Articles

Biological Properties of Two Models of Calcitonin Gene Related Peptide with Idealized Amphiphilic α-Helices of Different Lengths[†,‡]

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ABSTRACT: Previous studies on calcitonin gene related peptide (CGRP) have demonstrated that it has the characteristics of an amphiphilic peptide, and from an examination of the sequence, we have proposed that it contains an amphiphilic α-helix. We have synthesized two analogues of CGRP which have different lengths of idealized amphiphilic α-helical secondary structure. The first model, CGRM-1, has been substituted with residues generating an idealized amphiphilic α-helix in the region between residues 8 and 25, equivalent to approximately five turns of an α-helix. This peptide is not an agonist in any of our bioassays, but it does bind with low affinity to rCGRP receptors in crude liver membranes. Our second model, CGRM-2, has an idealized amphiphilic α-helix between residues 8 and 18, which is equivalent to approximately three turns of an α-helix. In an in vitro rat vas deferens assay, this peptide is an agonist with a potency one-fourth that of the native hormone. However, the potency of CGRM-2 in an adenylate cyclase assay is much lower, only $^1/_{140}$th the potency of CGRP. Both model peptides display amphiphilic characteristics commensurate with their design. We conclude that there is an amphiphilic α-helix in rCGRP between residues 8 and 18 and that this helix terminates in the vicinity of residue 18.

Many peptide hormones have regions of amphiphilic secondary structure which play important roles in their functions (Kaiser & Kezdy, 1983, 1984). We have previously synthesized and studied several analogues of calcitonin in an effort to probe the amphiphilic properties of that hormone and have demonstrated the importance of an amphiphilic α-helix to its function (Moe et al., 1983; Moe & Kaiser, 1985; Green et al., 1987). It was during our studies of calcitonin that we first became interested in the structure–function relationships of the recently discovered peptide calcitonin gene related peptide (CGRP).[1]

Calcitonin gene related peptide is a 37-residue peptide hormone, with a disulfide between cysteines at positions 2 and 7, and a carboxy-terminal phenylalanine amide. The existence of this peptide was first predicted from studies of alternate processing of RNA from the calcitonin gene (Amara et al., 1982). The peptide was subsequently found throughout the body, having been identified in the central nervous system and in peripheral nerves in close association with the cardiovascular system (Rosenfeld et al., 1983). CGRP has been shown to be one of the most common neuropeptides, having a particularly high occurrence in the spinal cord (Gibson et al., 1984). Binding sites for CGRP have been observed in many organs, including liver, spleen, heart, and peripheral blood vessels, and it has been shown to circulate in the blood (Zaidi et al., 1986). Given its widespread occurrence, it is no surprise that CGRP appears to play a role in the function of a number of different physiological systems, among the most interesting of which is peripheral blood vessel dilation (Brain et al., 1985).

In our studies, we have consistently used the rat form of the peptide (rCGRP), and all specific references to the properties of the peptide refer to this form. However, since there are only four amino acid differences between rat and human CGRP, we except the gross structural features of the peptides to be similar. Furthermore, it was recently determined that there are two closely related forms of this hormone, α and β, in both rat (Rosenfeld et al., 1984) and human (Steenbergh et al., 1985). The α and β forms differ by only one amino acid in the rat hormone and three amino acids in the human hormone. Our studies have all been with the α form of rCGRP.

When the sequence of CGRP was determined, it proved to have homologies to the calcitonins (Jonas et al., 1985), and subsequent work demonstrated that CGRP and salmon calcitonin I (sCT-I) cross-react with each other's receptors (Goltzman & Mitchell, 1985). Examination of the amino acid sequence of rCGRP suggests that it has potential to form an amphiphilic α-helix between residues 8 and 25 (Figure 1). Since our previous modeling studies with calcitonin had demonstrated the importance of an amphiphilic α-helix between residues 8 and 22, it seemed plausible that a longer amphiphilic α-helix might indeed be an important structural element in the longer peptide CGRP.

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[‡] This paper is dedicated to the memory of Dr. E. T. Kaiser, who was an inspiration to all who knew him.

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[1] Abbreviations: Boc, *tert*-butyloxycarbonyl; BSA, bovine serum albumin; Bzl, benzyl; CGRM-1, calcitonin gene related model 1; CGRM-2, calcitonin gene related model 2; DCC, dicyclohexylcarbodiimide; HOBt, hydroxybenzotriazole; rCGRP, rat calcitonin gene related peptide; SD, Sprague-Dawley; TFA, trifluoroacetic acid; TFE, trifluoroethanol; Tos, tosyl; Z, benzyloxycarbonyl.

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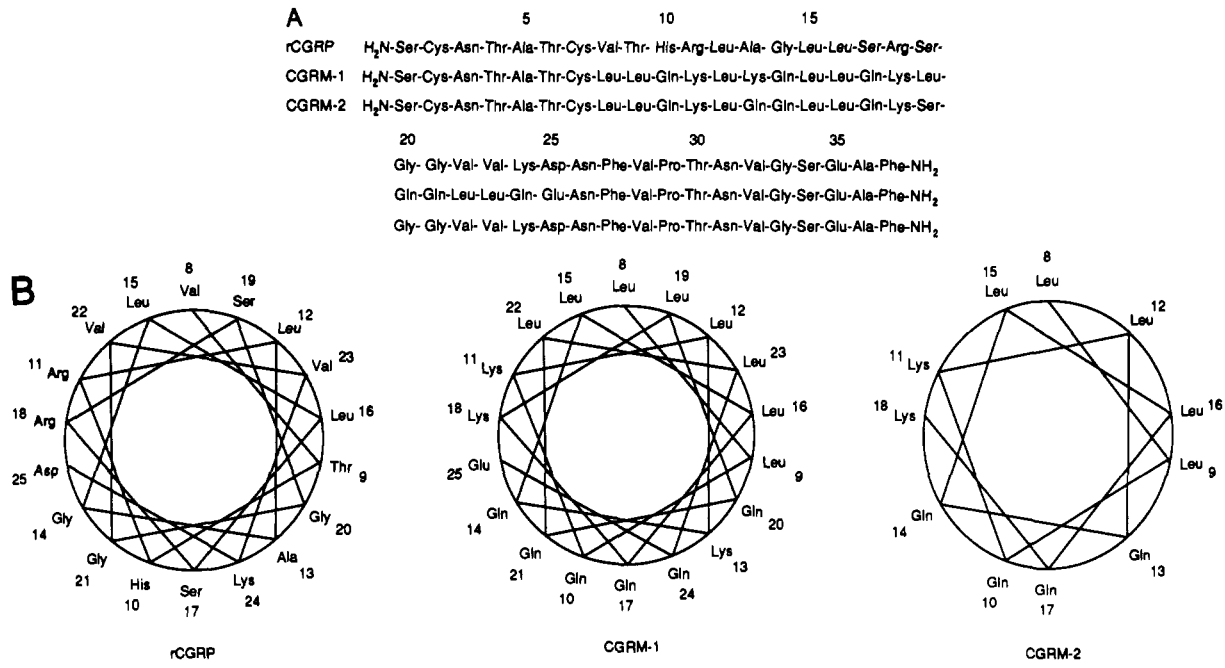

