Identification of β-Endorphin Residues 14–25 as a Region Involved in the Inhibition of Calmodulin-Stimulated Phosphodiesterase Activity†

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ABSTRACT: The inhibition of the calmodulin-mediated stimulation of bovine brain cyclic nucleotide phosphodiesterase activity (3′:5′-cyclic adenosine monophosphate 5′-nucleotidohydrolase, EC 3.1.4.17) by the 31-residue opiate peptide β-endorphin has been investigated. Using conditions in which porcine brain calmodulin (6 nM) is limiting (i.e., to give a 3-fold, Ca^{2+}-dependent stimulation of enzymic activity toward cyclic guanosine monophosphate), the domain of β-endorphin responsible for the inhibition was mapped by using a series of deletion peptides. β-Endorphin exhibited an ED_{50} of several micromolar under the conditions employed, and several amino-terminal deletion peptides were essentially as inhibitory as the parent peptide. Methionine enkephalin and various carboxy-terminal deletion peptides had no demonstrable effect at concentrations of 100–200 μM. Peptides 1–25 and 1–27 (C′ fragment) inhibited the calmodulin-dependent activity of phosphodiesterase, but higher concentrations were required than of β-endorphin. Studies using combined amino- and carboxy-terminal deletion peptides demonstrated that peptide 14–25 was the shortest peptide examined that was capable of inhibiting calmodulin stimulation of phosphodiesterase activity under the conditions used. There was no evidence to indicate that the amino-terminal region comprising residues 1–13 of β-endorphin contributes to the measured inhibition of calmodulin-stimulated enzymic activity. The circular dichroic spectra of calmodulin, β-endorphin, and mixtures of the two were obtained, and the ellipticity of the peptide-protein mixtures at 221 nm exceeded that expected by assuming simple additivity. This finding is consistent with a direct interaction of β-endorphin with calmodulin which seems to lead to enhanced helicity of one or both components. Using a spin-labeled calmodulin derivative, we found β-endorphin to have only subtle effects on the electron paramagnetic resonance spectrum. This finding, in conjunction with the circular dichroic spectra, suggests that the peptides do not act by inducing a calmodulin conformation like that in the Ca^{2+}-free state. Overall, our results demonstrate that the β-endorphin-mediated inhibition of calmodulin-dependent phosphodiesterase activity arises from a nonopiate portion of the molecule. Moreover, two naturally occurring β-endorphin-derived peptides, des-Tyr[1]-β-endorphin and C′ fragment, are inhibitory whereas others, α- and γ-endorphin, are not.

Calmodulin is a ubiquitous Ca^{2+}-binding protein of known amino acid sequence (Watterson et al., 1980). Under in vitro conditions, the Ca^{2+}-activated protein has been shown to stimulate the activity of a variety of enzymes including cyclic nucleotide phosphodiesterase[1] [cf. reviews by Cheung (1980), Klee & Vanaman (1982), and Van Eldik et al. (1982)] and to alter tubulin polymerization (Means & Dedman, 1980), perhaps through microtubule-associated proteins (Lee & Wolff, 1982).

Recently, Sellinger-Barnette & Weiss (1982) reported that the opiate peptides β-endorphin (see Figure 1 for the amino acid sequence) and dynorphin could block the activation of phosphodiesterase by calmodulin, there being no effect on the basal enzymic activity. They also found that similar concentrations of α-endorphin (β-endorphin residues 1–16), des-Tyr[1]-γ-endorphin (β-endorphin residues 2–17), and methionine enkephalin (β-endorphin residues 1–5) were without effect. From equilibrium dialysis experiments at 4 °C and pH 7.0 in a low ionic strength buffer, they suggested that the molecular basis of the inhibition resulted from a Ca^{2+}-dependent binding of β-endorphin to calmodulin characterized by an equilibrium dissociation constant of 4.6 μM and a stoichiometry of 1 mol of peptide/mol of protein. Using the fluorescence of dansylcalmodulin (0.17 mol of dye/mol of calmodulin) to monitor peptide interactions at 25 °C and pH 7.3, Malencik & Anderson (1982) also found evidence for Ca^{2+}-dependent-β-endorphin binding characterized by a K_d of 2–3 μM depending upon the Ca^{2+} concentration. In addition, they showed that (leucine and methionine) enkephalin had no effect on the fluorescence of the conjugated protein. A variety of peptide hormones and neuropeptides were screened, and several were identified that bound to calmodulin (e.g., substance P, glucagon, and adrenocorticotropic hormone) and others that did not (e.g., insulin, angiotensin, and others). This work was extended to include secretin, the vasoactive intestinal peptide, and the gastric inhibitory peptide which have an apparent affinity for calmodulin some 10–30 times that of β-endorphin (Malencik & Anderson, 1983). We have recently performed cross-linking studies and have demonstrated in a direct and unequivocal fashion the binding of β-endorphin, as well as selected peptides derived from the β-endorphin sequence, to calmodulin in a Ca^{2+}-dependent fashion (Giedroc et al., 1983a). In these studies at pH 7.5, we also showed that 0.2 M NaCl had no demonstrable effect on the association.

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[1] Abbreviations: AMP, adenosine 5′-monophosphate; CD, circular dichroism; ED_{50}, effective dose for 50% inhibition; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EPR, electron paramagnetic resonance; G, gauss; GMP, guanosine 5′-monophosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Hz, hertz; phosphodiesterase, 3′:5′-cyclic adenosine monophosphate 5′-nucleotidohydrolase (EC 3.1.4.17); Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; β_h-, β_o-, and β_p-endorphin, sequences corresponding to the human, ovine, and porcine β-endorphin peptides, respectively.

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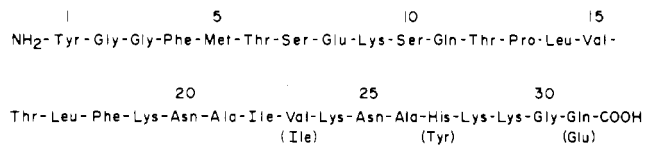

FIGURE 1: Amino acid sequence of porcine β-endorphin. The sequence of the human peptide is identical except where replacements are indicated by the residues in parentheses. Ovine β-endorphin is identical with porcine β-endorphin with the sole exception of an Ile replacement at position 23.

