Structure−Activity Studies on Position 14 of Human α-Calcitonin Gene-Related Peptide

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A structure−activity study was performed to examine the role of position 14 of human α-calcitonin gene-related peptide (h-α-CGRP) in activating the CGRP receptor. Interestingly, position 14 of h-α-CGRP contains a glycyl residue and is part of an α-helix spanning residues 8−18. Analogues [Ala^{14}]-h-α-CGRP, [Aib^{14}]-h-α-CGRP, [Asp^{14}]-h-α-CGRP, [Asn^{14}]-h-α-CGRP, and [Pro^{14}]-h-α-CGRP were synthesized by solid phase peptide methodology and purified by RP-HPLC. Secondary structure was measured by circular dichroism spectroscopy. Agonist activities were determined as the analogues' ability to stimulate amylase secretion from guinea pig pancreatic acini and to relax precontracted porcine coronary arteries. Analogues [Ala^{14}]-h-α-CGRP, [Aib^{14}]-h-α-CGRP, [Asp^{14}]-h-α-CGRP, and [Asn^{14}]-h-α-CGRP, all containing residues with a high helical propensity in position 14, were potent full agonists compared to h-α-CGRP in both tissues. Interestingly, replacement of Gly^{14} of h-α-CGRP with these residues did not substantially increase the helical content of these analogues. [Pro^{14}]-h-α-CGRP, predictably, has significantly lower helical content and is a 20-fold less potent agonist on coronary artery, known to contain CGRP-1 receptor subtypes, and an antagonist on pancreatic acini, known to contain CGRP-2 receptor subtypes. In conclusion, the residue in position 14 plays a structural role in stabilizing the α-helix spanning residues 8−18. The α-helix is crucial for maintaining highly potent agonist effects of h-α-CGRP at CGRP receptors. The wide variety of functional groups that can be tolerated in position 14 with no substantial modification of agonist effects suggests the residue in this position is not in contact with the CGRP receptor. [Pro^{14}]-h-α-CGRP may be a useful pharmacological tool to distinguish between CGRP-1 and CGRP-2 receptor subtypes.

Calcitonin gene-related peptide (CGRP)[1] was first predicted to exist as a result of alternative, tissue-specific processing of the calcitonin gene mRNA.[2] Subsequently, two forms (α and *β*) have been identified in human and rat.[3,4] It is widely distributed in the central nervous system (CNS) and the peripheral nervous system, especially in sensory nerves.[5] CGRP activates cell surface receptors to produce an assortment of biological actions. Based on the affinity of the antagonist analogue h-α-CGRP (8−37) (human form), CGRP receptors have been classified into two subtypes called CGRP-1 which has a high affinity for h-α-CGRP (8−37) and CGRP-2 which has a low affinity for h-α-CGRP (8−37).[6] Since CGRP is a potent vasodilator,[7−9] one important physiological role is thought to be control of the tone of resistance blood vessels.[9,10] Additionally, other characterized biological actions are inhibition of gastric acid secretion,[11] stimulation of pancreatic amylase secretion,[12] cardiac acceleration,[13] inhibition of insulin-stimulated glycogen synthesis,[14] and regulation of calcium metabolism.[15]

h-α-CGRP is a 37-residue peptide with a disulfide bridge between positions 2 and 7 and a C-terminal phenylalanyl amide residue (Chart 1). Its secondary and tertiary structures are less well defined. CD spectroscopy suggests that approximately 20% of the sequence, or 8−10 residues, adopts an α-helical structure in buffered aqueous solutions between pH 3.5 and 9.0.[16,17] The helical content can rise to 60% in the presence of SDS, TFE, or HFIP.[16−19] NMR studies[20,21] show the disulfide bond bridges a well-defined N-terminal loop, which is followed by an amphiphilic α-helix approximately 10 residues long. The helix ends in a *β*-turn involving residues 19−22. The remaining C-terminal polypeptide chain has little secondary structure. The amphiphilic α-helix has been proposed to play a major role in the interaction of CGRP with its receptor.[19,20] Indeed, the greater potency of chicken CGRP over that of h-α-CGRP has been attributed to the presence of an aspartyl residue in position 14 of the chicken form versus a glycyl residue, a helix breaker, in the human form.[22,23] In the present study a detailed structure−function analysis was performed to further elucidate the role of this position. Secondary structure was determined by CD spectroscopy, and function was measured as the ability to stimulate amylase secretion from isolated guinea pig pancreatic acini and to relax porcine coronary arteries.

