.

tested, with half-maximal binding occurring at 15–20 μM. However, as many as five or six sites may exist for interaction with the peptide since technical limitations prevented saturation from being rigorously determined.

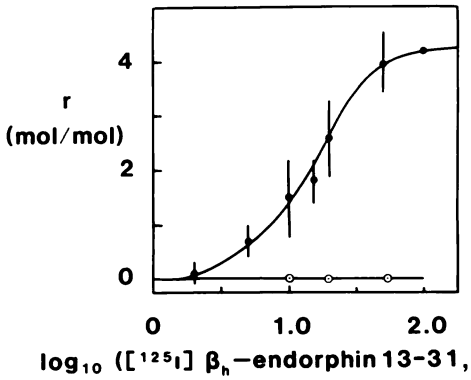

FIG. 5. *Summary of [^{125}I]β-endorphin 13-31 binding to calmodulin*

Equilibrium binding data, collected as in Fig. 4, are presented as a binding ratio (r = moles of peptide/mole of calmodulin) as a function of the log of the equilibrating [^{125}I]β-endorphin 13-31 concentration in the presence of $CaCl_2$ (●) or EGTA (⊙). Where an error bar is given, the mean ± standard deviation of r was taken from four independent experiments.

The evidence for weak positive cooperativity is not compelling and could be explained by small differences in the affinities of unlabeled and iodinated peptide for calmodulin. If, however, some degree of apparant positive cooperativity does exist, it could arise either from interaction of binding sites on calmodulin or from peptide-peptide interactions in solution. Further studies are necessary to clarify this issue.

It is noteworthy that irreversible cross-linking experiments with the peptide and protein resulted in the formation of only 1:1 and 2:1 peptide/protein complexes; a large excess of cross-linker (e.g., 1.6 mM) gave only traces of 3:1 oligomer with higher order structures simply not being observed (data not shown). It is pertinent, however, to emphasize the dependence of cross-linking on both binding and subsequent chemical reactivity of proximate nucleophilic groups with the reagent to form a productive cross-link. For example, cross-linking to calmodulin may involve the first and third or fourth molecules of peptide sequentially bound at two sites; chemical reaction at other sites simply may not be able to occur. Alternatively, an apparent 2:1 stoichiometry from cross-linking may result from the binding at four distinct sites since there are no data to suggest that the same sites of cross-linking occur on any given calmodulin molecule; the lysyl residues on calmodulin which participate in cross-linking have not been identified. Thus, caution must always be exercised in using cross-linking alone as the major criterion for establishing maximum binding.

The present results demonstrate that the binding equilibrium between a β-endorphin peptide and calmodulin is more complex than originally thought from previous reports based on intact β-endorphin. For example, Malencik and Anderson (9) investigated the association of β-endorphin with a fluorescent protein derivative, dansylcalmodulin, by monitoring its enhanced fluorescence upon peptide binding in a Ca^{2+}-dependent manner. It was shown that fluorescence enhancement exhibited saturation behavior and appeared to fit expected changes if one assumes a stoichiometry of 1 mol/mol. However, it

is conceivable that the binding of subsequent peptides produces no further enhancement of the fluorescence of dansylcalmodulin above that which occurs concomitantly with the binding of the first peptide. Also, covalent attachment of the dye to calmodulin may block some of the β-endorphin binding sites. Sellinger-Barnette and Weiss (16) also reported, through equilibrium dialysis experiments with a low ionic strength buffer, that [^{125}I] β-endorphin and calmodulin associate with a limiting 1:1 stoichiometry; Scatchard treatment of their data indicated linearity with an apparent K_d of 4.6 μM. However, the highest concentration of peptide employed appeared to be 5.5 μM, and thus the additional binding sites would have been missed. Consequently, we believe that the approach used herein, along with the wide peptide concentration employed, overcomes the technical limitations and suppositions of the other studies.

