Structure–Activity Study of hCGRP$_{8-37}$, a Calcitonin Gene-Related Peptide Receptor Antagonist[†]

Murielle Mimeault,[‡] Rémi Quirion,[§] Yvan Dumont,[§] Serge St-Pierre,[‡] and Alain Fournier*[,‡]

Institut national de la recherche scientifique-Santé (INRS-Santé), Université du Québec, 245, Hymus Blvd, Pointe-Claire, Québec, Canada H9R 1G6, and Douglas Hospital Research Centre, McGill University, 6875, LaSalle Blvd., Verdun, Québec, Canada H4H 1R3. Received December 17, 1991

A structure–activity study was carried out to determine the importance of the N-terminal amino acids of hCGRP$_{8-37}$ in binding and antagonistic activity to CGRP receptors. Therefore, fragments of hCGRP$_{8-37}$ as well as analogs obtained by the replacement of residues 9–12 by L-alanine were synthesized by solid-phase peptide synthesis, using BOP as a coupling reagent. The affinities of the peptides to CGRP receptors were evaluated in the rat brain, guinea pig atrium, and guinea pig vas deferens membrane preparations. Their antagonistic activities were measured in the guinea pig atria and rat vas deferens bioassays. The pharmacological characterization showed that arginine-11 and leucine-12 play a crucial role for the affinity of hCGRP$_{8-37}$. Interestingly, it was observed that [Ala^{11}]hCGRP$_{8-37}$ was able to potentiate the twitch response of the electrically stimulated rat vas deferens. On the other hand, the substantial antagonistic potencies of analogs [Ala^9]-, [Ala^{10}]-, and [Ala^{12}]hCGRP$_{8-37}$, as compared to those of the fragments hCGRP$_{10-37}$, hCGRP$_{11-37}$, and hCGRP$_{12-37}$, suggest that the side chains of Thr-9, His-10, and Leu-12 assume mainly a structural role. Accordingly, the conformational characterization of these peptides by circular dichroism spectroscopy revealed that the residues 9–12 are important for the integrity of the amphiphilic α-helix of hCGRP$_{8-37}$.

Introduction

Calcitonin gene-related peptide (CGRP) is a 37-amino acid peptide containing a disulfide bond between cysteines 2 and 7 and a C-terminal phenylalanine amide (Figure 1). Existence of CGRP was first predicted from sequence analyses of the rat calcitonin gene.[1] Subsequent studies confirmed its existence and have shown, in humans, the presence of two forms of CGRP—α and β—which differ by only three amino acid residues.[2] During the past years, various biological actions have been attributed to CGRP, including vasodilation,[3,4] cardiac acceleration,[5,6] and inhibition of gastric secretion.[7] CGRP has also been shown to modulate effects of neurotransmitters such as acetylcholine[8] and substance P.[9] Immunohistochemical studies revealed that this variety of actions is correlated with a large distribution of binding sites for CGRP in the central nervous system[10,11] and peripheral tissues such as blood vessels[12] and heart.[13]

Structure–activity studies with synthetic analogs and fragments of CGRP provided evidence for the heterogeneity of CGRP receptors. Among others, the linear analog [Cys(Acm)^{2,7}]hCGRPα was shown to act as a potent agonist of hCGRPα in the inhibition of the twitch response of the electrically stimulated rat vas deferens whereas it had no effect on the inotropic and chronotropic responses of the guinea pig atrium.[14] The fragment hCGRP$_{8-37}$ also appeared as a potent antagonist of responses induced by hCGRPα in the guinea pig atrium but possessed a weak antagonistic activity in the rat vas deferens.[14,15] The structural requirements for binding and biological activity

of CGRP have also been partially identified. Indeed, the N-terminal amino acids of the molecule would be essential
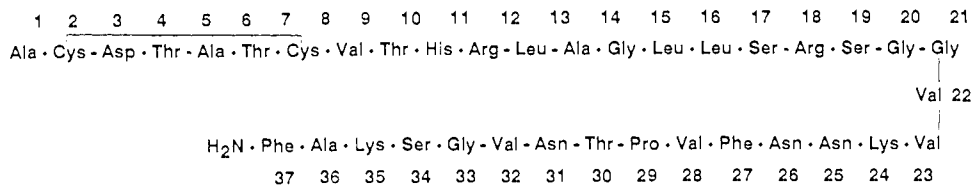
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Figure 1. Primary structure of human CGRPα.