FIGURE 1: (A) Amino acid sequences of the peptides α-rCGRP, CGRM-1, and CGRM-2. (B) Helical wheel diagrams of rCGRP (8–18), CGRM-1 (8–25), and CGRM-2 (8–18).

After preliminary studies on the physical characteristics of rCGRP which established that it has the characteristics of an amphiphilic peptide, we initially proposed a structure that included the presence of an amphiphilic α-helix between residues 8 and 25 (Kaiser et al., 1985). Our first model peptide, CGRM-1, has amino acid substitutions generating a five-turn idealized amphiphilic α-helix in this region (Figure 1). When our preliminary biological studies proved that CGRM-1 was not an effective analogue of CGRP, we were forced to reconsider our structural hypothesis. Reexamining the sequence of CGRP, we realized that by far the greatest amphiphilic α-helical potential lay in the region of the sequence between residues 8 and 18. Our second model, CGRM-2, has substitutions only in the region 8 and 18 and should have a shorter, three-turn, amphiphilic α-helix (Figure 1). Thus, CGRM-2 was expected to be less amphiphilic than CGRM-1. We report here the synthesis of these two peptide analogues of CGRP, a discussion of their physical and biological properties, and a reevaluation of the initially proposed model for the structure of CGRP in light of these results.

Materials and Methods

Synthetic rCGRP was purchased from BaChem Inc., Torrance, CA. Protected amino acids were obtained from Peninsula Laboratories, San Carlos, CA. *p*-Methylbenzhydrylamine resin was from U.S. Biochemicals Corp., Cleveland, OH. $Na^{125}I$ was obtained from New England Nuclear Corp., Boston, MA. [α-^{32}P]ATP (10–50 Ci/mmol) and [2,8-^{3}H]cAMP (115 Ci/mmol) were from Amersham, Arlington Heights, IL. Male Sprague-Dawley (SD) rats were purchased from Charles River Breeding Laboratories, Wilmington, MA. All other chemicals and supplies were of high purity, from commercial sources.

CGRM-1 and CGRM-2 were prepared on a *p*-methylbenzhydrylamine-substituted polystyrene resin cross-linked with 1% divinylbenzene (Hruby et al., 1977). The peptides were synthesized by using symmetric anhydrides of Boc-Arg(N^{g}-Tos), Boc-Cys(*S*-4-MeOBzl), Boc-Glu(O^{γ}-Bzl), Boc-Gly, Boc-Leu, Boc-Lys(N^{ϵ}-2-ClZ), Boc-Phe, Boc-Pro, Boc-Ser(OBzl), Boc-Thr(OBzl), and Boc-Tyr(O-2,6-Cl_2Bzl). For Boc-Asn (Mojsov et al., 1980) and Boc-Gln (König & Geiger, 1970), the HOBt/DCC coupling method was used. Synthesis was done in a Beckman 990 peptide synthesizer using a coupling and deprotection program similar to that employed by Yamashiro and Li (1978), except that Boc-Gln was deprotected using 6 N HCl in dioxane for 30 min (Beyerman et al., 1973). Side chain deprotection and cleavage from the resin of both peptides were by the standard HF (hydrogen fluoride) method.

The peptides were extracted from the resin with 20% acetic acid and lyophilized, yielding the crude products. Oxidation and formation of the disulfide-bridged loop in both peptides were performed as follows: 200 mg of crude product was dissolved in approximately 50 mL of 20% CH_3CN in 0.05 M potassium phosphate buffer, pH 8.0, with 20 equiv of reduced dithiothreitol. The peptides were left to reduce overnight under N_2, diluted into 4 L of 0.05 M potassium phosphate buffer, pH 8.0, and oxidized with stirring, by the slow addition of potassium ferricyanide (in the same buffer) until yellow color persisted. The dilute solutions of the oxidized peptides were adjusted to pH 6.5 and loaded onto a Vydac C4 preparative reverse-phase HPLC column. The crude peptides adhered to the column and were eluted by using a gradient of 20–70% CH_3CN with 0.1% TFA. The collected peptides were lyophilized and purified by two passes through a Vydac C4 semipreparative reverse-phase HPLC column using an isocratic elution system of either 58% CH_3CN or 39% CH_3CN plus 0.1% TFA (for CGRM-1 and CGRM-2, respectively).