In contrast to the results of Sellinger-Barnette & Weiss (1982) and Malencik & Anderson (1982), our results demonstrated that 2 mol of peptide/mol of calmodulin was the limiting species formed (Giedroc et al., 1983a).

In an attempt to define more specifically the region(s) of the opiate peptide involved in this interaction, we have investigated the effects of a number of deletion peptides derived from the amino acid sequences of porcine, human, and ovine β-endorphin on the Ca^{2+}-dependent stimulation of phosphodiesterase activity by calmodulin. Recently, we found that a nonopiate fragment of human β-endorphin (peptide 14–31) was capable of inhibiting the calmodulin-mediated stimulation of phosphodiesterase (Puett et al., 1983). Interestingly, in the present work, a dodecapeptide, corresponding to residues 14–25 in β_p-endorphin, was found to be necessary and sufficient to cause the inhibition. We previously suggested that this same region of β-endorphin was capable of forming an amphipathic helix and was responsible for the increased affinity of β-endorphin to the brain opiate receptor relative to methionine enkephalin (Hammonds et al., 1982). Circular dichroic spectroscopy of calmodulin and selected peptides was also used in the present study to assess helicity changes concomitant with the purported interaction, and electron paramagnetic resonance spectroscopy was used to monitor effects of β-endorphin on the spectrum of spin-labeled calmodulin.

Materials and Methods

Chemicals and Reagents. Guanosine, GTP, and *Crotalus atrox* venom were from Sigma Chemical Co., St. Louis, MO, and cyclic [^{3}H]GMP (specific activity of 8.3 Ci/mmol) was from New England Nuclear Corp., Boston, MA. 2-Chloro-10-(3-aminopropyl)phenothiazine hydrochloride was kindly provided by Dr. Albert Manian (NIH, Bethesda, MD). The spin-label probe 3-(2-iodoacetamido)-2,2,5,5-tetramethylpyrrolidinyl-1-oxy was purchased from Syva Corp., Palo Alto, CA. D-Ala^2-methionine enkephalinamide was from Calbiochem. Other chemicals and reagents were obtained from standard suppliers.

β-Endorphin and Derived Peptides. β_p-Endorphin, its various deletion peptides, β_o-endorphin peptide 1–27, and β_h-endorphin peptide 2–31 were synthesized by using solid-phase methods, purified, and characterized as described earlier (Ling, 1977). The amino acid sequences of porcine, ovine, and human β-endorphin [cf. Li (1978)] are given in Figure 1.

Preparation and Spin-Labeling of Porcine Brain Calmodulin. Frozen porcine brain was thawed, stripped of dura mater (0.7–1.3 kg), minced, and homogenized in 2–2.4 L of 50 mM Tris-HCl, pH 7.5, containing 5 mM EDTA and 0.125 mg/mL soybean trypsin inhibitor. Following centrifugation (1500*g*, 1 min), the pellet was discarded, and the supernatant was centrifuged at 16000*g* (15 min). The supernatant was saved, and the pellet was resuspended in 1 L of the above buffer and recentrifuged, and the two supernatants were combined. Ammonium sulfate was added to the combined supernatants to give 50% saturation (pH 7.4). The suspension was stirred for 16–20 h at 4 °C and then centrifuged at 10000*g* (1 h), and the supernatant was titrated to pH 4.2 with 2 N H_2SO_4 in ammonium sulfate at 50% saturation. This suspension was stirred for 5–8 h at 4 °C and then centrifuged at 10000*g* (1 h); the pellet was suspended in 10 mM Tris-HCl, pH 7.5, containing 10 mM $CaCl_2$ and then dialyzed against this buffer.

The calmodulin fraction was then applied to a chlorpromazine–Sepharose affinity column (Jones, 1981) at 4 °C, and calmodulin and other Ca^{2+}-dependent phenothiazine-binding proteins were eluted with 10 mM EDTA. The calmodulin-containing fraction was dialyzed, lyophilized, and chromatographed on a DEAE-Sephadex A-50 column which was equilibrated with 10 mM Tris-HCl, pH 7.5, containing 2 mM β-mercaptoethanol and 0.3 M NaCl and then developed with a 0.3–0.6 M linear NaCl gradient essentially as described by Jamieson & Vanaman (1979). Following dialysis, purified calmodulin was characterized by $NaDodSO_4$–polyacrylamide gel electrophoresis in the presence of urea, isoelectric focusing gel electrophoresis, amino acid composition, and stimulation of phosphodiesterase activity.

The protein (0.242 μmol) was reacted with 4.84 μmol of 3-(2-iodoacetamido)-2,2,5,5-tetramethylpyrrolidinyl-1-oxy in the presence of a 0.1 M sodium succinate buffer (pH 5.8) containing 0.1 mM $CaCl_2$ and 7.1% ethanol (total reaction volume = 0.7 mL) for 26 h at 37 °C. These are conditions known to modify 2–3 mol of methionine/mol of calmodulin (Hewgley & Puett, 1980). Spin-labeled calmodulin was separated from free spin-label via chromatography on a 1 × 30 cm column of Sephadex G-25 equilibrated and developed with 0.1 M ammonium bicarbonate, pH 7. The tubes comprising the protein fraction were pooled, dialyzed exhaustively against water, and lyophilized, with an overall yield of 88%.

Phosphodiesterase Preparation and Assay. The phosphodiesterase preparation from brain was based on the methods described by Cheung & Lin (1974) and Watterson et al. (1976). Briefly, fresh bovine brain was homogenized as described for the preparation of calmodulin. The fraction precipitated by 55% saturation with ammonium sulfate (pH 7.2) was chromatographed on a DEAE-Sephadex A-50 column (2.5 × 40 cm) which was equilibrated and developed for 1 column volume with a buffer consisting of 10 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM dithioerythritol, and 0.2 M NaCl. A linear ionic strength gradient (500 mL in each chamber) was then run from 0 to 0.5 M ammonium sulfate in the starting buffer. The activatable enzyme was located in the second phosphodiesterase fraction.