Table I. Characterization of hCGRPα and hCGRP₈₋₃₇ and Its Fragments and Analogs by Analytical HPLC, Capillary Electrophoresis, and Amino Acid Analysis

peptide	HPLC[a] Tr (min)	CE[b] Tm (min)	amino acid analysis[c]											
			Asx	Ser	Gly	His	Arg	Thr	Ala	Pro	Val	Leu	Phe	Lys
hCGRPα	16.25	9.89	3.95	3.00	4.26	0.93	2.06	4.10	4.32	1.00	5.13	3.33	2.13	2.12
hCGRP₈₋₃₇	13.90	8.59	2.70	2.88	4.31	1.00	2.14	2.16	2.11	1.04	5.22	2.93	2.15	2.23
hCGRP₉₋₃₇	13.35	7.96	3.00	2.74	4.34	1.01	2.14	1.89	2.13	1.06	4.00	3.03	2.07	2.12
hCGRP₁₀₋₃₇	13.09	7.73	2.70	2.75	4.19	1.07	2.03	0.97	2.20	1.10	4.03	3.15	2.13	2.07
hCGRP₁₁₋₃₇	13.08	7.53	2.84	2.97	4.19	0.00	2.00	1.10	2.17	1.12	3.91	3.28	2.09	1.90
[Ala⁹]hCGRP₈₋₃₇	13.35	8.13	2.76	2.70	4.40	0.90	2.02	0.98	3.03	1.10	5.15	2.82	2.20	2.11
[Ala¹⁰]hCGRP₈₋₃₇	13.82	8.80	2.88	2.90	4.13	0.00	2.08	1.90	3.10	1.01	5.35	2.92	2.20	2.16
[Ala¹¹]hCGRP₈₋₃₇	13.55	8.21	3.02	3.00	4.19	0.99	1.12	1.91	3.29	1.10	5.38	3.00	1.87	2.24
[Ala¹²]hCGRP₈₋₃₇	13.14	8.23	2.89	2.77	4.26	1.04	2.08	2.05	3.21	1.00	4.97	2.14	1.98	2.24

[a] Retention times in minutes following the analytical HPLC conditions described in the Experimental Section. [b] Migration times in minutes following the capillary electrophoresis conditions described in the Experimental Section. [c] Amino acid analyses from hydrolysates (distilled 6 N HCl containing 0.1% phenol in evacuated sealed tubes at 110 °C for 24 h). Cys was not determined.

for biological repsonse while the central and C-terminal segments would allow the formation of the appropriate conformation required for the interaction with the receptor.[14,16]

Although progress was made in characterizing CGRP receptors, still little is known on the relationship between the secondary and tertiary structures of CGRP and its activity. Structural studies of the region between amino acids 8–18 suggested the presence of an amphiphilic α-helix which would be terminated by a β-turn ensuring the reversibility of the peptide chain.[17-19] As reported for several peptides, including calcitonin,[20,21] neuropeptide Y,[22] and β-endorphin[20] modeling studies proposed for CGRP a determinant role for the amphipathic region of the molecule in the interaction of the peptide with the membrane receptor[23,24] and the triggering of the biological response.[17,25] As a matter of fact, in previous studies, we showed that the successive deletion of residues 9–11 resulted in fragments with poor affinity and antagonistic activity to CGRP receptors.[14,26]

The present study was undertaken to determine if amino acids 8–12 of hCGRP₈₋₃₇ assume only a structural role in forming the amphiphilic α-helix or if they interact specifically with the receptor. Therefore, we synthesized a series of fragments of hCGRP₈₋₃₇ by the solid-phase peptide synthesis method in which N-terminal residues 8 to 10 were successively deleted. Analogs in which amino acids 9–12 were substituted by L-alanine were also synthesized in order to help in estimating the importance of these residues in hCGRP₈₋₃₇. Binding assays and bioassays of fragments and analogs were carried out on a variety of tissues for evaluating the effect of these modifications on affinity and antagonistic potency.

Finally, a conformational characterization of the derivatives was also performed by circular dichroism spectroscopy in order to establish the correlation between the affinity and the helical content of the molecules.