Results and Discussion

All peptides were made by Merrifield's solid phase peptide synthesis methodology as described in a previous report.[24] Briefly, *t*-Boc/benzyl-protected amino acid derivatives were coupled as preformed hydroxybenzo-

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Chart 1. Primary Structure of h-α-CGRP

Ala[1]-Cys[2]-Asp[3]-Thr[4]-Ala[5]-Thr[6]-Cys[7]-Val[8]-Thr[9]-His[10]-Arg[11]-Leu[12]-Ala[13]-Gly[14]-Leu[15]-Leu[16]-Ser[17]-Arg[18]-Ser[19]-Gly[20]-Gly[21]-Val[22]-Val[23]-Lys[24]-Asn[25]-Asn[26]-Phe[27]-Val[28]-Pro[29]-Thr[30]-Asn[31]-Val[32]-Gly[33]-Ser[34]-Lys[35]-Ala[36]-Phe[37]-NH_2

Table 1. Potency, Helical Content, and $\Delta\Delta G$ Values of h-α-CGRP and Its Position 14-Substituted Analogues

	guinea pig pancreatic acini		porcine coronary artery		α-helix content (%)		$\Delta\Delta G^d$
peptide	EC_{50} (nM)	RP[a]	EC_{50} (nM)	RP[b]	water[c]	50% TFE[c]	(kcal/mol)
h-α-CGRP	7.7 ± 2.7	1.00	3.84 ± 1.13		17.2	41.4	0.00 (Gly)
[Ala[14]]-h-α-CGRP	5.5 ± 1.3	1.40	6.87 ± 0.90	0.84	21.1	47.0	−0.77 (Ala)
[Aib[14]]-h-α-CGRP	15.9 ± 0.5	0.49	6.01 ± 0.86	0.55	22.5	35.5	−0.69 (Aib)
[Asp[14]]-h-α-CGRP	16.5 ± 4.3	0.47	7.58 ± 0.96	0.42	14.9	41.1	−0.15 (Asp)
[Asn[14]]-h-α-CGRP	28.7 ± 8.8	0.27	6.13 ± 0.85	0.50	15.5	39.9	−0.07 (Asn)
[Pro[14]]-h-α-CGRP	>1000	<0.01	72.40 ± 0.92	0.05	12.3	30.7	3.00 (Pro)

[a] RP: relative potency = EC_{50}(h-α-CGRP)/EC_{50}(analogue) when comparing data obtained in separate groups of cells (n = 5). [b] RP: relative potency = EC_{50}(h-α-CGRP)/EC_{50}(analogue) when comparing data obtained in paired rings from the same animal (n = 4). [c] α-Helix contents deconvoluted using Selcon.[32] [d] Calculated by substitution of amino acids into a guest position of a synthetic peptide which forms helical dimers in equilibrium with randomly coiled monomers. Equilibrium constants were derived to determine ΔG values. $\Delta\Delta G$ values were calculated by subtracting ΔG for each peptide from ΔG for the Gly-peptide.[34]

triazole active esters to *p*-MBHA resin. Peptides were freed of protecting groups, cleaved from the resin by the low−high TFMSA method of Tam,[25] and cyclized in high dilution with $K_3Fe(CN)_6$.[26] h-α-CGRP was purified, without incident, by gel filtration, low-pressure cation-exchange chromatography, and semipreparative RP-HPLC in an overall yield of 4.4%. Analogues [Aib[14]]-h-α-CGRP and [Pro[14]]-h-α-CGRP were purified to apparent homogeneity using this procedure.

During a second synthesis of h-α-CGRP, a strong cation-exchange HPLC column was used in place of the dextran-based low-pressure cation-exchange column. This silica-based HPLC column is extremely useful for the separation of naturally derived and synthetic peptides.[27] Analytical RP-HPLC showed h-α-CGRP content of the lyophilized material obtained from this column was 50%, a significant improvement over the 39% content previously found in the corresponding material isolated from the CM dextran chromatography. Semipreparative RP-HPLC from the second synthesis yielded h-α-CGRP in an improved overall yield of 5.2%. Subsequent purifications of h-α-CGRP and purification of analogues [Ala[14]]-h-α-CGRP, [Asp[14]]-h-α-CGRP, and [Asn[14]]-h-α-CGRP employed this cation-exchange HPLC column.