By reverse-phase HPLC, the purity was estimated to be >95%, and often >97%. On the basis of the amounts of crude peptides, the yields of pure peptide averaged approximately 15%. Amino acid analysis (hydrolysis in 6 N HCl for 24 h) of pure CGRM-1 yielded the following (calibrated to Val, since there are no Val–Val bonds in this molecule): Asx, 3.22 (3); Thr, 3.06 (3); Ser, 2.04 (2); Pro, 0.95 (1); Glx, 8.46 (8); Gly, 1.32 (1); Ala, 2.13 (2); Val, 2.00 (2); Cys, 1.58 (2); Leu, 7.62 (8); Phe, 1.93 (2); Lys, 2.70 (3). For CGRM-2, the results are (calibrated to Phe) as follows: Asx, 3.98 (4); Thr, 2.72 (3); Ser, 3.13 (3); Pro, 0.91 (1); Glx, 5.08 (5); Gly, 2.92 (3); Ala, 2.06 (2); Val, 2.95 (4); Cys, 1.46 (2); Leu, 4.48 (5); Phe,

2.00 (2); Lys, 2.64 (3). The value for Val was low, possibly due to the presence of Val–Val bonds in the peptide, which are resistant to hydrolysis. Mass spectral analysis using the ^{252}Cf fission fragment ionization method (Chait et al., 1981) was performed on both peptides. The result for CGRM-1 was an $(M + H)^+$ peak at 4147.3 (calculated = 4146.7; $\Delta = +0.6$), and for CGRM-2, it was an $(M + H)^+$ peak at 3937.6 (calculated = 3937.4; $\Delta = +0.2$).

Amino acid analysis (hydrolysis in 6 N HCl for 24 h) of rCGRP from BaChem yielded the following (calibrated to Phe): Asx, 4.05 (4); Thr, 3.67 (4); Ser, 3.84 (4); Pro, 0.94 (1); Glx, 1.11 (1); Gly, 4.35 (4); Ala, 3.08 (3); Val, 4.09 (5); Cys, 1.58 (2); Leu, 2.68 (3); Phe, 2.00 (2); His, 1.06 (1); Lys, 1.48 (1); Arg, 2.30 (2). (As with CGRM-2, the value for Val was low.) Mass spectral analysis using the ^{252}Cf fission fragment ionization method on the commercial rCGRP yielded an $(M + H)^+$ peak at 3806.5 (calculated = 3806.2; $\Delta = +0.3$). rCGRP from BaChem was checked for purity by C18 reverse-phase analytical HPLC, using a solvent system of 0.2 M phosphate buffer, pH 2.6, in the aqueous phase, and CH_3CN as an organic modifier. In this system, the peptide eluted as a single peak of greater than 99% purity.

Film balance studies on all peptides were performed employing a subphase of 0.16 M KCl/0.1 M Tris-HCl, pH 7.4, in glass-distilled water, on a Lauda film balance with an Apple IIE controller interface. Surface-active contaminants were removed by aspirating the surface of the buffer after bubbling air through the solution. Peptide was applied to the surface of the buffer from stock solutions with concentrations of approximately 0.2 mM (as determined by amino acid analysis), forming a monolayer at the air–water interface. This monolayer was then compressed by a moving barrier, yielding a force vs area isotherm.

Circular dichroism spectra of rCGRP, CGRM-1, and CGRM-2 were observed on an Aviv Model 60ds spectropolarimeter. Either the spectra were recorded in filtered 0.16 M KCl/0.02 M potassium phosphate buffer, pH 7.4, or alternately this buffer system plus 50% trifluoroethanol (TFE), which is a structure-inducing solvent (Greff et al., 1976). The spectrometer cells were soaked in a 2% aqueous poly(ethylene glycol) (M_r 15K–20K) solution for 1 h and rinsed with distilled water before use. For determination of the secondary structures of the peptides, spectra were recorded between 250 and 195 nm, at relatively high concentrations and in short path-length cells (0.5–1 mm). For low-concentration scans, 2-cm path-length cells were used. Concentrations of the stock solutions were determined by amino acid analysis; dilute peptide solutions made from the stock solutions had their concentrations confirmed by UV absorbance.

For molecular weight determination by ultracentrifugation, peptide or protein was dissolved in a buffer of 0.16 M KCl/0.02 M sodium phosphate, pH 7.4 with 7 mg/mL dextran T-40 added. Concentrations of peptides and of the protein used as a standard, egg white lysozyme from chicken (*N*-acetylmuramoylhydrolase; EC 3.2.1.17), were as follows: rCGRP, 4.8×10^{-4} M; CGRM-1, 1.4 mM; CGRM-2, 0.5 mM; lysozyme, 1.4×10^{-5} M. For each experiment, 100 μL of the respective solution was spun in a Beckman Spinco Airfuge at approximately 90 000 rpm for 24 h. The relative peptide concentration in each successive 10-μL aliquot was determined by derivatizing with fluorescamine after dilution into 2 mL of 0.08 M borate buffer, pH 9.0, and measuring the fluorescence intensity (λ_{ex} 390 nm, λ_{em} 480 nm) on a Perkin-Elmer 650-40 fluorescence spectrometer. Analysis of the concentration gradient generated gave the apparent molecular weight (Pollet et al., 1979).

rCGRP and the model peptides were tested in an isolated tissue assay for their ability to inhibit the electrically induced contractions of rat vas deferens as described by Ohhashi and Jacobowitz (1985). Paired vasa deferentia cut to a 1-cm length were suspended in 12-mL organ baths in a Krebs solution at 37 °C bubbled constantly with 95% O_2/5% CO_2 and stimulated by square voltage pulses of 0.5-Hz duration at 50 V for 1 ms. Vasa deferentia were stretched to an initial resting tension of 1*g* and were given approximately 1 h of stimulation, until a constant response was seen, before peptides were added to the bath. The peptides were applied in sequentially increasing concentrations at 5-min intervals, or until the change in contraction force (resulting from addition of peptide) had equilibrated, without washing between doses. Results are presented in Figure 3 as percent contraction vs log peptide concentration. CGRM-1 was tested for action as an antagonist by studying the effects of added rCGRP on rat vas deferens pretreated with 2.5 μM CGRM-1. CGRM-1 was added to the vas deferens 10 min before initiation of an experiment; in all other ways, the experiment was performed as above.

The two model peptides, CGRM-1 and CGRM-2, were compared with rCGRP in a competitive liver membrane binding assay (Nakamuta et al., 1986). The chloramine T method was used to iodinate native rCGRP with $Na^{125}I$ (Hunter & Greenwood, 1962) to a specific activity of approximately 50 μCi/μg. Iodinated rCGRP was purified by ion-exchange chromatography on SP-Sephadex C-25 and stored until needed in 100-μL aliquots at −20 °C. Liver sections were removed from Sprague-Dawley rats (125–150-g rat weight) and homogenized with a 10-fold volume of 5 mM Tris-HCl buffer (pH 7.4). The crude liver homogenate was centrifuged at 50000*g* for 30 min. The resultant pellet was washed by suspension in the above buffer and centrifugation as above. This process was repeated a total of 3 times. The pellet either was used on the day of preparation or was suspended in the above buffer containing 0.25 M sucrose and stored at −80 °C (with no effect on the assay of using frozen membranes). On the day of use, the frozen pellet was washed twice as above. The final pellets were suspended in 50 mM Tris-HCl at pH 7.4.