Results and Discussion

The fragments and analogs were synthesized by solid-phase peptide synthesis using BOP as a coupling reagent.[27]

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Table II. Relative Affinities of hCGRPα and hCGRP$_{8-37}$ Fragments and Analogs for [^{125}I]hCGRPα Binding Sites in Atrium, Brain, and Vas Deferens Membrane Preparations

peptide	atrium (guinea pig)		brain (rat)		vas deferens (guinea pig)	
	IC$_{50}^a$ (nM)	RA^b	IC$_{50}$ (nM)	RA	IC$_{50}$ (nM)	RA
hCGRPα	2.0 ± 0.3	100	2.4 ± 0.3	100	1.7 ± 0.1	100
hCGRP$_{8-37}$	2.3 ± 0.6	87	0.6 ± 0.4*	400	0.5 ± 0.2*	340
hCGRP$_{9-37}$	2.3 ± 0.3	87	0.9 ± 0.4*	267	0.5 ± 0.3*	340
hCGRP$_{10-37}$	9 ± 2*	22	10 ± 4*	24	9.0 ± 0.7*	19
hCGRP$_{11-37}$	20.2 ± 0.7*	10	17.9 ± 0.5*	13	16 ± 4*	11
[Ala^9]hCGRP$_{8-37}$	6.9 ± 0.9*	29	6.8 ± 0.7*	35	8 ± 1*	21
[Ala^{10}]hCGRP$_{8-37}$	3.0 ± 0.2*	67	11 ± 2*	22	12 ± 2*	14
[Ala^{11}]hCGRP$_{8-37}$	25 ± 1*	8	33 ± 4*	7	21.9 ± 0.9*	8
[Ala^{12}]hCGRP$_{8-37}$	11.2 ± 0.8*	18	22 ± 8*	11	14.5 ± 0.9*	12

^{a} IC$_{50}$: concentration of peptide producing a 50% inhibition of maximum binding. The IC$_{50}$ values are the mean ± SE of triplicate determinations from three separate experiments. ^{b}RA: relative affinity. *p < 0.05 as compared to hCGRPα.

Table III. Relative Potencies of Fragments and Analogs of hCGRP$_{8-37}$ on Responses Induced by hCGRPα in Different Tissues

| peptide | left atrium (guinea pig) inotropic response | | right atrium (guinea pig) | | | | vas deferns (rat) twitch response | |
| | | | inotropic response | | chronotropic response | | | |
	EC$_{50}^a$ (nM)	RP^b (%)	EC$_{50}$ (nm)	RP (%)	EC$_{50}$ (nM)	RP (%)	EC$_{50}$ (nM)	RP (%)
hCGRPα	8.7 ± 1		7.9 ± 0.2		19 ± 2		1.4 ± 0.3	
in presence of 1 μM:								
hCGRP$_{8-37}$	194 ± 11	100	267 ± 26	100	290 ± 35	100	6.7 ± 0.3*	100
hCGRP$_{9-37}$	215 ± 13	111	225 ± 6*	84	180 ± 35*	62	7.6 ± 0.1*	113
hCGRP$_{10-37}$	65 ± 2*	34	114 ± 9*	43	77 ± 7*	27	3.2 ± 0.1*	48
hCGRP$_{11-37}$	30 ± 2*	15	89 ± 6*	33	65 ± 5*	22	1.4 ± 0.1	21
[Ala^9]hCGRP$_{8-37}$	117 ± 6*	60	193 ± 11*	72	238 ± 66	82	2.4 ± 0.3*	36
[Ala^{10}]hCGRP$_{8-37}$	95 ± 2*	49	179 ± 7*	67	129 ± 41*	44	3.2 ± 0.4*	48
[Ala^{11}]hCGRP$_{8-37}$	58 ± 3*	30	106 ± 5*	40	83 ± 20*	29	[3.1 ± 0.3*]^c	46
[Ala^{12}]hCGRP$_{8-37}$	89 ± 7*	46	116 ± 4*	43	116 ± 24*	40	2.2 ± 0.4	33

^{a} EC$_{50}$: concentration of hCGRPα required to produce 50% of the maximal response in absence or in presence of 1 μM of fragments and analogs of hCGRP$_{8-37}$. This value is mean ± SE of data obtained from 3 to 15 individual tissues. ^{b}RP: relative potency. *p < 0.05 as compared to hCGRP$_{8-37}$. ^{c}The analog [Ala^{11}]hCGRP$_{8-37}$ was shown to increase, in a concentration-dependent manner, the twitch response of the rat vas deferens stimulated electrically (see Figure 4).