Purified products from all syntheses had satisfactory amino acid compositions and correct masses as determined by FAB-MS. Peptides were judged to be >98% pure by isocratic analytical C_{18} and C_4 RP-HPLC.

The far-UV CD spectra of h-α-CGRP and its position 14 analogues were obtained in 10 mM phosphate buffer. The characteristic pattern for α-helix includes a $n\pi^*$ transition at 222 nm and both components of the $\pi\pi^*$ transition at 192 and 208 nm. All three bands are present in the spectra of h-α-CGRP and all analogues, although they are quite weak for the aqueous samples. The water spectra also exhibit a significant amount of "random coil", evident by the blue shift of the negative maxima toward 200 nm.[28,29] Three different methods were used to determine the content of secondary structural characteristics in h-α-CGRP and its analogues, with particular interest in the α-helix. The methods used included the calculation of helix from $[\theta]_{222}$,[30] Prosec,[31] and Selcon.[32] All three methods resulted in varying α-helix content (data not shown). However, all three techniques ranked helix content in exactly the same order. The results from Selcon are shown in Table 1. In agreement with previous studies, h-α-CGRP had 17.2% helical content which is predicted to span residues 8−18.[16,20] The CD spectra of h-α-CGRP in 50% TFE/50% water (v/v), a solution known to stabilize nascent α-helices in peptides including h-α-CGRP,[16,17] were also obtained. The spectrum revealed the classical α-helical spectral characteristics mentioned above. As expected, α-helical content in h-α-CGRP increased to 41.4%. The increase may reflect α-helix formation in the C-terminal region of h-α-CGRP in addition to the 8−18 residues described above.[16]

The agonist properties of h-α-CGRP and its analogues were determined in porcine coronary artery and guinea pig pancreatic acini. h-α-CGRP relaxed isolated coronary arteries, precontracted with KCl, in a dose-dependent manner with a potency of 3.84 nM. This potency was similar to our previously reported potencies for the effects of h-α-CGRP on rat mesenteric artery and guinea pig pancreatic acini.[24]

[Ala[14]]-h-α-CGRP, [Aib[14]]-h-α-CGRP, [Asp[14]]-h-α-CGRP, and [Asn[14]]-h-α-CGRP were all full agonists on pancreatic acini and coronary artery. On pancreatic acini the most potent analogue, [Ala[14]]-h-α-CGRP, was equipotent with h-α-CGRP and [Asn[14]]-h-α-CGRP was the least potent with an EC_{50} value of 28.7 nM. When tested in paired rings taken from the same artery, [Ala[14]]-h-α-CGRP was also the most potent analogue on coronary artery, being equipotent with h-α-CGRP with an EC_{50} value of 6.87 nM. Potencies of [Aib[14]]-h-α-CGRP, [Asp[14]]-h-α-CGRP, and [Asn[14]]-h-α-CGRP were approximately 2-fold lower than that of h-α-CGRP. No correlation was found between the rank order of potency of analogues on pancreatic acini and coronary artery. Clearly, substitution of Gly[14] for Ala, Aib, Asp, or Asn does not abolish the agonist properties of h-α-CGRP at the CGRP receptor in these two tissues nor substantially change the potency of the resulting agonists.