The binding equilibria reported herein for the interaction of calmodulin with β-endorphin 13-31 are intriguing when one considers two recent reports which reexamine the Ca^{2+}-dependent binding of the phenothiazine, chlorpromazine, by calmodulin (7, 29). It was initially suggested that this drug associated with a stoichiometry of approximately 2 mol/mol of calmodulin (11); however, Marshak *et al.* (29) showed that calmodulin binds 5–6 molecules of chlorpromazine in a complex equilibrium. We have also obtained similar results, but we found an apparent maximum binding ratio of 7–8 (7). Moreover, we found that the binding of chlorpromazine was quantitatively reduced in covalent β-endorphin 13-31/calmodulin (1:1) complexes and was insignificant in the 2:1 peptide/calmodulin complex. Lukas *et al.* (28) also found that phenoxybenzamine-calmodulin adducts bound less chlorpromazine than native calmodulin, and their results indicated phenothiazine binding sites in helical regions of the second and fourth Ca^{2+}-binding sites. Finally, Jackson and Puett (30) found that acylation of lysines 75 and 148 with a fluorenyl-containing spin label greatly diminished trifluoperazine binding. Thus, various types of calmodulin antagonists appear to possess some commonality in their binding sites on calmodulin, and at least some of these bind in a cooperative-like fashion. These results are particularly intriguing in view of the tertiary structure of calmodulin which is characterized by two globular lobes separated by an extended helix (2).

