Calcitonin Gene-Related Peptide Analogues with Aza and Indolizidinone Amino Acid Residues Reveal Conformational Requirements for Antagonist Activity at the Human Calcitonin Gene-Related Peptide 1 Receptor

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Calcitonin gene-related peptide antagonists have potential for the treatment and prevention of disease states such as non-insulin-dependent diabetes mellitus, migraine headache, pain, and inflammation. To gain insight into the spatial requirements for CGRP antagonism, three strategies were employed to restrict the conformation of the potent undecapeptide antagonist, [D^{31},P^{34},F^{35}]$CGRP_{27-37}$. First, aza-amino acid scanning was performed, and ten aza-peptide analogues were synthesized and examined for biological activity. Second, (3*S*,6*S*,9*S*)-2-oxo-3-amino-indolizidin-2-one amino acid (I^2aa) and (2*S*,6*S*,8*S*)-9-oxo-8-amino-indolizidin-9-one amino acid (I^9aa) both were introduced at positions 31−32, 32−33, 33−34, and 34−35, regions of the backbone expected to adopt turns. Finally, the conformation of the backbone and side-chain of the C-terminal residue, Phe^{35}-Ala^{36}-Phe^{37}-NH_2, was explored employing (2*S*,4*R*,6*R*,8*S*)-9-oxo-8-amino-4-phenyl-indolizidin-9-one amino acid (4-Ph-I^9aa) as a constrained phenylalanine mimic. The structure−activity relationships exhibited by our 26 analogues illustrate conformational requirements important for designing CGRP antagonists and highlight the importance of β-turns centered at Gly^{33}-Pro^{34} for potency.

Introduction

Calcitonin gene-related peptide (CGRP,[a] Figure 1) is a 37 amino acid peptide produced in two forms (α and β) by an alternative splicing of calcitonin mRNA.[1] Both α and β human CGRP are present in the central and peripheral nervous systems, and although they differ by three amino acids, they produce similar biological activity.[1−3] CGRP controls many distinct physiological responses including actions on the cardiovascular system and the central nervous system, reproductive organs, skeletal muscles, calcium metabolism, insulin regulation, and gastric secretion.[4]

Two pharmacologically distinct receptors have been proposed to mediate CGRP actions.[5−7] The best characterized $CGRP_1$ receptor is composed of the calcitonin receptor-like receptor (CRLR), a seven transmembrane domain protein, and the receptor-activity-modifying proteins (RAMPs) that determine the pharmacological selectivity of the receptor. Indeed, whereas the CRLR/$RAMP_1$ complex leads to the $CGRP_1$ receptor, association of CRLR with either $RAMP_2$ or $RAMP_3$ leads to adrenomedulin (AM) receptors.[8−10] Although less well characterized, a third protein, the receptor component protein (RCP), has been proposed to act as a chaperone protein required for receptor CGRP function.[11,12] A second pharmacologically distinct CGRP receptor, $CGRP_2$, has been reported to occur in reproductive organs[13−17]and to be less sensitive to $CGRP_{8-37}$ antagonism. To this date, the molecular composition of this receptor remains unknown and its existence as a tractable distinct entity remains controversial.

The very potent vasodilatory effects of CGRP originally suggested that CGRP receptors agonists could represent clinically valuable compounds for the treatment of severe hypertension, cerebrovascular vasospasm, and Raynaud's syndrome; however, drawbacks including lack of efficacy and tachycardia have limited their development.[18−20] CGRP antagonists, however, remain attractive candidates for treating a variety of indications, including, non-insulin-dependent diabetes mellitus, migraine headache, pain, inflammation, and morphine-induced analgesia. For example, a dipeptide analogue (Figure 1) has recently entered into clinical trials for the treatment of migraine.[21,22]

The conformation of CGRP has been shown by NMR and CD spectroscopy to comprise an amino-terminal disulfide-bonded loop containing residues 2−7 that nucleates an α-helical region between residues 8 and 18 which unfolds, *via* a turn about residues 19−21, into a less ordered sequence that may adopt turn conformations.[23−28] Initial examination of CGRP by NMR spectroscopy in 1:1 trifluoroethanol:water identified the presence of a turn-like conformation about residues 19−21.[25] Examination of CGRP in DMSO by NMR spectroscopy and computational analysis provided further evidence to suggest a γ-turn between the amide carbonyl of Ser^{19} and the amide NH of Gly^{21}.[27] Spectroscopic analysis of the C-terminal [But-Cys^{18}]h-β-$CGRP_{19-37}$ fragment in DMSO also revealed a turn conformation in the Ser^{19}-Gly^{21} region as well as indicated a second type I β-turn in the region of Asn^{31}-Val^{32}-Gly^{33}.[28] Although this second turn was not observed in spectroscopic studies of the parent CGRP peptide, a comparison with a C-terminal fragment of CGRP and two different agonists, both constrained by a disulfide ring, one comprised at the C- and the other at the N-terminal fragment, indicated that the Val^{32}-Gly^{33}-Ser^{34} sequence may adopt an inverse γ-turn.[29,30]

The analysis of abridged CGRP analogues has shown that N-terminal fragments usually activate the receptor[31] and that the C-terminal fragments act as antagonists of CGRP action.[32−38]

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[a] Abbreviations: CGRP, calcitonin gene-related peptide; CRLR, calcitonin receptor like receptor; RAMP, receptor activity modifying proteins; AM, adremodulin receptor; BTD, bicyclic turn dipeptide; BTC, bis(trichloromethyl)carbonate; TIS, triisopropylsilane; HBTU, *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; Iaa, indolizidinone amino acid.

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hα-CGRP	ACDTATCVTHRLAGLLSRSGGVVKNNFVPTNVGSKAF-NH_2
hβ-CGRP	AC**N**TATCVTHRLAGLLSRSGG**N**VK**S**NFVPTNVGSKA**F**-NH_2
hα-$CGRP_{8-37}$	VTHRLAGLLSRSGGVVKNNFVPTNVGSKAF-NH_2
[But-Cys^{19}]$CGRP_{19-37}$	C(tBu)GGVVKNNFVPTNVGSKAF-NH_2
[BTD^{19-20}]$CGRP_{8-37}$	VTHRLAGLLSR(**BTD**)GVVKNNFVPTNVGSKAF-NH_2
[BTD^{33-34}]$CGRP_{8-37}$	VTHRLAGLLSRSGGVVKNNFVPTNV(**BTD**)KAF-NH_2
[BTD^{19-20}, BTD^{33-34}]$CGRP_{8-37}$	VTHRLAGLLSR(**BTD**)GVVKNNFVPTNV(**BTD**)KAF-NH_2
hα-$CGRP_{27-37}$	FVPTNVGSKAF-NH_2
[D^{31}, P^{34}, F^{35}]$CGRP_{27-37}$	FVPT**D**VG**PF**AF-NH_2

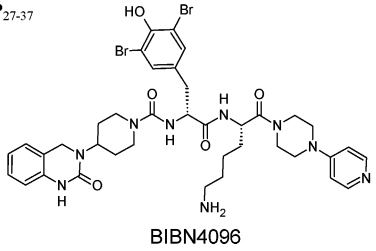

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Figure 1. Structures of hα,*β*-CGRP, peptidic and nonpeptidic CGRP antagonists.