No quantitative correlation was found between analogues' helical content and their potencies, in either tissue. Table 1 shows that in aqueous solutions helical content ranged from 22.5% in [Aib[14]]-h-α-CGRP to

14.9% in [Asp^{14}]-h-α-CGRP. Predictably, helical content rose in all analogues in solutions containing 50% TFE (Table 1), however, not proportionally. While [Aib^{14}]-h-α-CGRP had the highest helical content in aqueous solution, it had the lowest helical content in 50% TFE/50% water. [Ala^{14}]-h-α-CGRP had the highest helical content in 50% TFE/50% water. This was the only analogue with a greater helical content than h-α-CGRP under these conditions. Two measures of amino acid helical propensity do correlate well with the α-helix content observed in these analogues. The incidence of these amino acids in helical structures[33] and the $\Delta\Delta G$ values[34] shown in Table 1 both give identical trends for the helical propensity. Surprisingly, replacement of Gly^{14} of h-α-CGRP with residues that have much higher propensities for adopting helical conformations does not always result in peptides with greater helical content than h-α-CGRP. While Ala in position 14 increased helical content in aqueous and 50% TFE/50% water solutions, Asp and Asn in position 14 actually lowered the helical content, albeit marginally in both solutions. Presumably, the remaining components of the α-helix of h-α-CGRP infer sufficient stability to accommodate and maintain the Gly^{14} of h-α-CGRP in a helical conformation.

Atypical is [Aib^{14}]-h-α-CGRP, which has the highest helical content of all analogues in aqueous conditions and the lowest helical content of the above analogues in 50% TFE/50% water solution. Aib is capable of forming both 3_{10}-helix and α-helix.[35,36] Distinguishing 3_{10}-helix and α-helix has proven difficult in CD spectroscopy due to small differences in energy, possible length-dependent transitions from one form to the other, and amino acid compositional effects.[28,37,38] Although it should be possible to distinguish an extended 3_{10}-helix from an α-helix by CD spectroscopy,[39,40] quantitating relatively small amounts in a single peptide is problematic.[28,41] Studies using Fourier transform infrared spectroscopy[42] to distinguish these helix types failed because the concentration of the peptide in water needed for this experiment (approximately 25 mg/mL) was beyond the solubility limit of all analogues including [Aib^{14}]-h-α-CGRP. Thus, the inconsistencies in the Aib analogue may be the result of the inability to distinguish subtle structural variations at position 14.

The lower potency of [Asp^{14}]-h-α-CGRP in our studies is not entirely consistent with previous studies of Asp^{14}-containing CGRP peptides. Chicken CGRP, which contains an aspartyl residue in position 14, relaxes perfused rat mesenteric vascular beds with a 10-fold higher potency than h-α-CGRP.[23] However, chicken CGRP is only 2.5-fold more potent in increasing cAMP production in KS-4 cells[22] and slightly more potent in lowering serum calcium levels in vivo[15] compared with h-α-CGRP. Replacement of the glycyl^{14} residue of h-α-CGRP with aspartic acid, namely, [Asp^{14}]-h-α-CGRP, resulted in only a 2.5-fold increase in the potency of cAMP production and increased in vivo hypocalcemic activity.[15,22] These previously published potencies of Asp^{14}-containing CGRP peptides do not differ substantially (less than 10-fold) from the potencies found in our studies. Differences that do exist may be due to varying receptor numbers in different tissues, species variants of the CGRP receptor, or disproportional proteolytic breakdown of peptide analogues by tissue-derived proteases. In addition, the somewhat higher potency of chicken CGRP over h-α-CGRP may be a result of differences in primary structure at positions 3, 15, and 23.[15] Previous data are consistent with our finding that replacement of Gly^{14} of h-α-CGRP for a residue with a high helical propensity, such as Asp, has little effect on agonistic effects at the CGRP receptor.

Introduction of the helix breaker proline into position 14 causes a significant reduction in the helical content in both aqueous solutions and 50% TFE/50% water solutions (Table 1). Presumably, the prolyl residue disrupts the α-helix spanning residues 8−18 of h-α-CGRP. Interestingly, the helical content is similar to that found in h-α-CGRP (8−37),[43] a CGRP receptor antagonist. The lower helical content of [Pro^{14}]-h-α-CGRP had a profound effect upon the actions of this analogue. As shown in Table 1, [Pro^{14}]-h-α-CGRP did not stimulate amylase secretion from pancreatic acini but was a full agonist on coronary arteries. Its vasodilatory potency was 20-fold lower than that of h-α-CGRP. On pancreatic acini, 10^{-6} M [Pro^{14}]-h-α-CGRP caused a parallel rightward shift of the dose−response curve of h-α-CGRP. Thus, [Pro^{14}]-h-α-CGRP acts as a weak antagonist. From these data we calculated the affinity of [Pro^{14}]-h-α-CGRP as an antagonist which gave a K_B value of 325 nM.