The syntheses were carried out according to synthetic methods that we recently described.[28] All couplings, including those of Boc-asparagine and Boc-histidine (tosyl) were performed with BOP reagent.[29] Cleavage of peptide–resin and removal of the protecting groups from the amino acid side chains were achieved in the presence of liquid hydrogen fluoride (HF). Satisfactory peptide preparations were obtained. However, an important side reaction occurred during the acid cleavage and gave rise to two major peaks with retention times of 13.9 and 14.1 min, respectively. This side reaction was also observed with the other analogs of this series. The presence in the hCGRP$_{8-37}$ sequence of numerous serine and threonine residues suggested that intramolecular N → O acyl shift[30,31]

might be responsible for the splitting of the major peak. Therefore, we verified the effect of an alkaline treatment on the crude material. As shown by analytical HPLC, the treatment transformed the side product eluting at 14.1 min into the material eluting at 13.9 min. Highly purified (≥98%) peptide preparations were obtained, and yields, although they were not optimized, were satisfactory. The characterization of peptides by analytical HPLC capillary electrophoresis and amino acid analysis assessed the excellent quality of the synthetic peptide preparations (Table I).

As we reported previously, the binding experiments carried out in atrium, brain, and vas deferens membrane with hCGRP$_{8-37}$ and some shorter fragments showed that the valine-8 residue is not important for the affinity.[26] In the aforementioned membrane preparations, the relative affinities of the fragments hCGRP$_{8-37}$ and hCGRP$_{9-37}$, for [^{125}I]hCGRPα binding sites, are very similar (Table II). However, as shown with hCGRP$_{10-37}$ and hCGRP$_{11-37}$ (Table II), the successive deletion of the threonine and histidine residues found at positions 9 and 10, respectively, resulted in important losses of affinity in all membrane preparations. The substitution of threonine-9 or histidine-10 by L-Ala also decreased the affinity of hCGRP$_{8-37}$ for the CGRP receptors. However, in contrast to what was measured in the guinea pig vas deferens and rat brain membrane preparations, [Ala^{10}]hCGRP$_{8-37}$ is characterized in the guinea pig atrium by a substantial affinity (relative affinity of 67%), thus suggesting that the His-10 side chain is not an essential feature for the binding of hCGRP$_{8-37}$ to CGRP receptors found in the guinea pig atrium. The replacement of arginine-11 or leucine-12 by L-alanine resulted in significant losses of affinity for [^{125}I]hCGRPα binding sites, since mean relative affinities of only ≈8%

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Table IV. Molecular Ellipticity of hCGRP$_{8-37}$ and Its Fragments and Analogs, at 208 and 222 nm, As Determined by Circular Dichroism Spectroscopy

peptide	molecular ellipticity $-[\theta]_{208/222}{}^{a}$ (deg–cm^2 dmol^{-1})	peptide	molecular ellipticity $-[\theta]_{208/222}{}^{a}$ (deg–cm^2 dmol^{-1})
hCGRP$_{8-37}$	11111/8251	[Ala^9]hCGRP$_{8-37}$	11470/8145
hCGRP$_{9-37}$	11604/8162	[Ala^{10}]hCGRP$_{8-37}$	10684/7941
hCGRP$_{10-37}$	9056/4865	[Ala^{11}]hCGRP$_{8-37}$	5942/4920
hCGRP$_{11-37}$	6055/3178	[Ala^{12}]hCGRP$_{8-37}$	10189/6493

^{a} Mean residue molecular ellipticity of the peptide at 208 and 222 nm, determined in 40% HFIP/phosphate buffer, pH 7.0 (see Experimental Section).
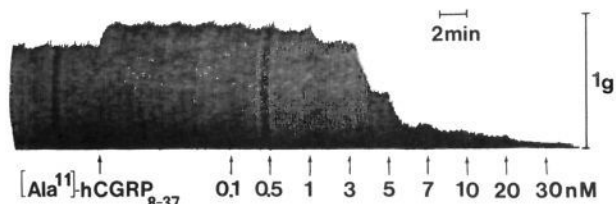

Figure 2. Inhibition of the twitch response of the isolated rat vas deferens produced by hCGRPα. This trace shows the potentiation of contractility produced by the analog [Ala^{11}]-hCGRP$_{8-37}$, at a concentration of 1 μM. The values indicate the final concentration of hCGRPα in nanomolar.

and ≈14% were obtained with [Ala^{11}]hCGRP$_{8-37}$ and [Ala^{12}]hCGRP$_{8-37}$, respectively (Table II). Moreover, in our previous study utilizing hCGRP$_{12-37}$, in the rat brain membrane preparation,[14] a relative affinity of 10% was measured. These data suggest that the Arg-11 and Leu-12 side chains play a particular role in the recognition phenomenon of hCGRP$_{8-37}$ by the receptors found in these preparations.