Phosphodiesterase activity was measured with cyclic [^{3}H]GMP as substrate essentially as described by Wells et al. (1975). The assay volume was 0.25 mL and contained 48 mM Tris-HCl buffer, pH 7.5, 0.8 mg/mL bovine serum albumin, 2 mM $MgCl_2$, and 1 μM cyclic GMP (10^5 cpm of cyclic [^{3}H]GMP). Calmodulin (6 nM unless stated otherwise) and peptides were added together or separately, and measurements were made in triplicate at each dose point. Unless stated otherwise, the level of calmodulin (with 0.1 mM $CaCl_2$) was chosen to give a 3.0–3.2-fold activation of the basal activity which was about 0.2 pmol/min. Incubation was for 30 min at 30 °C, and the reaction was terminated by addition of 0.025 mL of a solution containing 0.1 M Tris-HCl, pH 7.5, with 50 mM EDTA, 30 mM theophylline, and 10 mM each of cyclic GMP and cyclic AMP. The enzymic conversion of cyclic [^{3}H]GMP to 5′-[^{3}H]GMP was followed by formation of [^{3}H]guanosine from the 5′-nucleotide using the 5′-nucleotidase activity in *Crotalus atrox* venom (10 mg/mL for 10 min). Quaternary aminoethyl-Sephadex was used to separate

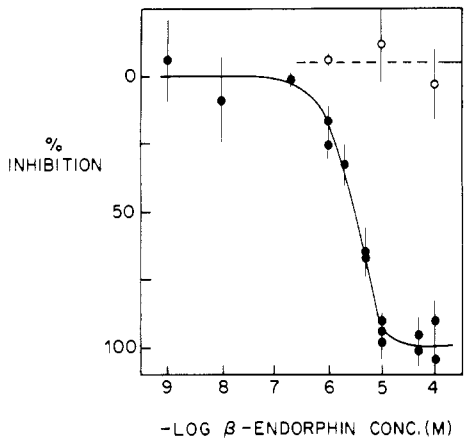

FIGURE 2: Inhibition of the calmodulin-stimulated phosphodiesterase activity by β_p-endorphin. The calmodulin concentration was 6 nM (●), and enzymic conditions were such that phosphodiesterase activity was increased about 3-fold with no added peptide. Triplicate measurements were made at each peptide concentration, and the results are shown as the mean ± SEM from several independent assays. Results are also shown with no added calmodulin (○); i.e., these points denote the percent inhibition or stimulation of basal activity by β_p-endorphin.

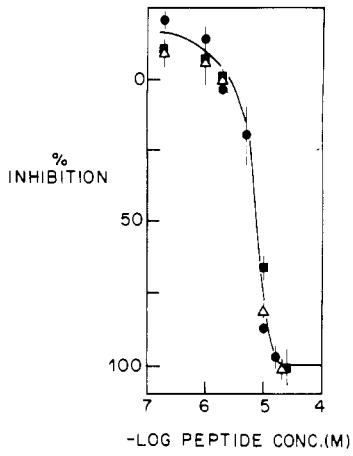

FIGURE 3: Inhibition of the calmodulin-stimulated phosphodiesterase activity by amino-terminal deletion peptides of β-endorphin: (●) β_h-endorphin peptide 2–31; (△) β_p-endorphin peptide 8–31; (■) β_p-endorphin peptide 12–31. The conditions are the same as those described in Figure 2. In the absence of calmodulin, peptides 2–31 (17 μM), 8–31 (21 μM), and 12–31 (25 μM) exhibited no significant effect on basal activity, the respective percent inhibitions being 0.5 ± 7.6, 4.4 ± 4.7, and 11.2 ± 4.7.

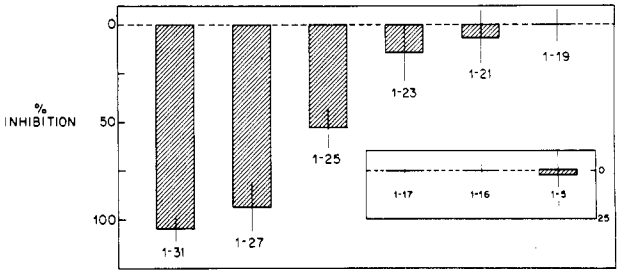

FIGURE 4: Inhibition of the calmodulin-stimulated phosphodiesterase activity by β_p-endorphin and several derived carboxy-terminal deletion peptides (peptide 1–27 is the sole exception and is based on the sequence of β_o-endorphin) at a concentration of 100 μM under the conditions described in Figure 2. Peptide 1–5 denotes the potent analogue of methionine enkephalin, D-Ala^2-methionine enkephalinamide.

[^{3}H]guanosine which was collected and counted.

The results are presented as the percent inhibition of calmodulin-stimulated phosphodiesterase activity. Thus, 0% inhibition by a given peptide means that the stimulated enzymic activity is unchanged (i.e., remains at a 3–3.2-fold increase over the basal value), while 100% inhibition denotes a reduction of the stimulated activity to the basal level. We have recently shown that essentially the same basal activity is obtained in the presence of $CaCl_2$ or EGTA (Puett et al., 1983).

Circular Dichroism. CD spectra were obtained at 23 °C by using a Cary 60 spectropolarimeter equipped with a CD attachment. The spectra of calmodulin (0.2 mg/mL), porcine β_p-endorphin (0.2 and 0.4 mg/mL), and mixtures of calmodulin and peptide were determined in a 50 mM Tris-HCl, pH 7.5, buffer containing either 2 mM EGTA or 2 mM EGTA plus 3.3 mM $CaCl_2$ to give an effective free Ca^{2+} concentration of 1.3 mM. The ellipticity was recorded between 200 and 260 nm in a fused quartz cell of 2 mm path length with a time constant of 3 s and a full-scale deflection of 0.1°. Since the peptides and the protein are optically active, the results are reported as the measured ellipticity. This is necessary with the mixtures since one does not know a priori the molar basis to which reduced ellipticity could be referred.

CD spectra of β_p-endorphin peptides 14–23 and 14–25 were determined in a 50 mM Tris-HCl, pH 7.5, buffer and in 90% (v/v) trifluoroethanol by using a peptide concentration of 0.1 mg/mL and cells with path lengths of 0.5 and 2 mm. These results are presented as molar ellipticity.

Electron Paramagnetic Resonance. All EPR spectra were determined by using a Varian E-112 spectrophotometer equipped with a TE_{102} microwave cavity (Varian E-231). Individual samples were prepared from a stock solution of spin-labeled calmodulin in 50 mM Tris-HCl, pH 7.5, containing 2 mM EGTA and incubated overnight at 4 °C and then loaded into a standard flat cell. For constant microwave power scans, the instrument was operated at a microwave power of 5 mW, and X-band frequency (~9.41 GHz) was used. Full spectrum scans were made at 100 G. For power saturation experiments, the microwave power was varied from 0.5 to 100 mW at constant modulation amplitude (typically 0.5 G), and 10-G scans of the +1 peak were obtained at a gain of 3.2×10^4 to calculate the peak heights. If necessary, these were normalized to a particular gain setting since, at constant microwave power and modulation amplitude, the factor relating two particular gain settings is identically proportional to peak heights at each gain setting (unpublished results).