CGRP-stimulated release of amylase from guinea pig pancreatic acini has been reported to be mediated by the CGRP-1 receptor subtype,[44] while relaxation of large coronary arteries is mediated by the CGRP-2 receptor subtype.[9] In our studies, the CGRP receptor selective antagonist h-α-CGRP (8−37) had a high affinity in pancreatic acini (K_B = 16.6 nM),[45] consistent with CGRP-1 receptors, and a low affinity in porcine coronary artery (K_B = 4.75 μM),[46] consistent with CGRP-2 receptors. These data are in agreement with previous studies of CGRP receptor subtypes in these tissues. [Pro^{14}]-h-α-CGRP is an agonist at CGRP-2 receptors in porcine coronary artery but has only antagonist properties in guinea pig pancreatic acini. While species differences, differences in metabolism, or other factors may explain these results, these data suggest that [Pro^{14}]-h-α-CGRP may be a useful pharmacological tool to differentiate between CGRP-1 and CGRP-2 receptor subtypes.

In conclusion, replacement of Gly^{14} of h-α-CGRP with Ala, Aib, Asp, or Asn, all residues with a high helical propensity, results in highly potent agonists of h-α-CGRP with similar secondary structure. Agonist potencies did not differ significantly, precluding a quantitative correlation with helical content. The lack of correlation between rank order of potency in each tissue, once again, may be the result of varying receptor numbers in different tissues, species variants of the CGRP receptor, or disproportional proteolytic breakdown of peptide analogues. The wide variety of functional groups tolerated in position 14 of these analogues suggests that this position is not in contact with the receptor. These residues stabilize the bioactive conformation of the peptide by contributing to the stability of the helix spanning residues 8−18. This helix is essential for high-potency agonist effects at the CGRP receptor on pancreatic acini and coronary artery. Disruption of the helix causes a large decrease in potency on coronary artery and antagonist effects on pancreatic acini. [Pro^{14}]-h-α-CGRP may be a useful pharmacologi-

cal tool for the characterization of CGRP-1 and CGRP-2 receptor subtypes.

Experimental Section

N-α-Boc amino acid derivatives were purchased from Bachem (Torrence, CA) and Applied Biosystems (Foster City, CA). Reactive side chains of amino acids were protected as follows: Arg, mesitylene-2-sulfonyl (Mts); Asp, benzyl ester (Bzl); Cys, *p*-methoxybenzyl (4-MeOBzl); His, dinitrophenyl (Dnp) or (benzyloxy)methyl (Bom); Lys, 2-chlorocarbobenzoxy (2-Cl-Z); Ser, benzyl ether (Bzl); Thr, benzyl ether (Bzl); Trp, formyl (CHO); Tyr, 2-bromocarbobenzoxy (2-Br-Z). Different batches of *p*-methylbenzhydrylamine (*p*-MBHA) resin, from Applied Biosystems, were used with substitutions varying from 0.62 to 0.77 mmol/g. All solvents and reagents for peptide syntheses were peptide synthesis grade from Applied Biosystems and Fisher Biotechnology (Pittsburgh, PA). Thioanisole, EDT, *m*-cresol, and DMS were purchased from Aldrich (Milwaukee, WI), TFA and diethyl ether were from Fisher, TFMSA was from Applied Biosystems, and DTT and Amberlite IRA68 were purchased from Sigma (St. Louis, MO). All chemicals were used as supplied. Low-pressure chromatography was accomplished on an ISCO chromatography system (Lincoln, NE) with continuous monitoring of eluent at 254 nm. Gel filtration was performed using a glass column (2.5 × 90 cm) packed with Bio-Gel P6 from Bio Rad (Richmond, CA). Low-pressure ion-exchange chromatography was performed using a glass column (1.6 × 15 cm) packed with CM Sephadex C25 from Pharmacia (Piscataway, NJ). Ion-exchange HPLC was performed on a Waters Corp. Inc. 625LC instrument using a PolySULFOETHYL Aspartamide column (1 × 20 cm) supplied by the Nest Group (Southboro, MA). Flow rate was 4 mL/min, and the eluent was continuously monitored at 220 nm. RP-HPLC was performed using a Waters Corp. Inc. 600E instrument. Semipreparative RP-HPLC was performed on a Vydac 218TP510 C_{18} column (1 × 25 cm) at a flow rate of 4 mL/min. Analytical RP-HPLC was performed on a Vydac 218TP54 C_{18} column (0.46 × 25 cm), a Vydac 214TP54 C_4 column (0.46 × 25 cm), and a Waters Corp. Inc. Delta Pak HPI C_{18} 300 Å column (0.39 × 15 cm). The flow rate was 1 mL/min, and the eluent was continuously monitored at 220 nm. Water was obtained from a Barnstead Nanopure system, and solvents for HPLC were Optima grade from Fisher. TFA for HPLC was supplied by Pierce (Rockford, IL). Eluents used for ion-exchange HPLC were as follows: A, 100 mM NaH_2PO_4/Na_2HPO_4, pH 7.0; B, acetonitrile; C, water; D, 1 M $NaCl_{aq}$. Mixtures of these eluents were employed to form an increasing linear salt gradient in the presence of a phosphate buffer (20 mM, pH 7.0) and 20% acetonitrile. Eluents used for RP-HPLC were (A) 0.1% TFA in water and (B) 0.09% TFA in acetonitrile/water (60/40, v/v). Amino acid analyses were performed on a Beckman 116 instrument modified to a one-column system. Samples were hydrolyzed in 6 M constant boiling hydrochloric acid (Pierce) under vacuum for 24 h at 110 °C. Cysteine was determined as cysteic acid.