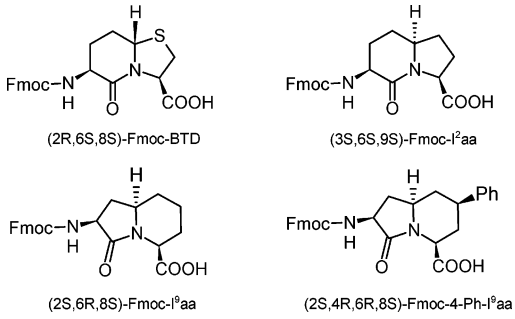

Figure 2. Representative indolizidinone amino acids.

In particular, $CGRP_{27-37}$ has been shown to be the shortest C-terminal peptide with a natural sequence having significant antagonist activity. Extensive structure-activity relationship studies of this peptide led to the discovery of [D^{31},P^{34},F^{35}]-$CGRP_{27-37}$, an antagonist with nanomolar affinity at the $hCGRP_1$ receptor, representing a 100-fold increased affinity compared to the unmodified CGRP segment.[39−41] Additionally, removal of its first two N-terminal residues associated with the substitution of three residues at position 31, 34, and 35 did not dramatically alter receptor binding and has furnished [D^{31},P^{34},F^{35}]-$CGRP_{29-37}$, an antagonist displaying only a 5-fold decrease of binding affinity compared with $CGRP_{27-37}$.[42] Examination of [D^{31},P^{34},F^{35}]$CGRP_{27-37}$ and related analogues by CD spectroscopy revealed that increases in ordered structure correlated with high binding affinity and suggested that replacement of the original Ser^{34} by proline reinforced a turn already present in the unmodified segment.[42] Studies of analogues of $CGRP_{27-37}$ indicated that Thr^{30}, Val^{32}, and Phe^{37} also play critical roles for antagonist potency on $hCGRP_1$, and computational and NMR spectroscopic studies suggested the participation of Thr^{30} in a turn conformation.[42] Two alternative turn conformations have also been proposed to be important for antagonist activity of C-terminus fragments: a possible *γ*-turn around Pro^{34} in [D^{31},P^{34},F^{35}]$CGRP_{27-37}$ and a left-handed helical turn from V^{32} to A^{36} in [D^{31},A^{34},F^{35}]$CGRP_{27-37}$. More recently, the introduction of the turn inducing thiaindolizidinone amino acid (BTD, Figure 2) at positions 19, 20 and 33, 34 of the antagonist hα-$CGRP_{8-37}$ produced constrained analogues that exhibited similar antagonist activity ($pA_2 = 6.0$ to 6.2) as the parent peptide (pA_2 ($CGRP_{27-37}$) = 6.0).[17,43] This observation has led to a suggestion that the active conformer possesses two *β*-bends at positions 18−21 and 32−35.

Exploring further the conformational requirements for the activity of peptide based CGRP antagonists, we have employed three strategies for restraining the geometry of the potent undecapeptide [D^{31},P^{34},F^{35}]$CGRP_{27-37}$. Initially, aza-amino acid scanning of the C-terminal peptide, i.e., sequential replacement of amino acid residues in the peptide sequence by their aza-amino acid counterparts, was performed by the synthesis of five aza-analogues of [D^{31},P^{34},F^{35}]$CGRP_{27-37}$ (antagonist **1**) and five aza-analogues of [D^{31},P^{34},F^{35}]$CGRP_{29-37}$ (antagonist **2**). This approach was taken to probe the existence, location, and importance of turn structures in antagonists **1** and **2**, because aza-peptides, peptide analogues in which the α-carbon of one or more of the amino acid residues is replaced with a nitrogen atom, retain normal side-chain functions and exhibit propensity for adopting the central positions of a *β*-turn conformation.[44−47] Moreover, aza-peptides of peptides **1** and **2** were examined because of their potential to increase resistance to proteases and provide mimics with longer duration of action relative to the natural peptide.[48−53]

In the second phase of our study, indolizidin-2- and -9-one *N*-(Fmoc)amino acids (Fmoc-I^2aa and Fmoc-I^9aa) were employed to induce restricted turn conformations in the backbone at positions 31−32, 32−33, 33−34, and 34−35, to characterize the geometry responsible for activity. X-ray structural analyses of *N*-(Boc)amino indolizidin-2- and -9-one methyl esters (Boc-I^2aa-OMe and Boc-I^9aa-OMe)[54] have indicated that the central dihedral angles constrained within the heterocycle were similar to the ideal values for the $i + 1$ and $i + 2$ residues of a type II′ *β*-turn. By NMR spectroscopy and computational analysis of model peptides, I^2aa has been shown to adopt the $i/i + 1$ positions as well as the $i + 1/i + 2$ positions of a *β*-turn and the $i/i + 1$ positions of a *γ*-turn.[55,56] Similar preferences for both the $i/i + 1$ positions and the $i + 1/i + 2$ positions of *β*-turn conformations have been previously observed when the thiaindolizidinone amino acid (BTD, Figure 3) was introduced respectively into cyclic hexapeptide mimics of tendamistat[57] and into the antibiotic cyclic peptide gramicidin S.[58] The synthesis of peptides respectively possessing I^2aa and I^9aa residues was intended to identify the region and backbone dihedral angle geometry of an active turn.

In the final strategy for constraining peptides **1** and **2**, the importance of the backbone and side-chain geometry of the aromatic residues in the C-terminal peptide was explored by the use of (2*S*,4*R*,6*R*,8*S*)-4-phenyl-indolizidin-9-one *N*-(Fmoc)-amino acid (Fmoc-4-Ph-I^9aa) as a constrained Phe derivative.