Results

Inhibition of Calmodulin-Stimulated Phosphodiesterase Activity. The effects of β_p-endorphin on the Ca^{2+}-dependent calmodulin activation of phosphodiesterase are shown in Figure 2. That this inhibition does not depend upon the portion of β-endorphin responsible for opiate activity (i.e., residues 1–5) is demonstrated by the finding that the amino-terminal deletion peptides 2–31, 8–31, and 12–31 are effective inhibitors (Figure 3). The slightly higher ED_{50} for these deletion peptides compared to β-endorphin is probably within the interassay variation. Preliminary data indicate that peptide 20–31 is only weakly inhibitory; e.g., it exhibited 14 ± 10% inhibition at 70 μM. Dose–response curves were obtained on a number of carboxy-terminal deletion peptides, and even at doses of several hundred micromolar, peptides lacking eight or more carboxy-terminal residues either were without effect or were only weakly inhibitory under the assay conditions (data not shown). For purposes of illustration, the effects of a series of carboxy-terminal deletion peptides at 100 μM on calmodulin-stimulated enzymic activity are shown in Figure 4. As the peptide length is extended from methionine enkephalin (peptide 1–5) to peptide 1–23, there is no significant effect on the

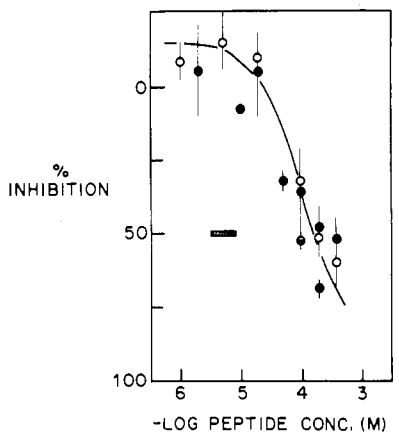

FIGURE 5: Inhibition of the calmodulin-stimulated phosphodiesterase activity by β_p-endorphin-derived peptides (○) 1–25 and (●) 14–25. The conditions were the same as those described in Figure 2. The shaded area at 50% inhibition denotes the range of ED_{50} values observed for β_p-endorphin and the amino-terminal deletion peptides shown in Figures 2 and 3.

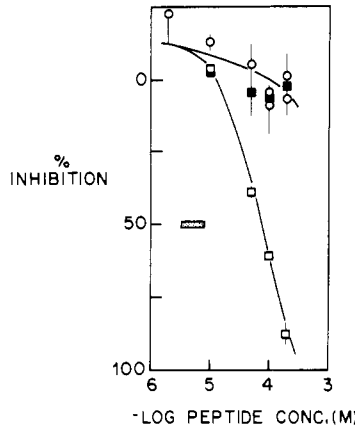

FIGURE 6: Inhibition of the calmodulin-stimulated phosphodiesterase activity by combined amino-terminal and carboxy-terminal deletion peptides of β_p-endorphin: (■) 10–19; (○) 14–23; (□) 14–27. The conditions were as described in Figure 2, and the shaded area at 50% inhibition was defined in the legend to Figure 5.

stimulated activity. The carboxy-terminal deletion peptides 1–25 and 1–27 are, however, effective inhibitors, with the latter having an apparent greater potency. A dose–response curve for peptide 1–25 is given in Figure 5, and it can be seen that the ED_{50} is considerably greater than those of β-endorphin and amino-terminal deletion peptides lacking 1, 7, and 11 residues (cf. Figures 2 and 3). This result suggests that at least some of the six carboxy-terminal residues of β-endorphin interfere with the association of calmodulin and phosphodiesterase. Also shown in Figure 5 are data for the combined amino- and carboxy-terminal deletion peptide 14–25. These results emphasize again that residues 1–13 of β-endorphin apparently contribute little to the macromolecular interaction. Results for other combined amino- and carboxy-terminal deletion peptides (two decapeptides, 10–19 and 14–23, and one dodecapeptide, 14–27) are presented in Figure 6. Peptide 14–27 has about the same potency as peptide 14–25 (cf. Figure 5), although the dodecapeptide may exhibit a somewhat lower ED_{50}. Under these conditions, peptides 10–19 and 14–23 are ineffective even at 200 μM.

The effects of β_p-endorphin and all peptides used in Figures 2–6 on basal enzymic activity were examined at multiple doses. Even at high doses (e.g., 100 μM and greater), there was no significant effect on basal activity (cf. Figure 2 and Table I; also other unpublished results). These results are consistent

Table I: Cyclic Nucleotide Phosphodiesterase Activity in the Presence of β-Endorphin Deletion Peptides[a]

peptide	A_p/A_0[b]
Carboxy-Terminal Deletions	
1-5	1.00 ± 0.15
1-16	1.08 ± 0.10
1-17	1.14 ± 0.10
1-19	1.02 ± 0.15
1-21	1.03 ± 0.16
1-23	1.04 ± 0.19
1-25	1.13 ± 0.21
1-27	1.01 ± 0.14
Combined Amino- and Carboxy-Terminal Deletions	
10-19	1.00 ± 0.07
14-23	1.00 ± 0.16
14-25	1.29 ± 0.26
14-27	0.88 ± 0.05

[a] The assays were performed as described in the text with no calmodulin being added. All peptides were present at 100 μM except peptide 1-5, which is the synthetic methionine enkephalin analogue (NH_2-Tyr-D-Ala-Gly-Phe-Met-NH_2) and was present at 200 μM. All peptides are based on the sequence of β_p-endorphin except 1-27 which is derived from the sequence of β_o-endorphin. [b] The results are presented as the ratio of the enzymic activity in the presence of peptide (A_p) to that in the absence of peptide (A_0). The mean ± SEM is shown (n was at least 3 and in some cases 6).

with the findings of Sellinger-Barnette & Weiss (1982) on β-endorphin and the derived peptides consisting of α-endorphin, des-Tyr^1-γ-endorphin, and methionine enkephalin, although the highest dose they used appeared to be 50 μM. Also in agreement with their study, we found that the inhibition of stimulated enzymic activity could be overcome by additional calmodulin (data not shown).