Solid Phase Peptide Synthesis. All peptides were made by Merrifield's solid phase methodology as described previously.[24] *N*-α-Boc amino acid derivatives were coupled to *p*-MBHA resin in a 4-fold excess using HOBt/DCC as coupling reagents, in NMP. The coupling reactions were monitored by the quantitative ninhydrin test.[47] The first 21 cycles were single-coupled, and the last 15 cycles were double-coupled to maintain yields in excess of 99%. After the twentieth coupling, the peptide-resin was dried and half was used for the rest of the synthesis. Once the desired sequence was assembled, His(Dnp)-containing peptide-resins were treated with thiophenol (20 equiv) in DMF to remove the Dnp group[48] and the final Boc group was removed with a solution of TFA in DCM (1/1, v/v). Finally, the peptides were deprotected and cleaved from the resin by the low−high TFMSA method of Tam using the scavengers thioanisole, EDT, *m*-cresol, and DMS.[24,25] The crude linear peptides were then stirred in a solution of ammonium acetate (50 mM, pH 8.5, 100 mL) and DTT (5 mM) overnight before being diluted to 1 L with degassed water. An excess of a solution of potassium ferricyanide (0.01 M) was added dropwise, and the solution was stirred for an additional 30 min. The pH was lowered to approximately 4.0 with glacial acetic acid, and anion-exchange resin (Amberlite IRA-68 Cl^- form, 30 mL settled volume) was added with stirring. After stirring for 30 min the suspension was filtered through a bed of anion-exchange resin (30 mL settled volume), and the bed was washed with 30% aqueous acetic acid (3 × 50 mL). The combined filtrates and washings were concentrated to approximately 300 mL, by azeotropic distillation of water with *n*-butanol on a rotary evaporator, and lyophilized.

Optimized Purification of h-α-CGRP. h-α-CGRP was synthesized according to the procedures described above. The crude cyclized peptide was gel filtered on the Bio-Gel P6 column eluted at a flow rate of 22 mL/h. Fractions containing the desired peptide were pooled and lyophilized. A sample of the lyophilized material peptide (100 mg) was loaded onto a PolySULFOETHYL Aspartamide column previously eluted with 20% A, 20% B, 60% C, and 0% D. The peptide was eluted with a linear gradient to 20% A, 20% B, 45% C, and 15% D over 60 min. Fractions containing the desired peptide were collected and lyophilized. The peptide was finally purified to apparent homogeneity by semipreparative C_{18} RP-HPLC with a linear gradient of 0−36% B for 10 min followed successively by 36−50% B for 60 min, 50−100% B for 10 min, and 100% B for 10 min. Fractions containing a single component were combined and lyophilized to yield 42 mg (5.2%) of a white fluffy powder.