Incubations were performed in microfuge tubes containing an assay mixture of 50 mM Tris-HCl (pH 7.4), 1% BSA, 0.1% bacitracin, 0.025% lactose, and 2 KIU/mL aprotinin, plus ^{125}I-rCGRP (approximately 20 000 cpm), varying amounts of unlabeled peptide, and crude membrane preparation (in a range of 1.6–1.9 mg of protein per tube), in a total volume of 0.5 mL. The tubes were shaken at 4 °C for 2 h. The assay was terminated by centrifuging the tubes in a Beckman microfuge for 4 min and then counting the pellets. Specific binding as a percent of total binding averaged 80% for these experiments (nonspecific binding was measured in the presence of 1.8×10^{-6} M rCGRP, and total binding was measured in the absence of cold peptide ligand). The results are reported in Figure 4 as percent binding of specifically bound ^{125}I-rCGRP vs log of added peptide.

Stimulation of adenylate cyclase in partially purified spleen membranes was studied using a membrane preparation as described by Sigrist et al. (1986) with an assay similar to that reported by Marcus et al. (1971). The reaction was in a final volume of 100 μL containing 25 mM Tris-HCl (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 5 mM $MgCl_2$, 1 mM [α-^{32}P]ATP (specific activity 90–130 cpm/pmol), 1 mM [2,8-^3H]cAMP (41 000 cpm per

tube), and an ATP-regenerating system of 20 μg/mL myokinase, 20 mM creatine phosphate, and 0.2 mg/mL creatine kinase, plus approximately 13 μg of membrane protein and varying amounts of peptide.

The reaction was started by the addition of membrane and was run at 37 °C for 20 min. Termination of the reaction was by the addition to the sample of 100 μL of ice-cold "stop" solution containing 40 mM ATP, 10 mM cAMP, and 1% sodium dodecyl sulfate, followed by boiling of the sample for 4 min. cAMP was isolated according to the procedure of Birnbaumer et al. (1976). The mean basal activity was 83.5 pmol of cAMP formed mg^{-1} min^{-1}, and the mean maximal stimulation obtained in the presence of 1.0×10^{-7} M rCGRP was 206 pmol of cAMP produced mg^{-1} min^{-1}. Results are presented in Figure 5 as (net activity of adenylate cyclase/net maximal activity adenylate cyclase) vs log peptide concentration.

Results

Design of the Model CGRP Peptides. A section of rCGRP was modeled by examining the sequence, hypothesizing an amphiphilic structure, and replacing the native amino acids with a combination of Leu for residues in hydrophobic regions and Gln and Lys for residues in hydrophilic regions. In general, we attempted to maintain the charge balance of the portions modeled (specifically, we replaced Arg residues in hydrophilic regions with Lys residues). The residues Leu, Gln, and Lys were chosen because of their high propensity for forming α-helical secondary structure (Chou & Fasman, 1978). The region 8–18 in CGRM-2 has the same sequence as the corresponding sequence in CGRM-1, except that residue 13 has the more conservative substitution from native rCGRP of Ala_{13}-Gln_{13}, rather than the substitution Ala_{13}-Lys_{13} made in CGRM-1.

Film Balance. Like other amphiphilic substances, many peptides and proteins form insoluble monolayers at the air–water interface. At low surface pressures, between approximately 0 and 2 dyn/cm, the force vs area isotherms of these monolayers can be analyzed according to the equation of state:

$$\pi(A - A_\infty) = n_c RT \quad (1)$$

where π is the force (in dynes per centimeter), A is the area of the monolayer in centimeters squared, A_∞ is the limiting area of the monolayer extrapolated to zero surface pressure, n_c is the apparent number of moles, R is the gas constant, and T is the temperature in degrees kelvin (Adamson, 1967). Equation 1 is a two-dimensional form of the ideal gas law. At higher surface pressures, an empirically based modification of eq 1 must be used:

$$\pi[(A - A_\infty)(1 - \kappa\pi)] = n_c RT \quad (2)$$

where κ is simply a constant reflecting the compressibility of the monolayer (Fukushima, 1980). The isotherms were analyzed between force values of 5 and 10 dyn/cm by nonlinear least-squares fit to eq 2.

The three peptides all form insoluble monolayers at the air–water interface. Collapse pressures are as follows: 11 dyn/cm for rCGRP [as previously reported (Kaiser et al., 1985)]; 15 dyn/cm for CGRM-2; and 30 dyn/cm for CGRM-1. Analysis of the force vs area isotherm yielded parameters for rCGRP of A_∞/molecule = 818 Å^2 (and A_∞/residue = 22.1 Å^2), κ = 0.017 cm/dyn, and n/n_c = 4.1. CGRM-2 gives values of A_∞/molecule = 855 Å^2 (A_∞/residue = 23.1 Å^2), κ = 0.016 cm/dyn, and n/n_c = 3.2. The corresponding values for CGRM-1 are A_∞/molecule = 696 Å^2 (A_∞/residue = 18.8 Å^2), κ = 0.015 cm/dyn, and n/n_c = 5.1.