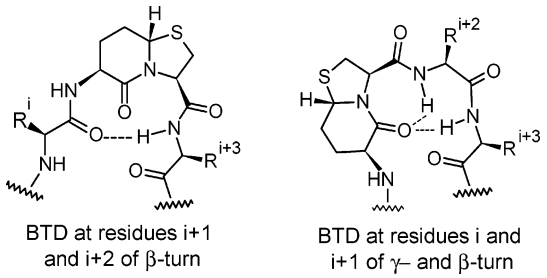

Figure 3. Positioning of BTD in peptide turns.

Introduction of 4-Ph-I[9]aa at positions 34−35 and 36−37 was used to explore the possibility of a turn region with a specifically oriented aromatic ring for the Pro-Phe and Ala-Phe regions. In summary, this application of aza-peptides and peptides constrained with different indolizidinone amino acids was focused on defining the significance of turn geometry for peptide activity and furnishing leads for the design of more effective mimics.

Results

Chemistry. Aza-peptides. Aza-Phe[27] (**8**), aza-Pro[34] (**5**), and aza-Phe[35] (**6** and **11**) peptide analogues were prepared as previously reported by using N-(Boc)-aza[1]-dipeptide building blocks. The respective *N*-Boc-Aza[1]-dipeptides were first synthesized in solution and subsequently incorporated into aza-peptides **26**−**29** by using a Boc protection strategy on oxime resin.[59,60] After cleavage of the peptide from the resin with saturated NH_3 in MeOH:DCM and removal of the side chain protecting groups [Thr(OBn) and Asp(OBn)] by hydrogenolysis, the Boc group of the final peptide was removed using 25% TFA in DCM, and aza-peptides **5**, **6**, **8**, and **11** were purified by RP-HPLC to furnish product suitable for biological analysis in 10−20% yields based on initial loading.

Aza-Asp[31] (**3** and **9**) and aza-Ala[36] (**7** and **12**) analogues were synthesized on acid-labile Rink resin[61] as previously reported by using Fmoc-aza-amino acid chloride building blocks;[62] aza-Gly[33] (**4** and **10**) was introduced by way of its 1,3,4-oxadiazol-2(*3H*)-one.[63] Standard Fmoc/*t*Bu protocols with Fmoc amino acids and HBTU as coupling agent were used to elongate the resin-bound peptide.[64] After acylation with the aza-amino acid residue, the subsequent Fmoc amino acid was coupled by using BTC as coupling agent,[65] and peptide synthesis was continued. Attempts failed to acylate the aza-Asp(OtBu) residue on the resin-bound peptide using Fmoc-Thr(OtBu)-OH and a number of activation methods, such that it was introduced as its oxazolidine carboxylate analogue. After simultaneous cleavage of the aza-peptide from the support and deprotection of side chain protecting groups with a TFA/TIS/H_2O (95/2.5/2.5, v/v/v) solution, the aza-peptides were isolated by precipitation with Et_2O, dissolved in a 1:1 acetonitrile/H_2O solution, and lyophilized to white foams that were directly analyzed by RP-HPLC to assess purity. Samples requiring additional purification were isolated by preparative RP-HPLC.

Chemistry. Indolizidinone Peptides. The rigid dipeptide surrogates I[2]aa, I[9]aa, and 4-Ph-I[9]aa were synthesized according to literature procedures that delivered the Boc-protected analogues.[54,66,67] Subsequent N-deprotection and conversion to the Fmoc-amino acid was achieved as described for I[2]aa in the Experimental Section. Standard peptide chemistry using an Fmoc-protection strategy and the respective indolizidinone N-(Fmoc)amino acid (Fmoc-I[2]aa, Fmoc-I[9]aa, and Fmoc-4-Ph-I[9]aa) was performed on BHA resin modified with a Rink linker by way of an amino caproic acid spacer. After treatment with piperidine to remove the terminal Fmoc protection, the final peptides were treated with neat TFA for 45 min to cleave the resin and to deprotect amino acid side chains. The resin was washed twice with TFA, and the TFA layers were combined and treated with excess Et_2O to precipitate peptides. The peptides were removed by centrifugation, and the solution was evaporated to an oil, that was precipitated again with ether. The combined white solids were dried under vacuum, dissolved in 50% water/50% acetonitrile, and purified on semipreparative HPLC/MSQ using an Alltech C18 column (25 cm × 22 mm), a 20 mL/min flow rate, and a gradient of water:acetonitrile containing 0.1% of TFA. Purification furnished peptides in 23% average overall yields.

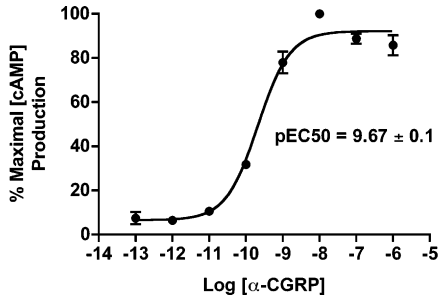

Figure 4. Dose−response curve of α-CGRP-promoted cAMP production. HEK293 stably expressing CRLR were challenged with increasing concentrations of α-CGRP, and cAMP levels were measured as described in the Experimental Section. Data are means ± SEM of three independent experiments. pEC_{50} is calculated from nonlinear regression fitting using GraphPad Prism 4.

Biological Examinations. Inhibition of Agonist-Promoted cAMP Production by CGRP(27−37) Analogues. To assess the ability of various $CGRP_{27-37}$ peptide analogues to inhibit agonist-promoted cAMP production by the $CGRP_1$ receptor, we used a HEK293 cell line that stably expresses human CRLR.[70] As shown in Figure 4, treatment of these cells with α-CGRP resulted in a dose-dependent accumulation of cAMP with an EC_{50} of ∼0.2 nM, which is similar to what was previously reported for the $CGRP_1$ receptor.[69] This indicated that the CRLR-HEK293 cells used herein contain sufficient endogenous RAMP-1 and RCP proteins for the generation of a functional $CGRP_1$ receptor. Therefore, this cell line was further used to test the effect of peptide analogues based on the $CGRP_1$ receptor antagonist [D[31],P[34],F[35]]$CGRP_{27-37}$ on α-CGRP-promoted cAMP production by $CGRP_1$. The inhibition potency and efficacy of individual peptide analogues are summarized in Table 2. The potency is expressed as pIC_{50} whereas the efficacy is presented as % inhibition of the cAMP production promoted by 0.5 nM α-CGRP.