The ability of hCGRP$_{8-37}$ and related analogs, as well as of shorter fragments, to antagonize the effects of hCGRPα was evaluated in the left and right guinea pig atria and the rat vas deferens pharmacological preparations. The concentration–response curves of the inotropic and chronotropic effects of hCGRPα was shifted significantly to the right in the presence of 1 μM hCGRP$_{8-37}$ or hCGRP$_{9-37}$. The EC$_{50}$ values of hCGRPα, obtained from the curves measured in the presence of fragments (1 μM), are given in Table III. The EC$_{50}$ values of hCGRPα, in the left and right atria, increased approximately 20-fold in the presence of these two fragments, while in the rat vas deferens the effect was much less important. As observed before with the binding experiments, the results show that the deletion of valine-8 does not seriously affect the antagonistic activity to CGRP receptors found in the guinea pig atrium. Interestingly, the conformational analysis of hCGRP$_{8-37}$ and hCGRP$_{9-37}$ by circular dichroism spectroscopy revealed that the removal of Val-8 did not modify the stability of the helical nucleus since the two fragments possess the same mean helical content (Table IV).

As measured with the fragment hCGRP$_{10-37}$, the deletion of threonine-9 has a detrimental effect on the antagonistic activity (relative potencies in the bioassays: ≈35%). As compared to the two other longer fragment homologs, this derivative is much less potent in inhibiting the biological responses of hCGRPα in the guinea pig atria. Threonine-9 thus appears to play a role in the expression of the antagonism to CGRP receptors in the guinea pig atria. This role does not seem to be associated with the participation of the residue-9 side-chain in the recognition phenomenon with the receptor since [Ala^9]hCGRP$_{8-37}$, an analog obtained by substituting threonine with L-alanine, exhibits a substantial antagonistic potency (relative potencies in the bioassays: ≈70%). The results suggest that the position 9 of hCGRP$_{8-37}$ would mainly play a structural role in the molecule. In accordance with this hypothesis is the CD data showing that [Ala^9]hCGRP$_{8-37}$ has a very similar mean helical content to that of fragment 8–37, while hCGRP$_{10-37}$ exhibits an important decrease of the molecular ellipticity (Table IV).

As shown by the poor antagonistic activity of hCGRP$_{11-37}$ (Table III) and hCGRP$_{12-37}$,[14] it appears that further deletions beyond histidine-10 are detrimental for

the antagonism exhibited by the fragment. These decreases in antagonistic activity as well as in binding capacity might be related to a poor stability of the remaining α-helix nucleus (Table IV). However, the relative potencies of the antagonistic activity of [Ala^{10}]-, [Ala^{11}]-, and [Ala^{12}]hCGRP$_{8-37}$ suggest that the stabilization of the secondary structure of the N-terminal portion of the molecule is not the only factor involved in the receptor interaction. Indeed, the α-helix content of [Ala^{10}]-hCGRP$_{8-37}$ is approximately the same as the nonsubstituted fragment. Nevertheless, its activity, as well as its binding affinity, are reduced in all preparations used. Therefore, we suspect that the His-10 side chain might participate in the recognition phenomenon of the ligand with the receptor or be involved in an intramolecular interaction ensuring the adequate folding of the peptide chain. This conclusion is also valid for [Ala^{12}]hCGRP$_{8-37}$ which exhibits a substantial α-helix content but shows poor binding and antagonistic properties. The data obtained with [Ala^{11}]hCGRP$_{8-37}$ are rather different and reflect the involvement of arginine-11 in the maintenance of the α-helix integrity. Indeed, the replacement of arginine by alanine, which is identified as a better helix-promoter,[32] should not seriously disturb the secondary structure. However, as shown by the mean residue molecular ellipticity, the α-helix content of the analog is about 60% that of the mother molecule, thus suggesting that Arg-11 participates in an interaction ensuring a better stability of the helical core of the molecule. The nature of the interaction is yet unclear. However, the absence of a negatively charged residue in the peptide sequence excludes the formation of an ionic bridge. A possible explanation is that arginine, a residue bearing a highly polar side chain, might be essential for the maintenance of the amphiphilic character of the N-terminal segment. The stability of the amphipathic α-helix would therefore depend on the favorable tendency of the participating residues to promote the α-helix secondary structure, and on their degree of hydrophilicity, in order to ensure an appropriate amphiphilic character of the helical segment of the molecule. This hypothesis, which is actually under investigation, would explain our previous observation[14] that fragment hCGRP$_{12-37}$ demonstrated limited binding affinity or antagonistic activity even at μM concentrations.