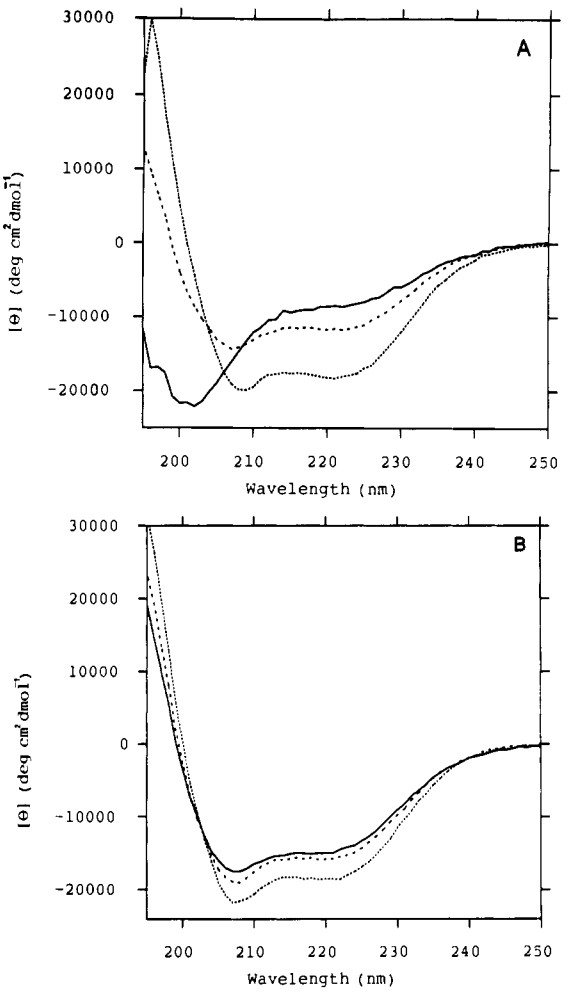

FIGURE 2: (A) CD spectra of rCGRP (—) (9.9×10^{-5} M), CGRM-1 (···) (4.8×10^{-5} M), and CGRM-2 (- - -) (7.8×10^{-5} M) in aqueous buffer. (B) CD spectra of rCGRP (—) (9.9×10^{-5} M)8 CGRM-1 (···) (4.8×10^{-5} M), and CGRM-2 (- - -) (7.8×10^{-5} M) in buffer plus 50% TFE.

The quantity n/n_c is the moles of peptide applied divided by the apparent number of moles of peptide obtained in eq 1. A_∞/molecule is the area the peptide occupies at the interface, and A_∞/residue is the area occupied per residue.

The ability to form stable monolayers with moderate to high collapse pressures has been correlated with the ability of a peptide to form a stable amphiphilic secondary structure (either α-helix or β-sheet) (DeGrado & Lear, 1985). The progressively increasing collapse pressures for the three peptides in the order rCGRP < CGRM-2 < CGRM-1 are consistent with, and probably result from, our design of CGRM-2 as slightly more amphiphilic, and CGRM-1 as much more amphiphilic (largely through an increase in amphiphilic α-helix length) than the native rCGRP. The values of κ are quite similar for the peptides. The limiting areas are most similar for rCGRP and CGRM-2, with CGRM-1 occupying the least amount of space at the air–water interface. The A_∞/residue values suggest that rCGRP and its analogues are proportionately larger at the air–water interface than the calcitonins studied by our group. Salmon calcitonin (sCT) and model calcitonins 1 and 2 (MCT-1 and MCT-2) had A_∞/residue values of 17.5, 13.6, and 11.3 Å^2, respectively (Moe, 1985). This is suggestive of a significantly different structure at the air–water interface for rCGRP and its analogues compared to sCT and its idealized amphiphilic analogues.

Circular Dichroism. The CD spectra (Figure 2) were analyzed between either 250 and 195 nm or 240 and 195 nm

Table I: Percent Secondary Structure Calculated from Circular Dichroism by the Method of Provencher and Glöckner (1981)

peptide	α-helix	β-sheet	β-turn	remainder
rCGRP (aq)	26	31	12	30
rCGRP (TFE)	48	22	3	27
CGRM-1 (aq)	75	0	0	25
CGRM-1 (TFE)	63	18	8	11
CGRM-2 (aq)	36	23	14	27
CGRM-2 (TFE)	51	16	6	27

[a] aq is aqueous buffer, and TFE is 50% trifluoroethanol.

(depending on the method), using four common methods for the estimation of secondary structure from CD spectra: Chang et al. (1978), Greenfield and Fasman (1969), Hennesey and Johnson (1981), and Provencher and Glöckner (1981). Methods of secondary structure calculation were rejected if they produced theoretical spectra that deviated significantly from the experimental spectra. Applying these criteria leads to the selection of the method of Provencher and Glöckner as the best for this group of peptides, and it has been suggested that this is the most accurate of the above methods (Wollmer et al., 1983). The estimates of secondary structure using the method of Provencher and Glöckner as implimented through the computer program CONTIN (Provencher, 1979) are shown in Table I.

In aqueous buffer, the relative amounts of α-helix calculated follow the series rCGRP < CGRM-2 < CGRM-1. Both CGRM-1 and CGRM-2 show an apparent concentration dependence in their CD spectra in aqueous buffer, while rCGRP does not. The mean residue molar ellipticity at 222 nm ($[\theta]_{222}$) is closely correlated with the percentage of α-helix in a peptide, and thus we report the following values of $[\theta]_{222}$ (in degrees centimeter squared per decimole) for the three peptides at high and low concentrations: the values for rCGRP vary from $[\theta]_{222} = -8546$ (9.9×10^{-5} M) to $[\theta]_{222} = -8065$ (3.6×10^{-7} M); those for CGRM-1 vary from $[\theta]_{222} = 18\,019$ (4.8×10^{-5} M) to $[\theta]_{222} = -15\,280$ (5.0×10^{-7} M); and those for CGRM-2 vary from $[\theta]_{222} = -11\,680$ (7.8×10^{-5} M) to $[\theta]_{222} = -6677$ (8.0×10^{-7} M). CGRM-2 shows the greatest change in this concentration range, with CGRM-1 showing a lesser change and rCGRP a small change (approximately 6%) that is probably not significant. This is in agreement with evidence from the ultracentrifuge studies that both model peptides aggregate in aqueous solution at higher concentrations. The differences in spectra of rCGRP and CGRM-2 in aqueous buffer are almost certainly due to this aggregation of the somewhat more amphiphilic CGRM-2 (which has a higher collapse pressure in our film balance studies) under these conditions. The CD spectra for rCGRP and CGRM-2 in 50% TFE are similar. Both are estimated to be approximately 50% α-helical, implying a similarity of structure, but this is more than the 30% α-helix expected in the structure.