Deletion of amino acids 27 and 28 of [D[31],P[34],F[35]]$CGRP_{27-37}$ did not affect its potency or efficacy for inhibiting cAMP production, indicating that amino acids 27 and 28 may not be essential for the antagonistic activity of the peptide. On the other hand, aza-amino acid scanning of the shorter $CGRP_{29-37}$ peptide resulted in total loss of cAMP inhibition when the respective residues (Asp[31], Gly[33],Phe[35], and Ala[36]) were replaced by an aza-amino acid, and a ∼6 fold reduction in potency in the case of Pro.[34] Aza-peptide analogues exhibited better potency in the longer peptides. For example, replacement of Phe[35] and Ala[36] of [D[31],P[34],F[35]]$CGRP_{27-37}$ resulted in only a 5 − 8-fold reduction in potency instead of promoting complete loss of activity in [D[31],P[34],F[35]]$CGRP_{29-37}$. Aza-residues at Phe[27] and Asp[31] did not significantly alter the potency nor efficacy relative to the parent peptide. Moreover, replacement of Gly[33] by its aza-amino acid counterpart resulted in an approximately 10-fold increase in potency.

Table 1. Analytical Data for the Peptide, Aza-peptide, and Azabicycloalkane-Containing Peptide Analogues Tested in This Study

no.	peptide	structure	HPLC t_{R_1}, min (system 1)	HPLC t_{R_2}, min (system 2)	M + 1 (calcd)	mass spectral analysis (M + 1)	purity (%) at 214 nm
1[a]	[D^{31},P^{34},F^{35}]CGRP$_{27-37}$	FVPTDVGPFAF-NH$_2$	18.4	22.7	1195.6	1196.0	>98
2[a]	[D^{31},P^{34},F^{35}]CGRP$_{29-37}$	PTDVGPFAF-NH$_2$	15.1	21.5	949.5	949.5	>99
3[b]	[azaD^{31},P^{34},F^{35}]CGRP$_{29-37}$	PTazaDVGPFAF-NH$_2$	14.2	26.2	950.5	950.5	86
4[b]	[D^{31},azaG^{33},P^{34},F^{35}]CGRP$_{29-37}$	PTDVazaGPFAF-NH$_2$	15.8	24.9	950.5	950.4	90
5[c]	[D^{31},azaP^{34},F^{35}]CGRP$_{29-37}$	PTDVGazaPFAF-NH$_2$	–	–	950.5	950.5	88
6[c]	[D^{31},P^{34},azaF^{35}]CG RP$_{29-37}$	PTDVGPazaFAF-NH$_2$	–	–	950.5	950.5	91
7[b]	[D^{31},P^{34},F^{35},azaA^{36}]CGRP$_{29-37}$	PTDVGPFazaAF-NH$_2$	16.3	25.2	950.5	950.4	96
8[c]	[azaF^{27},D^{31},P^{34},F^{35}]CGRP$_{27-37}$	azaFVPTDVGPFAF-NH$_2$	–	–	1196.6	1196.3	>99
9[b]	[azaD^{31},P^{34},F^{35}]CGRP$_{27-37}$	FVPTazaDVGPFAF-NH$_2$	16.3	27.8	1196.6	1196.6	86
10[b]	[D^{31},azaG^{33},P^{34},F^{35}]CGRP$_{27-37}$	FVPTDVazaGPFAF-NH$_2$	16.1	26.8	1196.6	1196.6	95
11[c]	[D^{31},P^{34},azaF^{35}]CGRP$_{27-37}$	FVPTDVGPazaFAF-NH$_2$	–	–	1196.6	1196.6	92
12[b]	[D^{31},P^{34},F^{35},azaA]CGRP$_{27-37}$	FVPTDVGPFazaAF-NH$_2$	17.5	27.4	1196.6	1196.5	95
13[a]	[I^{2}aa$^{31-32}$,P^{34},F^{35}]CGRP$_{29-37}$	PTI^{2}aaGPFAF-NH$_2$	14.5	20.6	915.5	915.7	>99
14[a]	[I^{9}aa$^{31-32}$,P^{34},F^{35}]CGRP$_{29-37}$	PTI^{9}aaGPFAF-NH$_2$	15.0	20.8	915.5	915.7	>98
15[a]	[D^{31},I^{2}aa$^{34-35}$]CGRP$_{29-37}$	PTDVGI^{2}aaAF-NH$_2$	8.2	17.7	885.4	885.3	91
16[a]	[D^{31},I^{9}aa$^{34-35}$]CGRP$_{29-37}$	PTDVGI^{9}aaAF-NH$_2$	9.4	17.8/18.1	885.4	885.3	60/30
17[a]	[I^{2}aa$^{31-32}$,P^{34},F^{35}]CGRP$_{27-37}$	FVPTI^{2}aaGPFAF-NH$_2$	17.8	22.4	1161.7	1161.2	86
18[a]	[I^{9}aa$^{31-32}$,P^{34},F^{35}]CGRP$_{27-37}$	FVPTI^{9}aaGPFAF-NH$_2$	18.6	22.5	1161.7	1162.0	87
19[b]	[D^{31},I^{2}aa$^{32-33}$,P^{34},F^{35}]CGRP $_{27-37}$,	FVPTDI^{2}aaPFAF-NH$_2$	16.3	26.3	1219.6	1219.6	>99
20[b]	[D^{31},I^{9}aa$^{32-33}$,P^{34},F^{35}]CGRP $_{27-37}$	FVPTDI^{9}aaPFAF-NH$_2$	16.9	26.8	1219.6	1219.6	>99
21[b]	[D^{31},I^{2}aa$^{33-34}$,F^{35}]CGRP $_{27-37}$	FVPTDVI^{2}aaFAF-NH$_2$	18.2	28.6	1221.6	1221.7	>99
22[b]	[D^{31},I^{9}aa$^{33-34}$,F^{35}]CGRP $_{27-37}$	FVPTDVI^{9}aaFAF-NH$_2$	17.4	28.3	1221.6	1221.7	>99
23[a]	[D^{31},I^{2}aa$^{34-35}$]CGRP$_{27-37}$	FVPTDVGI^{2}aaAF-NH$_2$	12.6	19.9	1131.6	1131.8	91
24[a]	[D^{31},I^{9}aa$^{34-35}$]CGRP$_{27-37}$	FVPTDVGI^{9}aaAF-NH$_2$	13.6	20.1	1131.6	1131.8	86
25[a,d]	[D^{31},Xaa$^{34-35}$]CGRP$_{29-37}$	PTDVGXaaAF-NH$_2$	16.6	21.7	961.5	961.7	>99
26[a,d]	[D^{31},P^{34},F^{35},Xaa$^{36-37}$]CGRP$_{29-37}$	PTDVGPFXaa-NH$_2$	17.0	22.5	987.5	987.7	>99
27[a,d]	[D^{31},Xaa$^{34-35}$]CGRP$_{27-37}$	FVPTDVGXaaAF-NH$_2$	20.1	23.1	1207.6	1207.4	>99
28[a,d]	[D^{31},P^{34},F^{35},Xaa$^{36-37}$]CGRP$_{27-37}$	FVPTDVGPFXaa-NH$_2$	21.0	23.8	1233.6	1233.7	92