CD of β-Endorphin–Calmodulin Mixtures. The binding, spectral, and cross-linking data that have been reported for calmodulin and β-endorphin clearly indicate a direct interaction of the opiate peptide with the protein (Sellinger-Barnette & Weiss, 1982; Malencik & Anderson, 1982; Giedroc et al., 1983a). This concept is also consistent with the present results which show that only the calmodulin-stimulated activity of phosphodiesterase is altered by the peptides; this, in turn, can be overcome by increasing the concentration of calmodulin.

In order to explore this putative interaction more thoroughly, we have measured the CD spectra of calmodulin, β_p-endorphin, and mixtures of the peptide with protein in Ca^{2+}-free and Ca^{2+}-containing solutions (Figure 7). When corrected to mean residue or molar ellipticity, the spectra for calmodulin ($\pm Ca^{2+}$) and β_p-endorphin agree with published CD spectra obtained under comparable conditions (Klee, 1977; Hammonds et al., 1982). Interestingly, the spectra of the β_p-endorphin–calmodulin mixtures exhibit more apparent ellipticity than can be accounted for on the basis of simple additivity. Moreover, there appears to be some enhancement even in the absence of Ca^{2+}, but the greatest effect is observed in the Ca^{2+}-containing solution. At the 10:1 molar ratio of peptide to protein in the presence of exogenous Ca^{2+}, the difference in ellipticity at 221 nm between the mixture and the sum of the peptide and protein can be attributed to induced helicity associated with the interaction. This difference is small, but significant. If it is assumed that calmodulin is saturated with peptide, i.e., 2 mol of peptide/mol of protein (Giedroc et al., 1983a), under the conditions corresponding to a 10:1 molar ratio[2] and that

[2] Using similar concentrations of peptide and protein in cross-linking studies [cf. Giedroc et al. (1983a)], we find a significant amount of the 2 mol of peptide–1 mol of calmodulin complex when a relatively high concentration of cross-linking reagent is present.

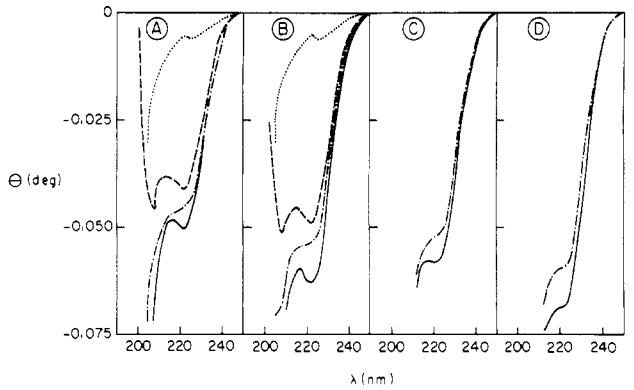

FIGURE 7: (A and B) CD spectra of 11.4 μM calmodulin (---), 57 μM β_p-endorphin (···), and a mixture of the peptide and protein at the same concentrations (—) and the calculated spectrum of the individual peptide and protein spectra (·-·-·). The buffer was 50 mM Tris-HCl, pH 7.5, and either 2 mM EGTA (A) or 2 mM EGTA + 3.3 mM $CaCl_2$ (B). Panels C and D depict mixtures of β_p-endorphin and calmodulin (same concentration as in panels A and B) but with a peptide:protein molar ratio of 10:1 (—); the calculated spectra based on additivity of the separate peptide and protein solutions are also given (·-·-·). The same buffer conditions apply to panels C and D as were given in panels A and B, respectively. The signal to noise ratios at 221 nm varied about 5:1 for the β_p-endorphin solutions and between 30:1 and 40:1 for the calmodulin-containing solutions. The 221-nm ellipticity differences between the mixtures and the values expected from simple additivity are (in units of millidegrees) (A) −4.3, (B) −8.9, (C) −6.2, and (D) −9.4.

$\Delta\theta$ is due only to helix formation by β_p-endorphin, an estimate can be made of the number of induced helical residues. Using the recent reference protein spectra reported by Chang et al. (1978) in which an average 10-residue α-helix in proteins exhibits a mean residue ellipticity ($[\theta]_{mwr}$) of -2.82×10^4 deg·cm^2/dmol at this wavelength, we measured a $\Delta\theta$ of -9.4×10^{-3} deg which corresponds to about 24 ± 3% helicity (when $[\theta]_{mwr}$ is referred to the number of peptide bonds in β_p-endorphin). This induced helicity reflects roughly 7 ± 1 residues adopting the helical conformation when bound to calmodulin. If, under the conditions used, the protein is not fully saturated with peptide, the estimated number of induced helical residues in the peptide would be even higher. Experimentally it is difficult to achieve meaningful measurements at higher molar ratios of peptide:protein due to the increased absorption.

CD of Combined Amino- and Carboxy-Terminal β-Endorphin Deletion Peptides. We have previously shown that β-endorphin and various deletion peptides exhibited little if any secondary structure in aqueous solution, but helicity could be induced in some of the peptides by the helicogenic solvent trifluoroethanol (Hammonds et al., 1982). Since no information is available on the conformational aspects of the combined amino- and carboxy-terminal deletion peptides of β-endorphin, we have determined the CD spectra of peptides 14–23 and 14–25 (i.e., peptides in which an apparent delineation occurs in inhibitory potency) in aqueous solution and in 90% trifluoroethanol. In the organic solvent, the spectra of both peptides were characterized by a negative extremum at 205 nm (assigned to a π–π^* transition) and crossover wavelengths to positive ellipticity between 195 and 198 nm (data not shown). The molar ellipticities in units of degrees per square centimeter per decimole were -13×10^4 (peptide 14–23) and -15.3×10^4 (peptide 14–25) at 205 nm and -6.6×10^4 (peptide 14–23) and -10×10^4 (peptide 14–25) at 218 nm. In a 50 mM Tris-HCl buffer (pH 7.5), both peptides exhibited considerably less ellipticity at all wavelengths above 210 nm; e.g., the molar ellipticity at 218 nm was about -3×10^4 deg·cm^2/dmol for both peptides. The spectra of these