The spectra of CGRM-1 in aqueous buffer and in 50% TFE are very similar. In TFE, CGRM-1 appears to show a slight decrease in α-helical secondary structure; however, in both environments, CGRM-1 displays the greatest amounts of α-helix of any of the peptides. The region of the peptide engineered for an amphiphilic α-helix structure is from residue 8 to 25, or 49% of the sequence; thus, the 63% α-helix estimated to be present in TFE is more than expected. It is interesting that all three peptides are estimated to have approximately 15–20% more α-helix in TFE than is accounted for by the putative amphiphilic α-helical regions. In conclusion, the CD spectra of the models in 50% TFE can be rationalized on the basis of our idealized structural models, with rCGRP and CGRM-2 being the most similar and CGRM-1 having the greatest percentage of α-helix.

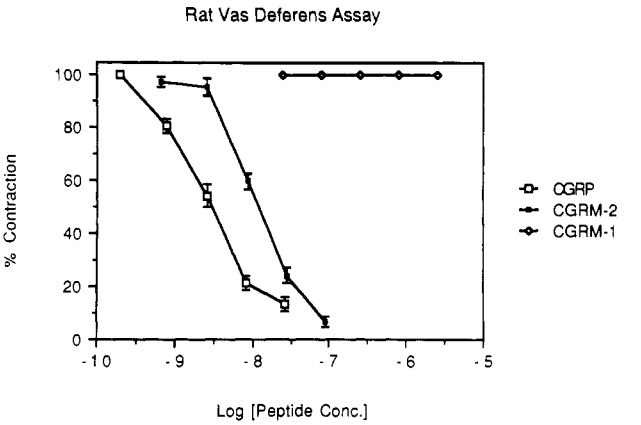

FIGURE 3: Inhibition of the electrically stimulated contraction of rat vas deferens smooth muscle by rCGRP (□), CGRM-2 (■), and CGRM-1 (◊). Each point is the mean of seven experiments. Error bars represent the SE.

Ultracentrifuge. All three peptides were centrifuged at high concentrations in order to observe the presence of absence of aggregation in aqueous buffer. rCGRP yields a molecular weight of 3279 ± 449 (0.86 times the expected molecular weight). CGRM-1 has a molecular weight of 14 201 ± 597 (3.3 times the expected molecular weight), while CGRM-2 gives a molecular weight of 9527 ± 943 (2.2 times the expected molecular weight). Lysozyme, analyzed as a control, gives a molecular weight of 13 998 ± 309 (0.98 times the expected molecular weight). This method is normally not considered accurate for peptides or proteins below a molecular weight of approximately 6000, which probably accounts for the anomalously low value for rCGRP, and thus the results for rCGRP are primarily evidence that the peptide does not aggregate into a larger molecular weight species. The results from this experiment are consistent with rCGRP existing as a monomer, CGRM-1 aggregating into a trimer and CGRM-2 aggregating into a dimer at the concentrations studied.

Rat Vas Deferens Assay. rCGRP is a potent inhibitor of electrically induced contractions of the isolated rat vas deferens. The results of this assay are presented in Figure 3. The order of potency of the peptides studied is rCGRP > CGRM-2. In the concentration range tested (20–20 000 nM), CGRM-1 is not an agonist. Half-maximal inhibition of contractions occurred at concentrations of rCGRP = 3.0 nM and CGRM-2 11.5 nM. Thus, in this assay, CGRM-2 is approximately one-fourth the potency of rCGRP. CGRM-1 was tested against CGRP in this assay for possible antagonism, but none was observed at concentrations of CGRM-1 of 2.5 μM.

Liver Binding Assay. Liver was chosen for binding studies because (1) it is representative of the visceral organs studied (Nakamuta et al., 1986) with similar K_d's and EC_{50} values for ^{125}I-rCGRP displacement from receptors to those reported for spleen and lung, (2) there are no limitations on tissue availability, and (3) it may be more relevant to systemic physiology than binding studies in the CNS. The results are shown in Figure 4 and parallel the results in the rat vas deferens assay with an important difference: CGRM-1 is active in this assay. Relative potencies are rCGRP > CGRM-2 > CGRM-1. Half-maximal inhibition of ^{125}I-rCGRP binding occurred at 3 nM rCGRP, 33 nM CGRM-2, and 520 nM CGRM-1. In this assay, CGRM-2 has approximately one-tenth the binding affinity of rCGRP. This contrasts with the relative agonist potencies in the rat vas deferens assay, where CGRM-2 has roughly one-fourth the potency of rCGRP.

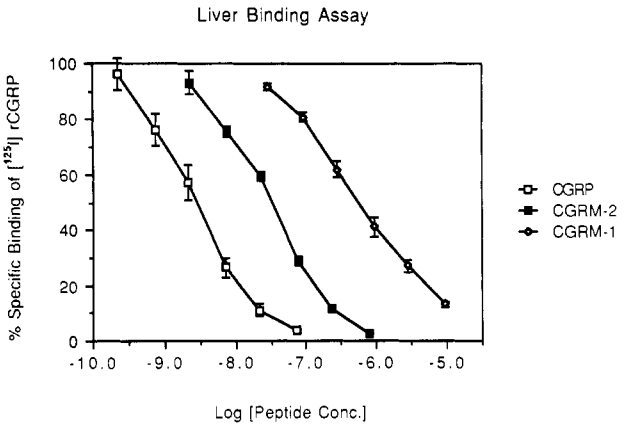

FIGURE 4: Competitive inhibition of ^{125}I-rCGRP binding to crude liver membranes by rCGRP (□), CGRM-2 (■), and CGRM-1 (◊). Each point is the mean of three triplicate determinations. Error bars represent the SE.