Analytical HPLC analyses were performed on an TARGA column from Higgins Analytical, Inc (4.6 × 250 mm, 5 μm, C_{18}) with a flow rate of 1.5 mL/min [a]using water (0.1% TFA)/CH_3CN (0.1% TFA) (system 1) eluents from 80/20 to 50/50 in 30 min and 50/50 to 10/90 in 5 min and water (0.1% TFA)/MeOH (0.1% TFA) (system 2) from 80/20 to 20/80 in 20 min, 20/80 to 10/90 in 5 min, [b]using a 40 min linear gradient from water (0.1% TFA) to CH_3CN (0.1% TFA) (system 1) or MeOH (0.1% TFA) (system 2). [c] See ref 59 for analytical datas. [d] Xaa = 4-Ph-I^{9}aa. [e] Two signals (2:1 ratio) corresponding to the same mass were observed, which may correspond to either a conformational or a configurational mixture.

Table 2. Antagonist Activities of [D^{31},P^{34},F^{35}]-CGRP$_{27-37}$ (**1**) and [D^{31},P^{34},F^{35}]-CGRP$_{29-39}$ (**2**) and Their Constrained Analogues on hαCGRP-Induced cAMP Accumulation in HEK293 Cells

no.	peptides	structure	pIC$_{50}$[b]	% inhibition[b]	n[c]
1	[D^{31},P^{34},F^{35}]CGRP$_{27-37}$	FVPTDVGPFAF-NH$_2$	6.15 ± 0.18	68 ± 4	4
2	[D^{31},P^{34},F^{35}]CGRP$_{29-37}$	PTDVGPFAF-NH$_2$	6.11 ± 0.18	81 ± 7	4
3	[azaD^{31},P^{34},F^{35}]CGRP$_{29-37}$	PTazaDVGPFAF-NH$_2$	N.A.[a]	N.A.	3
4	[D^{31},azaG^{33},P^{34},F^{35}]CGRP$_{29-37}$	PTDVazaGPFAF-NH$_2$	N.A.	N.A.	3
5	[D^{31},azaP^{34},F^{35}]CGRP$_{29-37}$	PTDVGazaPFAF-NH$_2$	5.35 ± 0.16	68 ± 8	3
6	[D^{31},P^{34},azaF^{35}]CGRP$_{29-37}$	PTDVGPazaFAF-NH$_2$	N.A.	N.A.	3
7	[D^{31},P^{34},F^{35},azaA^{36}]CGRP$_{29-37}$	PTDVGPFazaAF-NH$_2$	N.A.	N.A.	3
8	[azaF^{27},D^{31},P^{34},F^{35}]CGRP$_{27-37}$	azaFVPTDVGPFAF-NH$_2$	6.23 ± 0.34	69 ± 25	3
9	[azaD^{31},P^{34},F^{35}]CGRP$_{27-37}$	FVPTazaDVGPFAF-NH$_2$	6.42 ± 0.30	59 ± 3	3
10	[D^{31},azaG^{33},P^{34},F^{35}]CGRP$_{27-37}$	FVPTDVazaGPFAF-NH$_2$	7.17 ± 0.17	70 ± 6	3
11	[D^{31},P^{34},azaF^{35}]CGRP$_{27-37}$	FVPTDVGPazaFAF-NH$_2$	5.24 ± 0.26	69 ± 5	3
12	[D^{31},P^{34},F^{35},azaA^{36}]CGRP $_{27-37}$	FVPTDVGPFazaAF-NH$_2$	5.45 ± 0.38	51 ± 5	3
13	[I^{2}aa$^{31-32}$,P^{34},F^{35}]CGRP$_{29-37}$	PTI^{2}aaGPFAF-NH$_2$	N.A.	N.A.	3
14	[I^{9}aa$^{31-32}$,P^{34},F^{35}]CGRP$_{29-37}$	PTI^{9}aaGPFAF-NH$_2$	N.A.	N.A.	3
15	[D^{31},I^{2}aa$^{34-35}$]CGRP$_{29-37}$	PTDVGI^{2}aaAF-NH$_2$	N.A.	N.A.	3
16	[D^{31},I^{9}aa$^{34-35}$]CGR P$_{29-37}$	PTDVGI^{9}aaAF-NH$_2$	N.A.	N.A.	3
17	[I^{2}aa$^{31-32}$,P^{34},F^{35}]CGRP$_{27-37}$	FVPTI^{2}aaGPFAF-NH$_2$	N.A.	N.A.	3
18	[I^{9}aa$^{31-32}$,P^{34},F^{35}]CGRP$_{27-37}$	FVPTI^{9}aaGPFAF-NH$_2$	N.A.	N.A.	3
19	[D^{31},I^{2}aa$^{32-33}$,P^{34},F^{35}]CGRP$_{27-37}$,	FVPTDI^{2}aaPFAF-NH$_2$	N.A.	N.A.	3
20	[D^{31},I^{9}aa$^{32-33}$,P^{34},F^{35}]CGRP$_{27-37}$	FVPTDI^{9}aaPFAF-NH$_2$	N.A.	N.A.	3
21	[D^{31},I^{2}aa$^{33-34}$,F^{35}]CGRP$_{27-37}$	FVPTDVI^{2}aaFAF-NH$_2$	6.97 ± 0.17	70 ± 6	5
22	[D^{31},I^{9}aa$^{33-34}$,F^{35}]CGRP$_{27-37}$	FVPTDVI^{9}aaFAF-NH$_2$	N.A.	N.A.	3
23	[D^{31},I^{2}aa$^{34-35}$]CGRP$_{27-37}$	FVPTDVGI^{2}aaAF-NH$_2$	N.A.	N.A.	3
24	[D^{31},I^{9}aa$^{34-35}$]CGRP$_{27-37}$	FVPTDVGI^{9}aa AF-NH$_2$	N.A.	N.A.	3
25	[D^{31},Xaa$^{34-35}$]CGRP$_{29-37}$	PTDVGXaaAF-NH$_2$	N.A.	N.A.	3
26	[D^{31},P^{34},F^{35},Xaa$^{36-37}$]CGRP$_{29-37}$	PTDVGPFXaa-NH$_2$	N.A.	N.A.	3
27	[D^{31},Xaa$^{34-35}$]CGRP$_{27-37}$	FVPTDVGXaaAF-NH$_2$	N.A.	N.A.	2
28	[D^{31},P^{34},F^{35},Xaa$^{36-37}$]CGRP$_{27-37}$	FVPTDVGPFXaa-NH$_2$	N.A.	N.A.	3