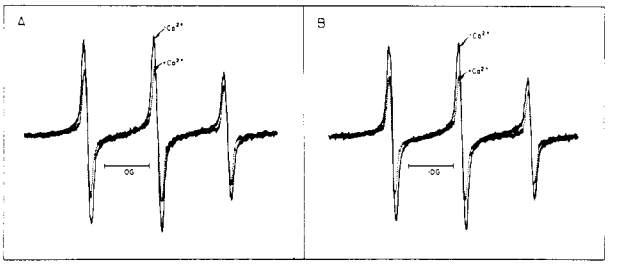

FIGURE 8: EPR spectra of 6.8 μM spin-labeled calmodulin in a 50 mM Tris-HCl buffer at pH 7.5 containing 0.2 M NaCl and either 2 mM EGTA (---) or 9.5 mM free Ca^{2+} (—), obtained by adding $CaCl_2$ to the 2 mM EGTA stock solution. (A) No added peptide. (B) In the presence of 500 μM β_p-endorphin. Similar spectra were obtained in the absence of NaCl (data not shown).

relatively short peptides in 90% (v/v) trifluoroethanol are not readily interpreted in terms of standard protein reference spectra obtained in aqueous solution [cf. Chang et al. (1978)], but they indicate that little helicity occurs. The much weaker calmodulin interaction of peptides 14–23 and 14–25, relative to β-endorphin, prohibited CD studies as performed above for mixtures of calmodulin and β-endorphin.

EPR of Peptide–Spin-Labeled Calmodulin Mixtures. EPR spectra of spin-labeled calmodulin are quite sensitive to exogenous Ca^{2+} (Figure 8). As initially shown by Hewgley & Puett (1980) for spin-labeled bovine brain calmodulin, there is a significant spectral collapse concomitant with Ca^{2+} binding. The spectra in Figure 8A of spin-labeled porcine brain calmodulin in the presence and absence of exogenous Ca^{2+} confirm this observation. These spectra were collected in the presence of 0.2 M NaCl to help ensure against potential artifactual electrostatic effects on the interacting nitroxides. Spectra obtained in the absence of 0.2 M NaCl invariably exhibited a greater spectral collapse upon addition of Ca^{2+} (unpublished results). The addition of β_p-endorphin to the spin-labeled calmodulin solutions had no major effect on the Ca^{2+}-mediated spectral collapse (Figure 8B). However, subtle, but reproducible, decreases were consistently observed in both the presence and absence of exogenous Ca^{2+}.

In order to better illustrate the slight perturbations in the EPR spectra of spin-labeled calmodulin due to association with β-endorphin, power saturation curves were obtained for both the Ca^{2+}-saturated and operationally defined Ca^{2+}-free states in the presence and absence of 500 μM β-endorphin (Figure 9). The results show that, under the conditions investigated, the effect of the peptide is to lower the height of each transition (only the +1 transition is shown), and this reduction in signal height is greatest in the presence of Ca^{2+}. For example, at 100 mW power, the reduction is 3% with only EGTA added and 6% with an excess of exogenous Ca^{2+}. In the absence of 0.2 M NaCl, β-endorphin leads to even greater reductions in peak height intensity (data not shown). Although the linear portion of power saturation curves are proportional to the reciprocal of the relaxation time (T_1) in simple systems, it is not justifiable to use this relationship in interacting spin systems.

Discussion

From this investigation into the molecular basis of the inhibition of calmodulin-stimulated phosphodiesterase activity by β-endorphin, several findings are of particular import. One is that several naturally occurring peptides, two of which are opiate peptides (β-endorphin and the C′ fragment) and one of which is a nonopiate peptide (des-Tyr^1-β-endorphin), are inhibitory. Other putative β-endorphin-derived peptides, α- and γ-endorphin (peptides 1–16 and 1–17, respectively), have

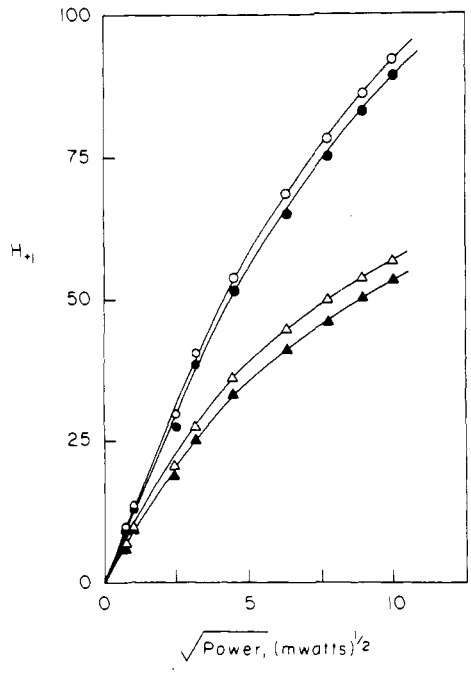

FIGURE 9: EPR power saturation curves of 6.8 μM spin-labeled calmodulin where the height (in arbitrary units) of the +1 transition (H_{+1}) is plotted as a function of the square root of microwave power. All solutions contained 50 mM Tris-HCl, pH 7.5, 0.2 M NaCl, and 2 mM EGTA. (○) Spin-labeled protein alone; (●) spin-labeled protein plus 500 μM β_p-endorphin; (△) spin-labeled protein alone with $CaCl_2$ added to give an effective free Ca^{2+} concentration of 9.5 mM; (▲) spin-labeled protein plus 500 μM β_p-endorphin with $CaCl_2$ added to give an effective free Ca^{2+} concentration of 9.5 mM. Similar effects of β_p-endorphin were noted on solutions ($\pm Ca^{2+}$) that lacked NaCl (data not shown).

no demonstrable effect, and the amino-terminal pentapeptide sequence (i.e., methionine enkephalin), as expected (Sellinger-Barnette & Weiss, 1982), is also without effect. Perhaps the most intriguing finding is that the dodecapeptide corresponding to residues 14–25 in β_p-endorphin is the shortest peptide found that yielded a significant inhibitory response under our standard conditions. The potency of the peptide was considerably less than that of β_p-endorphin but was equivalent to that of peptide 1–25. Our argument that the inhibitory potency of the peptides is in fact referrable to a Ca^{2+}- and calmodulin-dependent interaction is strengthened considerably by recent studies in which we showed that similar results are obtained with highly purified phosphodiesterase (Giedroc et al., 1983b).