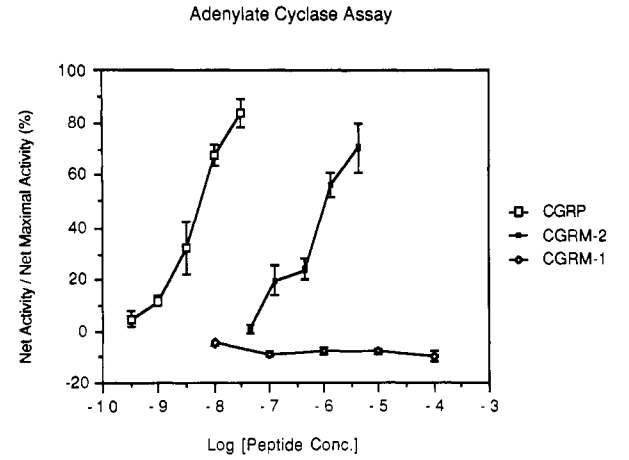

FIGURE 5: Stimulation of adenylate cyclase in spleen membrane fractions by rCGRP (□), CGRM-2 (■), and CGRM-1 (◊). Adenylate cyclase activity is presented as the enzyme activity above basal (net activity) divided by the maximal stimulated enzyme activity above basal (net maximal activity) × 100. Each point represents the mean of two determinations from two separate experiments. Error bars represent the SE.

Surprisingly, CGRM-1 binds to receptors, albeit with an affinity 170 times less than rCGRP.

Spleen Adenylate Cyclase Assay. This assay tests the ability of native rCGRP and our model peptides to activate adenylate cyclase in spleen. In spleen tissues and in rat aortic smooth muscle cells (Kubota et al., 1985), rCGRP is known to activate adenylate cyclase, yet in other tissues, most notably from the CNS, it does not appear to act via the adenylate cyclase system (Goltzman & Mitchell, 1985). The results of a spleen-based adenylate cyclase assay are shown in Figure 5. Again, there is a relative potency of rCGRP > CGRM-2 (with CGRM-1 not stimulating adenylate cyclase above basal levels of activity in the concentration range studied). Half-maximal stimulation of adenylate cyclase activity was observed with 5.6 and 784 nM rCGRP and CGRM-2, respectively. CGRM-2 displays is weakest relative potency in this assay, being approximately 140 times less potent than rCGRP. As can be seen in Figure 5, CGRM-1 appears to depress the activity of adenylate cyclase in the spleen membranes below basal levels. In a preliminary test of the antagonist potential of CGRM-1, we studied the ability of rCGRP to stimulate adenylate cyclase at concentrations up to 2.5×10^{-7} M in the presence of CGRM-1 (at a concentration of 4.8×10^{-5} M). There was apparent stimulation of adenylate cyclase activity by rCGRP, but the stimulation observed occurred at a relatively higher concentration of rCGRP than in the absence of CGRM-1 (data not shown). We again observed depression of activity of adenylate cyclase to below basal levels by CGRM-1, and this effect suggests that there is a possible disruption of the membrane-based adenylate cyclase system by the highly amphiphilic CGRM-1, which makes it difficult to interpret the results of the antagonism assay.

Discussion

In our initial analysis of the sequence of CGRP, we proposed an amphiphilic α-helical secondary structure in the sequence between residues 8 and 25, or for five turns of an α-helix, realizing that the strongest potential for α-helix formation was in the N-terminal segment, between residues 8 and 18. The reason for this is apparent from examination of the sequence: the residues following residue 17 are Arg-Ser-Gly-Gly-Val. It is well-known that glycine residues are "helix breakers" (Chou & Fasman, 1977), and the combination of two in a row is especially unfavorable for helix formation. When CGRM-1 proved to be a poor analogue of CGRP, not acting as an agonist in either the rat vas deferens assay or the adenylate cyclase assay, our suspicion fell on the 19–25 region of the native peptide as playing an important role in the function of CGRP. We therefore synthesized a new model, CGRM-2, with a shorter idealized amphiphilic α-helix of approximately three turns between residues 8 and 18 and, equally important, with retention of the native sequence between residues 19 and 25 (Ser-Gly-Gly-Val-Val-Lys-Asp). Our alternate hypothesis for the structure of CGRP was confirmed when this new peptide proved to be an agonist with rCGRP-like activity.

The monolayer and CD studies of the peptides support our hypothesis for the amphiphilic α-helix structure of rCGRP. In the pharmacological assays, we see that CGRM-2 is a better analogue of rCGRP than is CGRM-1. CGRM-2 is a potent agonist in the rat vas deferens assay, while CGRM-1 has no agonist or antagonist activity at concentrations of up to 1×10^{-6} M. In the liver-based binding assay, CGRM-2 shows a high affinity for the CGRP receptor, while CGRM-1 also binds, but with a much reduced affinity compared to rCGRP, and, finally, it is in the spleen-based adenylate cyclase assay that the weakest relative potency of CGRM-2 vs rCGRP is observed, and again we note the lack of any agonist ability of CGRM-1.

The observed variation in potency ratios in the three assay systems studied raises the question of whether this might be accounted for by receptor heterogeneity. These differences would normally be considered to be evidence of such if the potency ratios reflect only differences in receptor properties in the three tissues studied [for a discussion, see Kenakin (1984)]. Since these assays measure different properties in differing tissue preparations (an isolated whole tissue, a whole liver homogenate, and a partially purified membrane fraction), it is not necessarily true that the potency ratios in these assays reflect only the properties of the receptors. The relatively small differences in the potency ratios observed in the rat vas deferens assay, compared to the liver binding assay, are likely to be accounted for by variations in intrinsic efficacy of the ligands, or by different rates of ligand degradation in the two preparations [potency ratio differences between isolated tissue and binding studies have been reported; see Kenakin (1984)]. The spleen adenylate cyclase assay result is harder to explain, since the system appears to resemble the kidney binding assay, with the addition of receptor coupling to the second messenger adenylate cyclase complex, and yet shows a large difference in the ratio of the potencies of rCGRP and CGRM-2. This

observation could be accounted for by the presence of a different receptor in the spleen, but further experiments will be required to determine if this is true.