[a] N.A., not applicable: no inhibition was detected with peptide concentrations up to 100 μM. [b] Data are represented as mean ± SEM from at least three independent experiments. [c] Number of experiments.

The difference between the short and long CGRP analogue was particularly spectacular when considering the replacement of Gly^{33} that led to a complete loss of potency in [D^{31},P^{34},F^{35}]-CGRP$_{29-37}$ (**4**) but promoted a 10-fold increase in potency when introduced into [D^{31},P^{34},F^{35}]CGRP$_{27-37}$ (**10**). This indicates that the short and long analogues do not share identical binding

Table 3. Backbone Torsion Angles Observed in Various Ideal β-Turn and γ-Turn Types, in Dipeptide Surrogates I^2aa and I^9aa, and in Aza-amino Acid-Induced Turns

amino acid or dipeptide surrogate		φ_{i+1}	ψ_{i+1}	φ_{i+2}	ψ_{i+2}
−	ideal β I turn	−60	−30	−90	0
−	ideal β I turn	60	30	90	0
−	ideal β II turn	−60	120	80	0
−	ideal β II turn	60	−120	−80	0
−	ideal β III turn	−60	−30	−60	−30
−	ideal β III turn	60	30	60	30
	ideal β IV turn	−61	10	−53	17
−	ideal β V turn	−80	80	−80	80
−	ideal β V turn	80	−80	80	−80
−	ideal β VIa turn	−60	120	−90	0
	ideal β VIb turn	−120	120	−60	0
−	ideal β VIII turn	−60	−30	−120	120
−	classic γ turn	75	−64	−	−
−	inverse γ turn	−79	69	−	−
I^2aa ($i+1/I+2$)	β II type turn[a]	−	−176	−78	−
I^9aa ($I+1/i+2$)	β II type turn[a]	−	−141	−34	−
Aza-aa ($i+1$)	β I type turn[b]	−74	−23	−102	12
	β II type turn[b]	59	−153	−90	12
Aza-aa ($i+2$)	β II type turn[b]	−60	130	71	21

[a] Data obtained from X-ray analysis of Boc-I^2aa-OMe and Boc-I^9aa-OMe, see ref 51. [b] Data obtained from computational analysis of Ac-AzaGly/Ala-L-Ala-NHMe and Ac-L-Ala-AzaGly/Ala-NHMe, see ref 74.

modes with the receptor and suggests that the two peptides need to be considered independently for the rational design of more potent/efficacious ligands.

In the second part of this study, the effects of substituting indolizidin-2- and -9-one *N*-(Fmoc)-amino acids (Fmoc-I^2aa and Fmoc-I^9aa) at positions 31−32, 32−33, 33−34, and 34−35 of $CGRP_{27-37}$ and $CGRP_{29-37}$ were investigated. For both $CGRP_{27-37}$ and $CGRP_{29-37}$, the introduction of I^9aa at positions 31−32, 32−33, 33−34, and 34−35 resulted in a failure to inhibit α-CGRP-promoted cAMP production. Similarly, most of the I^2aa analogues were inactive, such as those with I^2aa at positions 31−32, 32−33, and 34−35 of both $CGRP_{27-37}$ and $CGRP_{29-37}$ peptides. Only in the case of substitution of I^2aa at positions 33−34 of $CGRP_{27-37}$ was activity found, and a ∼7 fold increase in potency was observed for inhibition of α-CGRP-promoted cAMP production.

Finally, substitutions of 4-Ph−I^9aa at positions 34−35 and 36−37 of $CGRP_{27-37}$ and $CGRP_{29-37}$ resulted in peptide analogues that failed to inhibit cAMP production by the $CGRP_1$ receptor. Treatment of the CRLR-HEK293 cells with $CGRP_{27-37}$ analogues (up to 1 μM) in absence of α-CGRP did not result in significant production of cAMP (data not shown), indicating that none showed partial agonistic activity toward $CGRP_1$.

Discussion

The introduction of aza-amino acids into a peptide may restrict peptide geometry by inducing turn conformations as a result of electronic interactions including dipole−dipole repulsion and resonance stabilization.[72,73] Indolidizidone dipeptides, for their part, create structural constraints by annulation and steric interactions that also favor turn geometry. Considering their different modes of action, the aza-scan and indolizidinone-scan were expected to serve as complementary methods for identifying the bioactive turn geometry of the active peptides. Biological activity exhibited by aza-Gly^{33} (**10**) and I^2aa^{33-34} (**21**) analogues of $[D^{31},P^{34},F^{35}]CGRP_{27-37}$ (**1**) showing respectively 10- and 7-fold increased antagonism potency, both with similar efficacy compared to their parent peptide (**1**), confirmed the importance of a turn conformation for antagonist activity at this position. In light of crystallographic and computational data of aza- and indolizidinone amino acids (Table 3), we can speculate that a type II′ beta-turn with Gly^{33}-Pro^{34} at the $i+1$ and $i+2$ positions may likely be the active antagonist conformation in the parent peptide.