[Ala^{14}]-h-α-CGRP. [Ala^{14}]-h-α-CGRP was synthesized by the general procedure. The crude cyclized peptide was subjected to gel filtration on the Bio-Gel P6 column eluted at a flow rate of 22 mL/h. Fractions containing the desired peptide were pooled and lyophilized. The gel-filtered material was loaded on a PolySULFOETHYL Aspartamide column which had been previously washed with a solvent mixture of 20% A, 20% B, 60% C, and 0% D. The product was eluted using a linear gradient to 20% A, 20% B, 45% C, and 15% D over 60 min. Fractions containing the desired peptide were pooled and loaded directly onto a semipreparative Vydac C_{18} column previously equilibrated with solvent A. The product was obtained using a linear gradient of 0−35% B for 10 min followed by 35−55% B for 80 min. Fractions containing the pure peptide were pooled and lyophilized.

[Aib^{14}]-h-α-CGRP. [Aib^{14}]-h-α-CGRP was synthesized according to the procedures already described. The crude cyclized peptide was gel-filtered on the Bio-Gel P6 column at a flow rate of 28 mL/h. All fractions containing the desired peptide were collected and lyophilized. The lyophilized material was purified by ion-exchange chromatography on the CM Sephadex C25 column with a linear gradient of 10−800 mM ammonium acetate (350 mL in each chamber) at a flow rate of 52 mL/h. Fractions containing the desired peptide were pooled and lyophilized. The peptide was finally purified by RP-HPLC on a semipreparative Vydac C_{18} column using a linear gradient of 0−36% B for 10 min followed by 36−50% B for 60 min. Fractions containing the pure peptide were collected and lyophilized.

[Asp^{14}]-h-α-CGRP. [Asp^{14}]-h-α-CGRP was synthesized by the previously described general procedures. The crude cyclized peptide was loaded onto the Bio-Gel P6 column which was then eluted at a flow rate of 19 mL/h. Fractions containing the desired peptide were collected and lyophilized. The lyophilized material was loaded onto the PolySULFOETHYL Aspartamide column previously equilibrated with a solvent mixture of 20% A, 20% B, 60% C, and 0% D. The product was eluted from the column using a linear gradient to 20% A, 20% B, 48% C, and 12% D over 60 min. Fractions containing the desired peptide were pooled and lyophilized. The peptide was finally loaded onto a semipreparative Vydac C_{18} column previously equilibrated with solvent A. The peptide was fractionated to apparent homogeneity using a linear gradient of 0−35% B for 10 min followed by 35−55% B for 80 min. Fractions containing the pure peptide were collected and lyophilized to yield a fluffy white powder.

[Asn^{14}]-h-α-CGRP. [Asn^{14}]-h-α-CGRP was made according to the general procedures described previously. The crude cyclized product was gel-filtered on the Bio-Gel P6 column

eluted at a flow rate of 19 mL/h, and fractions containing the desired peptide were pooled and lyophilized. This material was applied to a PolySULFOETHYL Aspartamide column previously equilibrated with a solvent mixture of 20% A, 20% B, 60% C, and 0% D. The peptide was eluted from the column using a linear gradient to 20% A, 20% B, 45% C, and 15% D over 60 min. Fractions containing the desired peptide were collected and loaded directly onto a semipreparative Vydac C_{18} column previously equilibrated with solvent A. The product was eluted using a linear gradient of 0−35% B for 10 min followed by 35−55% B for 80 min. Fractions containing the pure peptide were pooled and lyophilized.

[Pro^{14}]-h-α-CGRP. [Pro^{14}]-h-α-CGRP was synthesized by the general procedure. The crude cyclized material was first purified by gel filtration on the Bio-Gel P6 column eluted at a flow rate of 28 mL/h. Fractions containing the desired peptide were collected and lyophilized. This material was then chromatographed on the CM Sephadex C25 column with a linear gradient of 10−800 mM ammonium acetate (350 mL in each chamber) at a flow rate of 52 mL/h. Fractions containing the desired peptide were pooled and lyophilized. This material was loaded onto a semipreparative Vydac C_{18} column previously equilibrated with solvent A. The product was eluted using a linear gradient of 0−36% B for 10 min followed by 36−50% B for 60 min. Fractions containing the pure peptide were collected and lyophilized to yield a fluffy white powder.