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REFERENCES

1. Burgess, W. H., M. Schleicher, L. J. Van Eldik, and D. M. Watterson. Comparative studies of calmodulin, in *Calcium and Cell Function*, vol. IV (W. Y. Cheung, ed.). Academic Press, Inc., New York, 209–261 (1983).
2. Babu, Y. S., J. S. Sack, T. G. Greenhough, E. C. Bugg, A. R. Means, and W. J. Cook. Three-dimensional structure of calmodulin. *Nature* **315**:37–40 (1985).
3. Klee, C. B., and T. C. Vanaman. Calmodulin. *Adv. Protein Chem.* **35**:213–321 (1982).
4. Klevit, R. E., D. C. Dalgarno, B. A. Levine, and R. J. P. Williams. ^{1}H-NMR studies of calmodulin. The nature of the Ca^{2+}-dependent conformational change. *Eur. J. Biochem.* **139**:109–114 (1984).
5. Giedroc, D. P., D. Puett, N. Ling, and J. V. Staros. Demonstration by covalent cross-linking of a specific interaction between β-endorphin and calmodulin. *J. Biol. Chem.* **258**:16–19 (1983).
6. Giedroc, D. P., N. Ling, and D. Puett. Identification of β-endorphin residues 14–25 as a region involved in the inhibition of calmodulin-stimulated phosphodiesterase activity. *Biochemistry* **22**:5584–5591 (1983).
7. Giedroc, D. P., T. M. Keravis, J. V. Staros, N. Ling, J. N. Wells, and D. Puett. Functional properties of covalent β-endorphin peptide:calmodulin complexes. Chlorpromazine binding and phosphodiesterase activation. *Biochemistry* **24**:1203–1211 (1985).
8. Weiss, B., W. Prozialeck, M. Cimimo, M. S. Barnette, and T. L. Wallace. Pharmacologic regulation of calmodulin. *Ann. N. Y. Acad. Sci.* **356**: 319–345 (1980).
9. Malencik, D. A., and S. R. Anderson. Binding of simple peptides, hormones and neurotransmitters by calmodulin. *Biochemistry* **21**:3480–3486 (1982).
10. Maulet, Y., and J. A. Cox. Structural changes in melittin and calmodulin upon complex formation and their modulation by calcium. *Biochemistry* **22**:5680–5686 (1983).
11. Levin, R. M., and B. Weiss. Selective binding of antipsychotics and other psychoactive agents to the calcium-dependent activator of cyclic nucleotide phosphodiesterase. *J. Pharmacol. Exp. Ther.* **208**:454–459 (1979).
12. Malencik, D. A., and S. R. Anderson. Binding of hormones and neuropeptides by calmodulin. *Biochemistry* **22**:1993–2001 (1983).
13. Malencik, D. A., and S. R. Anderson. Peptide binding by calmodulin and its proteolytic fragments and by troponin C. *Biochemistry* **23**:2420–2428 (1984).
14. Comte, M., Y. Maulet, and J. A. Cox. Ca^{2+}-dependent high affinity complex formation between calmodulin and melittin. *Biochem J.* **209**:269–272 (1983).
15. Barnette, M. S., R. Daly, and B. Weiss. Inhibition of calmodulin activity by insect venom peptides. *Biochem. Pharmacol.* **32**:2929–2933 (1983).
16. Sellinger-Barnette, M., and B. Weiss. Interaction of β-endorphin and other opioid peptides with calmodulin. *Mol. Pharmacol.* **21**:86–91 (1982).
17. Hummel, J. P., and W. J. Dreyer. Measurement of protein-binding phenomena by gel filtration. *Biochem. Biophys. Acta* **63**:530–532 (1962).
18. Moroder, L., A. Hallett, E. Wunsch, O. Keller, and G. Wersin. Di-tert.-butyldicarbonatein vorteilhaftes reagenz zur einfuhrung der tert.-butyloxy carbonyl-schutzgruppe. *Hoope-Seyler's Z. Physiol. Chem.* **357**:1651–1653 (1976).
19. Tam, J. P., S. B. H. Kent, T. W. Wong, and R. B. Merrifield. Improved synthesis of 4-(Boc-aminoacyloxymethyl)-phenylacetic acids for use in solid phase peptide synthesis. *Synthesis* 955–957 (1979).
20. Merrifield, R. B., L. D. Vizioli, and H. G. Boman. Synthesis of the antibacterial peptide cecropin A(1-33). *Biochemistry* **21**:3020–3031 (1982).
21. Mojsov, S., A. R. Mitchell, and R. B. Merrifield. A quantitative evaluation of methods for coupling asparagine. *J. Org. Chem.* **45**:550–560 (1980).
22. Sarin, V. K., S. B. H. Kent, J. P. Tam, and R. B. Merrifield. Quantitative monitoring of solid phase peptide synthesis by the ninhydrin reaction. *Anal. Biochem.* **117**:147–157 (1981).
23. Ackers, G. K. Studies of protein ligand binding by gel permeation techniques. *Methods Enzymol.* **27**:441–445 (1973).
24. Hammonds, R. G., Jr., A. S. Hammonds, N. Ling, and D. Puett. β-Endorphin and deletion peptides. A correlation of opiate receptor affinity with helix potential. *J. Biol. Chem.* **257**:2990–2995 (1982).
25. Terwilliger, T. C., and D. Eisenberg. The structure of melittin. II. Interpretation of the structure. *J. Biol. Chem.* **257**:6016–6022 (1982).
26. Puett, D., D. P. Giedroc, S. Tollefson, and N. Ling. des-(1-13) Human β-endorphin interacts with calmodulin. *Peptides (N. Y.)* **4**:191–194 (1983).
27. Prozialeck, W. C., and B. Weiss. Inhibition of calmodulin by phenothiazines and related drugs. Structure-activity relationships. *J. Pharmacol. Exp. Ther.* **222**:509–516 (1982).
28. Lukas, T. J., D. R. Marshak, and D. M. Watterson. Drug-protein interactions: isolation and characterization of covalent adducts of phenoxybenzamine and calmodulin. *Biochemistry* **24**:151–157 (1985).
29. Marshak, D. R., T. J.. Lukas, and D. M. Watterson. Drug-protein interactions: binding of chlorpromazine to calmodulin, calmodulin fragments, and related calcium binding proteins. *Biochemistry* **24**:144–150 (1985).
30. Jackson, A. E., and D. Puett. Trifluoperazine binding to calmodulin and adducts. *Fed. Proc.* **44**:7982a(1985).

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