In contrast to the enhanced activity observed in I^2aa-analogue **21**, use of I^9aa amino acid as a Gly^{33}-Pro^{34} surrogate in $[D^{31},P^{34},F^{35}]CGRP_{27-37}$ (**1**) did not produce a compound with significant activity. Although such sensitivity to the change in heterocycle may suggest that a precise set of backbone torsion angles in the turn is a prerequisite for the biologically active conformer of $[D^{31},P^{34},F^{35}]CGRP_{27-37}$ (**1**), different steric effects of the two ring systems may also have differentially affected binding. Similarly, although the introduction of I^2aa at positions other than 33−34 and the use of other indolizidinone amino acids (I^9aa and 4-Ph−I^9aa) were not well tolerated by the $CGRP_1$ receptor, these results do not necessarily refute the presence of turns at these positions in the receptor-bound conformation of the parent peptide, because the steric bulk of the Iaa residues as well as the removal of key side chains may have interfered with binding in such cases. Interestingly, the aza-amino acid scan did produce other active compounds (aza-peptides **5**, **8**, **9**, **11**, and **12**) that displayed either equal or moderately lower potency than the parent peptide. This indicates that the introduced constraints did not prevent the analogues from adopting active conformers that may result from the presence and orientation of the amino acid side chains. Finally, the striking difference of the activities of the aza-Gly^{33} analogues of the longer and shorter peptide antagonists **1** and **2** may indicate that the two peptides bind the receptor in a different manner and as a consequence information gained from the scanning of the shorter peptide cannot be extrapolated to obtain information on the binding modalities of the longer peptides.

As mentioned, spectroscopic analyses of related C-terminal analogues have suggested a turn geometry about the residues from positions 31 to 36, including a type I β-turn in the region of Asn^{31}-Val^{32}-Gly^{33},[28] a possible γ-turn around Pro^{34} and a left-handed helical turn from V^{32} to A^{36}.[42,71] Consistent with this notion, insertion of the thiaindolizidinone BTD at positions 33, 34 of the antagonist hα-$CGRP_{8-37}$ did not affect antagonist activity, indicative of the presence of a type II′ turn centered at this position.[17,43] Recently, computational analysis, NMR spectroscopy, and the insertion of turn-inducing amino acid surrogates into $[D^{31},P^{34},F^{35}]$-$CGRP_{27-37}$ have suggested that Pro^{29} and Thr^{30} exist in a type I β-turn conformation and that a hydrophobic residue at position 29 enhances receptor affinity.[42,71] The presence of multiple turn conformations has, furthermore, led to the hypothesis of a polyproline-like structure for $[D^{31},P^{34},F^{35}]$-$CGRP_{27-37}$.[71] The presence of another turn located lower in the sequence and the possibility of a polyproline conformation are not, however, supported by our study involving the systematic use of aza- and indolizidinone amino acids. Support for a turn conformation was obtained for only the type II′ beta-turn with Gly^{33}-Pro^{34} at the $i+1$ and $i+2$ positions, as the active antagonist conformation in the parent peptide.

Conclusion

A better insight into the structural requirements for antagonist activity at the $CGRP_1$ receptor of the potent antagonist undecapeptide $[D^{31},P^{34},F^{35}]CGRP_{27-37}$ has been obtained by the use of aza-amino acid and indolizidinone dipeptide surrogates for identifying turn secondary structures responsible for peptide activity. The importance of a type II′ beta-turn centered at Gly^{33}-Pro^{34} has been illustrated by the respective 10- and 7-fold increase antagonism potency of the aza-Gly^{33} (**10**) and I^2aa^{33-34}

(**21**) analogues of [D^{31},P^{34},F^{35}]CGRP$_{27-37}$ (**1**). In addition, a difference of receptor binding modes has been indicated by the contrast in potency of aza-Gly^{33} analogues of **1** and **2**. The application of complimentary organic chemistry tools for conformational constraint such as aza-amino acids and indolidizidinone dipeptide surrogates has provided two new leads for the development of calcitonin gene-related peptide antagonists as well as insight into their active receptor bound conformation. Moreover, the improved metabolic stability and longer duration of action observed previously with aza- and indolizidinone amino acid analogues suggest that the aza-Gly^{33} and I^{2}aa^{33,34} analogues **10** and **21** offer interesting potential for developing more active antagonist peptide mimics with improved pharmacological profiles.

Experimental Section

According to our reported method, (3*S*,6*S*,9*S*)-indolizidin-2-one *N*-(Boc)amino acid (Boc-I^{2}aa) was prepared from L-glutamic acid in 23% overall yield, and (2*S*,6*R*,8*S*)-indolizidin-9-one *N*-(Boc)-amino acid (Boc-I^{9}aa) and (2*S*,4*R*,6*R*,8*S*)-4-phenyl-indolizidin-9-one *N*-(Boc)amino acid (Boc-4-Ph−I^{9}aa) were prepared from L-aspartic acid in 28% and 8% respective overall yield.[66,67] Subsequent conversion to their *N*-Fmoc amino acid counterparts was performed as described below. Solvents (DMF, CH_2Cl_2, and Et_2O) were dried using a GlassContour solvent dispensing system. *N*,*N*-Diisopropylethylamine (DIEA) was successively distilled from ninhydrin, and CaH_2 and was kept under argon atmosphere. Solid-phase synthesis was performed in SPE tubes (3 or 6 mL) using vortex agitation.

(3*S*,6*S*,9*S*)-2-Oxo-3-(*N*-(9-fluorenylmethoxycarbonyl)amino)-1-azabicyclo[4.3.0]nonane-9-carboxylic Acid ((3*S*,6*S*,9*S*)-Fmoc-I^{2}aa). A solution of (3*S*,6*S*,9*S*)-*N*-Boc-I^{2}aa (2 g, 6.7 mmol, prepared according to ref 54) in 50 mL of EtOAc was treated with dry HCl gas bubbles at room temperature until TLC analysis (eluent 5% AcOH in EtOAc) showed complete disappearance of the starting material (R_f = 0.36). The volatiles were removed *in vacuo*, and the remaining salt was collected and used without further purification. A solution of (3*S*,6*S*,9*S*)-I^{2}aa hydrochloride (120 mg, 0.51 mmol) in 10 mL of water was next treated with 84 mg (1 mmol) of $NaHCO_3$, followed by a solution of succinimidyl 9-fluorenylmethyl carbonate (171 mg, 0.51 mmol) in 10 mL of acetone. The reaction was monitored by TLC (eluent 5% AcOH in EtOAc) until complete consumption of the starting amine was observed. Citric acid was added to the reaction mixture, which was subsequently extracted with EtOAc (2 × 25 mL). The organic layers were combined, washed with saturated NaCl, and evaporated to a residue that was purified by column chromatography using 2% AcOH in EtOAc as eluent. The fractions containing the reaction product were combined and evaporated to give 175 mg (82%) of Fmoc-I^{2}aa: $[\alpha]_D$ −26.2° (*c* 1.16, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.58−1.69 (m, 3 H), 2.00−2.24 (m, 4 H), 2.41 (m, 1 H), 3.63 (br s, 1 H), 4.16 (q, 2 H, J = 7.1), 4.27 (m, 2 H), 4.48 (d, 1 H, J = 8), 5.99 (d, 1 H, J = 6.2), 7.04 (br s, 1 H), 7.23 (t, 2 H, J = 7), 7.31 (t, 2 H, J = 7), 7.54 (t, 2 H, J = 6.5), 7.68 (d, 2 H, J = 7); ^{13}C NMR ($CDCl_3$) δ 26.5, 26.9, 28.6, 32.0, 47.1, 50.3, 57.1, 58.8, 60.4, 67.1, 119.9, 125.1, 127.0, 127.6, 141.2, 143.7, 143.9, 156.2, 170.3, 173.4; HRMS calcd for $C_{24}H_{24}N_2O_5Na$ (MNa^+): 443.1583, found: 443.1581.