The poor binding and antagonistic activities of the Arg^{11}-substituted hCGRP$_{8-37}$ fragment appear to be related to its low helical content as compared to that obtained with hCGRP$_{8-37}$. Furthermore, this fragment exhibits at 222 nm a similar mean molecular ellipticity to that of hCGRP$_{10-37}$, a peptide with antagonistic potencies close to those measured for [Ala^{11}]hCGRP$_{8-37}$. However, the particular behavior of [Ala^{11}]hCGRP$_{8-37}$ in the rat vas

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deferens bioassay (Figure 2) suggests that arginine-11 might participate, in this pharmacological preparation, in the interaction phenomenon with the receptor. Indeed, as shown in Figure 2, the analog carrying an alanine at position 11 potentiates the twitch response of the rat vas deferens stimulated electrically. This effect, which is not observed with the other fragments and analogs of this study, is concentration-dependent (data not shown).

As reported before[14,15,26], the fragments are poor inhibitors of the twitch response induced by hCGRPα in the rat vas deferens. We observed that the Ala-containing analogs of hCGRP₈₋₃₇ are also weak antagonists of this effect (Table III). Therefore, this data is in agreement with our previous reports suggesting the existence of at least two classes of CGRP receptors.[14,15]

In conclusion, the pharmacological characterization of analogs of hCGRP₈₋₃₇ and related fragments has shown, for residues 9–12 of the sequence, more or less critical roles for an effective binding and antagonistic activity to CGRP receptors. Moreover, the conformational analysis of fragments and analogs by circular dichroism revealed that the deletion of theonine-9 and histidine-10, or substitution of arginine-11 or leucine-12 with L-alanine, gave rise, at various extents, to reductions of the α-helix content in the molecules. Taken together, the structural and biological studies suggest that these amino acid residues are involved mainly in the maintenance of the amphiphilic α-helix of hCGRP₈₋₃₇, a secondary structure which seems to be crucial for a good affinity of the ligand. Of these, arginine-11 appears as a residue playing a critical role since its deletion gives a peptide unable to potentially interact with the CGRP receptors. Furthermore, its substitution with Ala is accompanied by substantial losses of binding capacity and antagonistic activity, in parallel with a decrease in the α-helix stability. Arginine-11 also displays a particular activity in the rat vas deferens since the analog [Ala¹¹]-hCGRP₈₋₃₇ is able to potentiate the twitch response of the electrically stimulated vas deferens.

Experimental Section

Synthesis Procedures. Reagents and Solvents. Boc-protected amino acid derivatives and BOP reagent were purchased from Richelieu Biotechnologies (St-Hyacinthe, Québec, Canada). ACS-grade dimethylformamide and methylene chloride were obtained from Anachemia Canada Inc. (Ville St-Pierre, Québec, Canada) and biograde trifluoroacetic acid was purchased from Halocarbon (Hackensack, NJ). Diisopropylethylamine was obtained from Pfaltz and Bauer (Waterbury, CT) and was distilled from ninhydrin before use. Finally, benzhydrylamine resin (copolystyrene-1% divinylbenzene, 0.42 mequiv/g) was also from Richelieu Biotechnologies (St-Hyacinthe, Québec, Canada).

Peptide Synthesis and Cleavage. The peptides hCGRPα and hCGRP₈₋₃₇ and its fragments and analogs were synthesized according to the solid-phase peptide synthesis method following a protocol that we recently described.[28,29] A benzhydrylamine resin was used as the solid support, and BOP reagent was used for the coupling step. The syntheses were carried out with a homemade manual multireactor synthesizer. Side-chain protection of α-Boc-amino acids was as follows: Arg(Tos), Asp(OcHx), His(Tos), Lys(ClZ), Ser(Bzl), Thr(Bzl), and Cys(Acm). Peptides were cleaved from the polymeric solid support with liquid hydrofluoric acid (10 mL of HF/g) in the presence of m-cresol (1 mL/g) at 0 °C for 60 min. After precipitation and washings with anhydrous diethyl ether, the crude peptides were extracted with pure trifluoroacetic acid followed by evaporation.