Circular Dichroism Spectroscopy. Circular dichroism spectra were measured using an Aviv 62DS spectrophotometer equipped with a thermoelectric temperature control unit. Temperatures were regulated to within 0.1 °C. All spectra were measured in 1 mm quartz cuvettes at 25 °C. The concentration of CGRP analogues in aqueous solution was determined by ultraviolet absorbance measurements using an extinction coefficient of 3670 M^{-1} cm^{-1} at 215 nm.[16] The extinction coefficient was calculated based on the absorbance of a solution of h-α-CGRP whose concentration was measured using quantitative amino acid analysis. The various CGRP analogues were measured at 0.1 mg/mL both in 10 mM phosphate buffer (pH 7) and in 50% TFE. All spectra were corrected using individually calculated mean residue weights to determine mean residue ellipticities [θ].

Coronary Artery Assay. Left circumflex coronary arteries were removed from pig hearts, obtained immediately after sacrifice at a local slaughter house. The arteries were maintained in oxygenated Krebs buffer (NaCl, 125 mM; KCl, 5.5 mM; $CaCl_2 \cdot 2H_2O$, 2.5 mM; $MgCl_2 \cdot 6H_2O$, 1.2 mM; NaH_2PO_4, 1.25 mM; $NaHCO_3$, 25 mM; dextrose, 11.1 mM; $Na_2CaEDTA \cdot 2H_2O$, 0.029 mM) and cleaned of adhering fat and connective tissue. Rings (2 mm in length) were prepared, mounted in water-jacketed organ baths between two stainless steel pins passed through the lumen of the vessel, and bathed in Krebs solution maintained at 37 °C and gassed with 95% oxygen/5% carbon dioxide, pH 7.4. One pin was connected to a Grass FT.03 force transducer for measurement of isometric tension with a Grass polygraph. Rings were equilibrated at a resting tension of 6 g for 1 h and then maximally contracted with KCl (45 mM). The rings were thoroughly washed, re-equilibrated at the resting tension, and then contracted again with 20 mM KCl. The rings were then relaxed by cumulative addition of h-α-CGRP. Following the completion of the h-α-CGRP dose−response curve, the rings were washed, re-equilibrated for 1 h, and subsequently contracted to a similar level of tone as previously with KCl. The ability of position 14-substituted analogues to relax coronary artery rings was tested by then generating cumulative dose−response relaxation curves. Each analogue was tested in a single ring.

Pancreatic Acini Assay. Analogues were tested for their ability to stimulate amylase release from guinea pig pancreatic acini following methods described previously.[24]

Drugs. All stock solutions and dilutions of synthetic peptides were made in 0.9% (w/v) isotonic NaCl solution.

Data Analysis. Data were analyzed by the Graphpad Inplot 4.0 software. Dose−response curves were plotted and potency (EC_{50}) values for h-α-CGRP and its analogues calculated using nonlinear regression analysis. Statistical analysis of data was performed using the *t*-test application on the Graphpad Instat software.

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Supporting Information Available: Amino acid compositions, physicochemical data, and CD spectra of all h-α-CGRP analogues (4 pages). Ordering information is given on any current masthead page.

References

(1) Abbreviations and symbols are in accord with the recommendation of the IUPAC-IUB Commission on Biochemical Nomenclature in *J. Biol. Chem.* **1972**, *247*, 977. All optically active amino acids are the L variety unless otherwise stated. Other abbreviations include the following: AAA, amino acid analysis; Boc, *tert*-butyloxycarbonyl; CD, circular dichroism; CGRP, calcitonin gene-related peptide; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; DMS, dimethyl sulfide; DTT, dithiothreitol; EDT, ethanedithiol; FAB-MS, fast atom bombardment mass spectrometry; h, human; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; HOBt, *N*-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; NMP, *N*-methylpyrrolidone; NMR, nuclear magnetic resonance; *p*-MBHA, *p*-methylbenzhydrylamine; RP, reversed phase; TFA, trifluoroacetic acid; TFE, trifluoroethanol; TFMSA, trifluoromethanesulfonic acid.

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