From the various deletion peptides of β-endorphin examined, it seems safe to conclude that the amino acid sequence corresponding to residues 14–25 is necessary and sufficient for the inhibition response to be manifested with a reasonable efficacy, but the six carboxy-terminal residues of β-endorphin appear to contribute to the peptide–calmodulin interaction or interfere with the association of calmodulin and phosphodiesterase. It cannot be ascertained from the present data alone whether the β-endorphin carboxy-terminal residues contribute in a specific fashion either to the protein interaction or to stabilization of a particular peptide conformation. In particular, the tandem lysines at positions 28 and 29 are attractive candidates for electrostatic interaction. Alternatively, these terminal regions in β-endorphin may act as "inert spacers" which simply lengthen the peptide chain and perhaps make the potential helical region (Hammonds et al., 1982) or other conformations more probable.

As we have discussed elsewhere, the same region of β-endorphin (i.e., residues 14–25) appears to be responsible for the increased affinity of β-endorphin to the opiate receptor relative to methionine enkephalin and the α- and γ-endorphins (Hammonds et al., 1982). We suggested that this sequence was compatible with an amphipathic helix, although a "pure" amphipathic helix would involve only residues 14–22. Since β_p-endorphin peptide 14–23 associates with calmodulin (Giedroc et al., 1983a) but does not exhibit any demonstratable inhibition, we must conclude that either or both of residues 24 and 25 promote interference with calmodulin-mediated enzymic activity. From the present data, we cannot determine if the effect is specific for Lys and Asn, or if a simple "filler role" is involved.

In an attempt to estimate a possible contribution of a filler role for residues 24 and 25, we have used the formulation of Zimm & Bragg (1959) to estimate the helix fraction (f) as a function of the number of residues in an oligohomopeptide.[3] The two additional residues in peptide 14–25 relative to peptide 14–23 may serve to promote some helix formation, albeit limited, simply by the increase in chain length. However, the 218-nm ellipticity, reduced to peptide bond equivalency, of peptide 14–25 is no more than about 20% greater than that of peptide 14–23, indicating that the dodecapeptide may contain only somewhat more secondary structure than the decapeptide. Yet, the apparent efficacy of the dodecapeptide as an inhibitor is considerably greater than that of the decapeptide. These particular results suggest that the contribution of residues 24 and 25 in β-endorphin may involve more than just a filler role and that they may be necessary in a somewhat specific manner for eliciting effective inhibitory activity.

Our model of the amphipathic helix may also have validity with regard to the calmodulin interaction since we found an enhancement of helicity in mixtures of calmodulin with β_p-endorphin. The CD spectral change does not of course represent unequivocal evidence that the conformation of the bound peptide is helical since peptide binding may increase the helicity of calmodulin. The argument against this is that β-endorphin, although devoid of significant secondary structure in aqueous solution, has been shown to have a high helix potential (Yang et al., 1977; Hammonds et al., 1977, 1982; Hollosi et al., 1977; Wu et al., 1979, 1981; Graf et al., 1980; Jibson & Li, 1981). Indeed, the elegant studies of Taylor et al. (1982, 1983) suggest that many bioactivities of β-endorphin can be mimicked by a synthetic peptide analogue constructed to form an amphipathic α-helix encompassing residues 14–31 in aqueous solution. Assuming then that the CD change in the calmodulin–β_p-endorphin mixtures arises only from the bound peptide, we have estimated that approximately two turns of helix are formed concomitant with binding. A similar study with β_h-endorphin peptide 14–31 suggested that about one turn of helix was stabilized (Puett et al., 1983). These results are of course tentative due to the assumptions made. In particular, we do not know if the $\Delta\theta$ measured represents a maximum, although the relative change in $\Delta\theta$ from a 5:1 to 10:1 molar ratio of peptide:protein was relatively small (<6% increase), and cross-linking under conditions comparable to these can lead to appreciable amounts of the saturated complex. Clearly, there is no way from these studies to distinguish if the bound peptides have identical helicities or if binding at one site promotes higher helicity than binding at another site. In any case, our CD results strongly indicate that the peptide does

[3] Using values of the helix nucleation constant, σ (10^{-1} and 2×10^{-4}), and the helix propagation constant, s (1.05 and 1.2), that seem to encompass expected ranges [cf. Puett (1972)], we find that f is essentially 0 for all s and σ values with the decapeptide. With the dodecapeptide, f is also 0 with $s = 1.05$; however, with $s = 1.2$ and $\sigma = 10^{-1}$, $f = 0.13$.

not induce a conformation in calmodulin similar to that found in a Ca^{2+}-free state since this form has less helicity. This suggestion is consistent with the EPR results since the spectral intensity decreases upon the addition of β-endorphin to spin-labeled calmodulin solutions in the presence of Ca^{2+}. If the peptide were interfering with Ca^{2+} binding or inducing a conformation like that in the Ca^{2+}-free state, one would expect the intensity to increase.

Interestingly, the CD spectra suggest that some complexion occurs between calmodulin and β_p-endorphin even in the absence of exogenous Ca^{2+}. Our EPR results also support this observation since, under operationally defined Ca^{2+}-free conditions, β_p-endorphin was found to consistently diminish the magnitude of the peaks at a given microwave power. These data suggest the presence of an interaction between β_p-endorphin and (native and spin-labeled) calmodulin even in the absence of added Ca^{2+}, a finding in apparent contrast to reports from other laboratories (Sellinger-Barnette & Weiss, 1982; Malencik & Anderson, 1982) and to our cross-linking studies (Giedroc et al., 1983).[4] However, Sellinger-Barnette & Weiss (1982) did find limited binding of β-endorphin to calmodulin in the absence of Ca^{2+}, albeit nonsaturable under the conditions used. Also, reduced binding to a Ca^{2+}-independent site may fail to enhance the fluorescence of dansyl-calmodulin.

Since β-endorphin is a basic peptide and calmodulin is an acidic protein, one could suspect that the interaction, even in the presence of Ca^{2+}, is electrostatic in nature. There are, however, at least two arguments against this. One is that the two dodecapeptides β_p-endorphin peptides 20–31 and 14–25 contain three and two lysyl residues, respectively, and no acidic side chains. Yet, the former peptide is not an effective inhibitor while the latter is. An even more pertinent result is our recent demonstration that β_p-endorphin peptide 14–23, an extremely hydrophobic peptide, is effectively cross-linked to calmodulin (Giedroc et al., 1983) while β_p-endorphin peptide 20–31, under identical conditions, is not (unpublished results obtained in collaboration with Dr. J. V. Staros).