Cyclization of Linear hCGRPα and Alkaline Treatments of the Peptides. Crude [Cys(Acm)²·⁷]hCGRPα was dissolved in a degassed 80% acetic acid (AcOH) solution (1 mg of peptide/mL). An equal volume of iodine solution (50 equiv), dissolved in the same solvent, was added. The final solution was incubated for a period of 2.5 h with occasional shaking. Then, zinc dust was added until the obtention of a discolored solution. After filtration,

the solution was evaporated to a final volume of approximately 50 mL to which 250 mL of 0.06% TFA/H₂O solution was added before lyophilization. The alkaline treatment was carried out as follows: the peptide was dissolved in 0.06% TFA (500 mg of peptide/200 mL) and the pH of the solution was adjusted to 9.5 with concentrated NH₄OH. After an incubation period of 30 min with slow shaking, the pH of the peptide solution was adjusted to 2 with concentrated TFA.

Peptide Purification. The crude peptides were purified by preparative reversed-phase HPLC on a Waters Prep LC 3000 system equipped with a Waters 1000 Prep Pak Module and a Model 441 absorbance detector. Peptide solutions obtained after alkaline treatment were injected on a Delta Pak C₁₈ (15 μm, 300 Å) column (30 × 5.7 cm). The material was eluted with linear gradients of (A) H₂O containing 0.06% TFA and (B) CH₃CN (40%) in TFA/H₂O (0.06%). The gradients used were 0–45% (B) in 25 min, 45–80% (B) in 60 min, and 80–100% (B) in 20 min. The flow rate was constant at 64 mL/min, and detection was at 230 nm. Fractions were analyzed by analytical reversed-phase HPLC on a 600 Multisolvent Delivery System with a Lambda-Max Model 481 LC spectrophotometer. Analyses were carried out with a Vydac C₁₈ (10-μm) column (30 × 0.39 cm) and an eluant of (A) H₂O with 0.06% TFA and (B) CH₃CN in a linear gradient mode. The gradient used was 15–45% (B) in 15 min. The flow rate was maintained at 1.5 mL/min, and detection was at 230 nm. The fractions corresponding to the purified peptide were pooled and lyophilized.

Peptide Characterization. The peptides were characterized by amino acid analysis, analytical HPLC, and capillary electrophoresis (Table I). Peptides were hydrolyzed with 6 N HCl containing 0.1% phenol for 24 h at 110 °C. The samples were analyzed after drying and derivatization with PITC, according to the method described by Waters Chromatography. The analyses were carried out with a HPLC system comprising two Waters 510 pumps, a Waters 715 Ultra Wisp sample processor, a Waters TCM temperature controller coupled to a column heater module, and a Waters PICO.TAG amino acid analysis column. During the analysis, the column was kept at 30 °C, and the elution of the PTC amino acid derivatives was achieved with successive gradients of CH₃CN in a sodium acetate buffer, according to the operation table described by Waters. The system was controlled, and the data processed with the Waters Baseline 810 chromatography workstation software with the NEC-APC IV Power Mater computer.

Analytical HPLC was performed with the system described above, and the peptides were eluted with a linear gradient of (A) H₂O containing 0.06% TFA and (B) CH₃CN. The gradient used was 20–40% (B) in 20 min at a flow rate of 1.5 mL/min, and the detection was at 230 nm. Capillary electrophoresis was carried out with an Applied Biosystems 270A system using the following conditions: 20 mM sodium citrate buffer, pH 2.5; capillary 45 cm × 50 μm; voltage: 20 kV; T: 30 °C; injection: 3 s in vacuum mode and detection at 200 nm.

Circular Dichroism Spectroscopy. Circular dichroism (CD) spectra were recorded on a Jobin Yvon Dichrograph CD6. The instrument was calibrated with (+)-10-camphorsulfonic acid. Spectra were recorded with a 2-nm bandwidth, 0.5-nm steps, and an integration time of 0.5 s. They were corrected for solvent contribution. The peptide samples were contained between cylindrical quartz windows with a path length of 0.1 cm. The peptides were studied in 40% 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP)/phosphate buffer (20 mM, pH 7.0) mixture, at a concentration of 15 μM. The molecular ellipticities ([θ]λ) are reported in deg-cm²-dmol⁻¹ using the mean residue weight of each peptide for the calculations.