(2*S*,4*R*,6*R*,8*S*)-4-Phenyl-indolizidin-9-one *N*-(Fmoc)amino Acid (Fmoc-4-Ph-I^{9}aa). (2*S*,4*R*,6*R*,8*S*)-9-Oxo-8-(*N*-(Boc)-amino)-4-phenyl-indolizidin-9-one amino acid (400 mg, 1.07 mmol prepared according to ref 67) was dissolved in a 1:1 solution of TFA/DCM and stirred for 2 h. The volatiles were evaporated, and the residue was dissolved in and evaporated from toluene twice. The amino acid was treated with DIEA (0.74 mL, 400 mol %) and Fmoc-OSu (720 mg, 200 mol %) in dioxane (10 mL) for 16 h. The solution was evaporated to a residue that was partitioned between $CHCl_3$ (50 mL) and 10% citric acid solution (50 mL). The phases were separated, and brine (25 mL) was added to the aqueous phase, which was extracted with $CHCl_3$(4 × 25 mL). The combined organic layer was dried over MgSO4, filtered, and concentrated. The residue was purified by flash chromatography using $CHCl_3$/MeOH/AcOH (94/5/1) as eluent to provided (2*S*,4*R*,6*R*,8*S*)-9-oxo-8-(*N*-(Fmoc)-amino)-4-phenyl-indolizidin-9-one amino acid (330 mg, 63%). ^{1}H NMR ($CHCl_3$) δ 7.67 (d, 2H, J = 7.5 Hz), 7.51 (d, 2H, J = 7.4 Hz), 7.31 (t, 2H, J = 7.4 Hz), 7.24−7.18 (m, 4H), 7.13 (t, 1H, J = 7.2 Hz), 7.06 (d, 2H, J = 7.4 Hz), 5.61 (d, 1H, J = 5.3 Hz), 4.91 (d, 1H, J = 5.2 Hz), 4.30 (m, 2H), 4.14 (t, 1H, J = 6.8 Hz), 3.77 (s, 1H), 2.70 (m, 2H), 2.40 (d, 1H, J = 13.3 Hz), 2.02 (d, 1H, J = 12.6 Hz), 1.81 (m, 1H), 1.58 (q, 1H, J = 10.7 Hz), 1.58 (q, 1H, J = 12.4 Hz); ^{13}C NMR ($CHCl_3$) δ 173.2, 172.5, 156.4, 143.7, 141.3, 128.7, 127.7, 127.0, 126.8, 126.6, 125.1, 119.9, 67.0, 52.7, 52.0, 51.4, 47.0, 39.4, 38.1, 35.1, 33.1; $[\alpha]^{20}_D$ −14.3° (*c* 0.006, $CHCl_3$/MeOH: 1/1); MS (ESI) *m*/*z* 497.3 ($M + H^+$).

Peptide Synthesis. Aza-peptides were synthesized according to references 59 and 62. Indolizidinone peptides were synthesized on a 0.16 mmol scale on Rink linker with an amino caproic acid spacer attached to BHA (0.4 mmol/g) resin to obtain C-terminal amides after resin cleavage and deprotection. Side chains of threonine and aspartic acid were respectively protected as *tert*-butyl ether and ester. At the ninth amino acid residue (Pro), the resin was split in two parts. One part (0.08 mmol) was deprotected with piperidine/DMF solution (1/1, v/v) and cleaved from the resin using TFA/TIS/H_2O (95/2.5/2.5 v/v/v). The second part (0.08 mmol) was elongated by two more amino acids and cleaved. Peptides were assembled by stepwise addition of *N*-Fmoc amino acids following a typical Fmoc strategy[64] monitored by colorimetric Kaiser's test.[68] Natural amino acids (300 mol %) were coupled using HBTU (300 mol %) and DIEA (600 mol %) in DMF for 1 h. Indolizidinone amino acids were subjected twice to the coupling condition (100 mol % and 50 mol %) using HBTU (100 mol % and 50 mol %) and DIEA (200 mol % and 100 mol %). Final Fmoc deprotection was followed by treatment with TFA/TIS/H_2O (95/2.5/2.5 v/v/v) for 60 min to cleave the resin and deprotect amino acid side chains. The resin was washed twice with TFA, and the TFA layers were combined, concentrated to 1 mL, treated with excess Et_2O to precipitate peptides that were dissolved in a 1:1 acetonitrile/H_2O solution, and lyophilized to white foams that were purified on semipreparative HPLC/MSQ using an Alltech C18 column (25 cm × 22 mm) with a 20 mL/min flow rate and a gradient of water:acetonitrile containing 0.1% of TFA. Purification was made using semipreparative LC/MS and furnished peptides in 23% average overall yields.

cAMP Measurements. HEK293 cells stably expressing the human CRLR (Hilairet et al., 2001) were detached using PBS containing 5 mM EDTA and then resuspended in PBS containing 0.01% D-glucose and 0.75 mM IBMX (Sigma). The cells were then seeded into cAMP CatchPoint 384 black and clear bottom plates (Molecular Devices) at a density of 5000 cells/well. To determine the EC_{50} of α-cGRP, the cells were challenged with increasing concentrations of α-cGRP for 15 min at 37 °C. In competition experiments, the cells were first preincubated with the CGRP (27−37) peptide analogues for 15 min at 37 °C and then were stimulated with 0.5 nM of α-cGRP for 15 min at 37 °C. cAMP levels were measured using the CatchPoint fluorescence-based cAMP kit and according to the manufacturer's recommendation (Molecular Devices). At least three independent experiments in triplicate were done for each peptide, and data was analyzed by nonlinear curve-fitting using the software PRISM 4 from GraphPad.

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