Our results are not in total agreement with the interpretation by Malencik & Anderson (1982) that the active region of β_h-endorphin is located between residues 19 and 29. They arrived at this suggestion by reversing the sequence of β-endorphin and comparing this with other regions of other peptides or proteins that bind to calmodulin. A two-residue hydrophobic region separated by three amino acids from a three-residue, generally basic, region was identified. They postulated that the antiparallel alignment of β-endorphin is permissible provided that the interaction with calmodulin does not involve the peptide backbone. Although our results support the concept that carboxy-terminal residues on β-endorphin contribute to the interaction with calmodulin, an argument against the region β_h-endorphin 19–29 being the primary binding domain is our finding that the dodecapeptide β_p-endorphin peptide 20–31, at 70 μM and up to 200 μM (Giedroc et al., 1983b), had only negligible inhibitory properties to calmodulin-stimulated phosphodiesterase activity, while the dodecapeptide β_p-endorphin peptide 14–25 was significantly inhibitory at lower doses. Furthermore, the cross-linking results alluded to above correlate with the inhibitory efficacy of the peptides.

[4] The conformations of bound peptide and Ca^{2+}-depleted calmodulin may be such that the required proximity or orientation of appropriate complementary nucleophilic aminoacyl residues on each species is not manifested, thereby preventing the cross-linking reaction even when binding occurs.

In summary, our CD and EPR spectral data suggest that subtle, and presumably quite localized, conformational changes occur when β-endorphin associates with calmodulin. The peptide does not appear to mediate inhibition of calmodulin-stimulated phosphodiesterase activity by inducing a conformation in the protein similar to that which occurs in the Ca^{2+}-free state. Rather, it seems more likely that the peptide either blocks the binding domain of calmodulin for the enzyme or induces a conformation distinct from that of the Ca^{2+}-free form of the protein.

The physiologic relevance, if any, of the interaction of calmodulin with the naturally occurring peptides β-endorphin, C′ fragment, and des-Tyr[1]-β-endorphin remains to be demonstrated. The estimated K_d for β-endorphin binding to calmodulin (Sellinger-Barnette & Weiss, 1982; Malencik & Anderson, 1982) is in good agreement with the ED_{50} value obtained from our inhibition data; however, it is some 4 orders of magnitude greater than the value found for interaction of this opiate peptide with the brain opiate receptor (Hammonds et al., 1982). Even if this in vitro interaction of calmodulin with β-endorphin and derived peptides is not of physiologic importance, it is still of great interest in exploring structure–function relationships of both calmodulin and these neuropeptides under defined conditions.

Acknowledgments

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Registry No. EC 3.1.4.17, 9040-59-9; β_p-endorphin, 60149-45-3; NH_2-Tyr-D-Ala-Gly-Phe-Met-NH_2, 61090-95-7; β_p-endorphin peptide 1–16, 59004-96-5; β_p-endorphin peptide 1–17, 60893-02-9; β_p-endorphin peptide 1–19, 61669-59-8; β_p-endorphin peptide 1–21, 68078-29-5; β_p-endorphin peptide 1–23, 75222-01-4; β_p-endorphin peptide 1–25, 75222-00-3; β_o-endorphin peptide 1–27, 66954-40-3; β_p-endorphin peptide 10–19, 64847-02-5; β_p-endorphin peptide 14–23, 84741-72-0; β_p-endorphin peptide 14–25, 87281-35-4; β_p-endorphin peptide 14–27, 87281-36-5; β_h-endorphin peptide 2–31, 70037-07-9; β_p-endorphin peptide 8–31, 87308-14-3; β_p-endorphin peptide 12–31, 81539-46-0.

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Analysis of the Adrenal Angiotensin II Receptor with the Photoaffinity Labeling Method†

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ABSTRACT: The angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe, AT) receptor of bovine adrenocortex has been investigated with photosensitive analogues of AT. In a first series of experiments, we have shown that isolated cortical cells secrete aldosterone in a permanent and specific manner if they have been photolyzed in the presence of the photolabel [Sar^1,(4′-N_3)Phe^8]AT. This permanent stimulation is in contrast to the smooth muscle assays where under similar conditions a permanent and specific block was always observed. It is assumed that the irreversible occupation of the AT receptor produces this effect. In a second type of experiment, we have shown that the AT binding site on adrenocortical membranes can be specifically and irreversibly occupied under similar conditions and that this occupation can be prevented in a competitive manner by the presence of nonphotosensitive hormone. Using a radioactive label, [Sar^1,(3′-^{125}I)Tyr^4,(4′-N_3)Phe^8]AT, we have identified the AT receptor as a 300-kDa protein by means of gel filtration under nonreducing and nondenaturating conditions. Under reducing and denaturating conditions, a subunit of 60 kDa was found by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and gel filtration. The AT receptor is proposed to be a 300-kDa protein with one binding subunit of 60 kDa.

The isolation and purification of peptide hormone receptors are the goals of continuing research efforts. Until recently, all the successful isolations [e.g., Jacobs et al. (1977), Welton et al. (1977) and McIntosh et al. (1976)] were of receptors that retained hormone binding ability after solubilization of the cell membrane. This ability permitted the detection of the receptor protein fractions and its purification with affinity-mediated procedures (affinity chromatography, affinity centrifugation). Unfortunately, several peptide hormone receptors lose their binding ability upon total solubilization and cannot be isolated by these straightforward methods. For example, the homogenization, solubilization, and subsequent isolation of the angiotensin II (AT)[1] receptor has often been attempted, but with a few exceptions, preparations from adrenals and blood vessels have always lost AT affinity upon addition of detergent (Devynck et al., 1974; Forget & Heisler, 1979). AT and its receptor have affinities in the range of 10^{-9} M and rapid

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[1] Abbreviations: AT, angiotensin II, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe; RIA, radioimmunoassay; kDa, kilodaltons; BSA, bovine serum albumin; ^{3}H-AT, [Sar^1,(4′-NH_2,3′,5′-$^3H_2$)Phe^4]AT; N_3-AT, [Sar^1,(4′-N_3)Phe^8]AT; ^{125}I-N_3-AT, [Sar^1,(3′-^{125}I)Tyr^4,(4′-N_3)Phe^8]AT; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography.

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