Bioassays and Binding Experiments. Bioassays. Adult male Hartley guinea pigs (350–400 g) and adult male Sprague-Dawley rats (200–250 g) obtained from Charles River (St-Constant, Québec, Canada), were sacrificed by decapitation. The guinea pig heart and rat vas deferens were dissected carefully and immediately placed in oxygenated (95% O₂–5% CO₂) Krebs–Ringer (K-R) buffer solution, [(mmol/L): NaCl, (118), KCl (4.8), CaCl₂(2.2), KH₂PO₄ (1.2), MgSO₄ (1.2), NaHCO₃ (25), and glucose (5.5)] maintained at 37 °C. Left and right atria or the prostatic part of vas deferens were mounted on platinum electrodes in a double-jacketed tissue bath containing oxygenated Krebs–Ringer

buffer. The tissues were equilibred for 1 h at a tension of 0.5-1 g. During that period of time, the K-R buffer was replaced at every 10 min. The tissues were stimulated with square electrical pulses using a Grass Stimulator (Model S44). The stimulation parameters were as follows: amplitude, 8V; duration, 0.3 ms; and frequency, 3 Hz for the left atrium and amplitude, 60-90V; duration, 0.5 ms; and frequency, 0.15 Hz for the rat vas deferens. The responses were recorded on a Grass polygraph, Model 79D using a Grass force transducer Model FT03D (Quincy, MA). Concentration-response curves for the effect of hCGRPα were determined by increasing the peptide concentrations in a cumulative manner. The antagonistic activity of hCGRP$_{8-37}$ or its fragments and analogs was evaluated by adding 1 μM of peptide in the tissue bath, 10 min before the addition of hCGRPα.

Binding Experiments. Male Sprague-Dawley rats (200-250 g) and male Hartley guinea pigs (350-400 g) obtained from Charles River (St. Constant, Québec, Canada) were sacrificed by decapitation. The guinea pig atrium, rat brain (without the cerebellum), and guinea pig vas deferens tissues were isolated, and the membranes were prepared following the method described by Sexton et al.[33] Briefly, the tissues were homogenized with a 20-fold volume of ice-cold 25 mM Tris·HCl buffer pH 7.4 using a Brinkman polytron (setting 6, for 30 s), and the crude homogenate was centrifuged at 48000g for 20 min at 4 °C. The pellets were washed and resuspended in the original volume of buffer and centrifuged. This step was repeated three times, and the final pellets were resuspended in 50 mM Tris·HCl pH 7.4 containing 100 mM NaCl. The content of proteins in the isolated membranes was determined using the method of Lowry et al.[34] A volume of 100 μL of guinea pig atrium, rat brain, and guinea pig vas deferens membrane preparation, corresponding to 75 μg, 250 μg, and 50 μg of proteins, respectively, were then incubated for 2 h at 4 °C in the presence of increasing concentrations (10^{-11}-10^{-6}M) of hCGRPα, or fragments and analogs of hCGRP$_{8-37}$, in 400 μL of 50 mM Tris·HCl buffer containing 100 mM NaCl, 0.2% bovine serum albumin, 0.4 mM bacitracine (Sigma Chemical Co., St. Louis, MO), and 40 pM [^{125}I]hCGRPα (2000 Ci/mmol, Amersham Canada, Oakville, Ontario). After incubation, bound [^{125}I]-

hCGRPα was separated from free by rapid filtration under reduced pressure using a Brandel cell harvester (Model M-24-R, Gaithersburg, MD) through glass fiber filters (no. 32, Schleicher and Schuell), presoaked in a 0.1% polyethyleneimine solution. The tubes were rapidly rinsed with 10 mL of ice-cold buffer, and the radioactivity found on the filters was measured with a γ counter (1281 Compugamma, LKB, Rockville, MD). The specific binding of [^{125}I]hCGRPα to guinea pig atrium, rat brain, and guinea pig vas deferens membrane preparations was estimated by incubation with 1 μM of unlabeled hCGRPα. The specific binding represented approximately 70-90% of total binding at equilibrium.

Data Analysis. Binding data were analyzed using the software package EBDA, Ligand, Kinetic, and Lowry (Bio-Soft Elsevier, Cambridge, UK). Bioassay data were analyzed using σ plot functions. Values are expressed as mean ± SE. Comparisons were made using the one-way analysis of variance followed by student's t test for paired or unpaired values. The level of statistical significance was accepted as $p < 0.05$.

Supplementary Material Available: Figures showing the analytical HPLC of hCGRP$_{8-37}$ after (a) solid-phase synthesis with BOP reagent with HF cleavage, (b) an alkaline treatment, and (c) purification on preparative HPLC, figures showing the concentration-response curves of the increase of inotropic and chronotropic responses of the isolated guinea pig atrium, induced by hCGRPα alone or in presence of fragments or analogs of hCGRP$_{8-37}$, and a figure showing the concentration-response curve for the twitch response inhibition of the electrically stimulated rat vas deferens induced by hCGRPα in absence or in presence of fragment or analogs of hCGRP$_{8-37}$ (4 pages). Ordering information is given on any current masthead page.

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