Our working model of the structure of CGRP is currently as follows: there is an N-terminal disulfide-bridged loop between residues 2 and 7, followed by an amphiphilic α-helix between residues 8 and 18, which probably terminates in a chain reversal in the region 18–23. Currently, the structure of the carboxyl-terminal region between residues 24 and 37 is unclear, but since no fragment of CGRP has yet been found to be active biologically, it is likely to play an important role in this hormone's action. According to the predictive methods of Chou and Fasman (1978), the section of the peptide between 18 and 23 appears to have a high potential for β-turn formation, with the sequence Arg-Ser-Gly-Gly (18–21) having the highest β-turn potential. It seems likely that the amphiphilic α-helix of CGRP terminates in this section, possibly as a β-turn. A chain reversal in the vicinity of residues 18–23 may play a role in the formation of the peptide into a compact structure at the receptor.

Whether this segment serves a purely structural role (i.e., by forcing a chain reversal) or, alternatively, whether the side chains of these residues might interact specifically with binding sites on the receptor has yet to be determined. Since sCT-I is a weak agonist of CGRP, but has no sequence homology to rCGRP in the region 18–25, we favor the structural hypothesis. It is interesting that when the sequences of salmon calcitonin and CGRP are aligned for maximum homology, there is a requirement for a five-residue insert between residues 19 and 23 (Gibson et al., 1984)—and this is exactly the region of the molecule that our studies identify as crucial for potency. We are preparing further analogues to study the properties of this section of the molecule.

In conclusion, we suggest that our approach to modeling peptide structures allows systematic distinction between alternate hypotheses concerning the length of a secondary structural unit in a peptide hormone. Furthermore, we emphasize the connection to the larger calcitonin–CGRP peptide hormone family. Our studies of CGRP complement our growing understanding of the structure of calcitonin. Previous work has identified in calcitonin a requirement for an amphiphilic α-helix in the region 8–22, which we believe terminates on the C-terminal side of residue 22 (Moe & Kaiser, 1985; Green et al., 1987). It appears that there are common structural elements in these two peptides: both require amphiphilic α-helices beginning at residue 8 but having different lengths and termination sequences.

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Stability and Covalent Modification of P-Glycoprotein in Multidrug-Resistant KB Cells

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ABSTRACT: An antipeptide antibody (P7) to P-glycoprotein has been produced by immunizing rabbits with a synthetic peptide. Antibody P7 is directed against the amino-terminal region of P170 (residues 28–35). The antibody immunoprecipitates a 170-kDa P-glycoprotein from extracts of drug-resistant KB-V1 cells that is not present in the drug-sensitive cell line KB-3-1. Antibody P7 was used to quantitate the amount of P-glycoprotein present in drug-resistant KB lines at various levels of resistance and to demonstrate the presence of P-glycoprotein in NIH 3T3 cells transfected with a cloned *MDR*1 cDNA or human genomic DNA encoding *MDR*1. Pulse–chase labeling experiments demonstrated that P-glycoprotein is synthesized as a 140-kDa precursor which is slowly converted over 2–4 h to a 170-kDa glycoprotein. Tunicamycin treatment blocks the conversion of the precursor to the mature form, and removal of N-linked oligosaccharides with Endo F reduces the relative molecular weight of P-glycoprotein to 140K. The mobility of mature P-glycoprotein is unaffected by treatment with neuraminidase and Endo H. These data indicate that P-glycoprotein is N-glycosylated and contains little or no neuraminic acid. P-Glycoprotein is also phosphorylated, and the extent of phosphate incorporated is proportional to the amount of protein present in drug-resistant cells.

Multidrug resistance (MDR) is the tissue culture phenomenon whereby cells, selected for resistance to a single drug, develop cross-resistance to multiple, structurally unrelated agents (Pastan & Gottesman, 1987; Riordan & Ling, 1985). A similar multidrug resistance phenotype occurs in human tumors and presents a major obstacle to successful cancer chemotherapy. To study this problem, we have developed MDR tissue culture cell lines resistant to colchicine, adriamycin, and vinblastine (Akiyama et al., 1985; Shen et al., 1986a). The characteristic features of multidrug-resistant cells are (1) decreased drug accumulation due to increased drug efflux (Fojo et al., 1985a; Inaba et al., 1979; Willingham et al., 1986), (2) reversibility of multidrug resistance by verapamil (Inaba et al., 1979; Tsuruo et al., 1982; Willingham et al., 1986), and (3) increased expression of the *MDR*1 gene (Fojo et al., 1985b; Gros et al., 1986a; Riordan et al., 1985; Roninson et al., 1984, 1986; Scotto et al., 1986; Shen et al., 1986b; Van der Bliek et al., 1986). This gene encodes a 170000-dalton plasma membrane glycoprotein, termed P-glycoprotein or P170 (Kartner et al., 1983). Recently, multidrug resistance has been transferred to drug-sensitive cells by *mdr* gene sequences (Debenham et al., 1982; Gros et al., 1986b; Shen et al., 1986c) and *mdr* cDNA (Gros et al., 1986c; Ueda et al., 1987). These results indicate that the presence of P-glycoprotein confers multidrug resistance.

Sequence analysis of the *MDR*1 gene indicates that human P-glycoprotein is composed of 1280 amino acids and consists of 2 homologous halves (Chen et al., 1986; Gros et al., 1986d). Each half of the molecule has six hydrophobic transmembrane domains, and each has a nucleotide binding site. The homology of the nucleotide binding regions to several bacterial transport proteins (Chen et al., 1986; Gerlach et al., 1986; Gros et al., 1986d), taken together with the plasma membrane localization of the protein (Willingham et al., 1987) and its ability to bind chemotherapeutic agents (Cornwell et al., 1986), suggests that P-glycoprotein could function as an energy-dependent efflux pump.

To elucidate the steps in the synthesis of P-glycoprotein and to define functional domains of this protein, it would be useful to have a panel of monospecific antibodies to various regions of the molecule. To date, three monospecific antibodies have been reported: C219, which recognizes an intracytoplasmic region in the COOH-terminus (Kartner et al., 1985), and MRK16 (Hamada & Tsuruo, 1986) and 265/F4 (Lathan et al., 1985), which recognize external determinants of P-glycoprotein which have not been identified. In the current work, we have prepared an antipeptide antibody based on the deduced amino acid sequence of the cloned human P-glycoprotein and used it to study the biosynthesis of P-glycoprotein in multidrug-resistant human KB cells.

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