From Micromolar to Nanomolar Affinity: A Systematic Approach To Identify the Binding Site of CGRP at the Human Calcitonin Gene-Related Peptide 1 Receptor†

Beate Rist,‡ Michael Entzeroth,§ and Annette G. Beck-Sickinger*,‡

Department of Pharmacy, ETH Zürich, Zürich, Switzerland, and Department of Biochemical Research, Dr. Karl Thomae GmbH, Biberach, Germany

Received August 13, 1997⊗

CGRP Y^0-28-37 is known as a selective $CGRP_1$ receptor antagonist. In order to elucidate the essential requirements for its receptor interaction, we performed a variety of systematic approaches by modifying the C-terminal segments CGRP Y^0-28-37 and CGRP 27-37. N-Terminal and C-terminal segments have been synthesized, as well as chimeras which combine segments of CGRP, adrenomedullin, and amylin. Furthermore, we carried out an Ala scan, a Phe scan, a D-amino acid scan and a Pro scan of CGRP 27-37. Additionally, single amino acids were replaced by those with similar biophysical properties. Receptor binding studies of all analogs were performed at human neuroblastoma cells SK-N-MC, which selectively express the $hCGRP_1$ receptor. On the basis of the obtained results, we synthesized a series of ligands with multiple amino acid replacements in order to optimize the exchange at each position. This approach yielded to a series of high affinity ligands, including [D^{31},P^{34},F^{35}] CGRP 27-37 which exhibits a 100-fold increased affinity compared to the unmodified segment. So far, this is the smallest CGRP analog that shows affinity in the nanomolar range.

Introduction

Calcitonin gene-related peptide (CGRP) is a 37 amino acid peptide generated by alternative tissue-specific splicing of the primary transcript of the calcitonin gene.[1,2] In contrast to calcitonin (CT) which is predominantly expressed in the C-cells of the thyroid, two forms (α and β) of CGRP are produced in a variety of human tissues which are mostly of neuronal origin.[3,4] CGRP is colocalized with substance P in sensory nerves, with acetylcholine in motorneurones, and with various other transmitters in the brain (for review see ref 5). A variety of biological effects have been reported for CGRP such as peripheral blood vessel dilation, increase in heart rate, regulation of calcium metabolism, insulin secretion, reduction of gastric acid secretion, increase in body temperature, and decrease in food uptake.[5] These effects have been suggested to be mediated by at least two receptor subtypes ($CGRP_1$ and $CGRP_2$),[6] which are both G-protein coupled. The two receptors can be distinguished pharmacologically. The analog CGRP (8-37)[7−12] has been reported to be a sensitive antagonist at the $CGRP_1$ receptor. In contrast, potent agonism of the linear analog [Cys(acm)[2,7]]h-α-CGRP[7,13] (human CGRP) has been observed only at the $CGRP_2$ receptor. The $hCGRP_1$ receptor has been cloned.[14]

Although amylin, calcitonin, and adrenomedullin structurally resemble CGRP, the peptides mainly mediate different physiological effects. Calcitonin and CGRP are not known to cross-react crucially at each others' receptors.[15−17] It is still unclear whether amylin acts on its own receptor subtypes or via CT- and CGRP-related receptors.[17−19] For adrenomedullin a highly specific receptor has been identified,[20] but little is known about the physiological importance of its cross-reactivity at the $CGRP_1$ receptor.[21−24]

In spite of the many and diverse biological functions ascribed to CGRP, its structural requirements at the receptor level are not well investigated. It has been suggested that the N-terminal disulfide loop (residues 2−7) is followed by an amphiphilic α-helix between residue 8 and 18. Thereafter a poorly defined turn in the region 18-23 is indicated, leading into the C-terminal region which is predominantly disordered.[25−28] The presence of the α-helix in aqueous solution is supported by several CD[26,28,29] and ^1H NMR studies.[25,27,28] Breeze et al. determined the structure of hCGRP by ^1H NMR in a mixed-solvent system (50% TFE, 50% H_2O, pH 3.7). The C-terminus was less defined, lacking a stable structure.

Structure−activity studies of reduced size CGRP analogs showed that the C- and N-terminal region of the hormone interact independently with their receptors. N-Terminally truncated analogs like CGRP 19-37, CGRP 23-37, or CGRP 28-37 bind to the receptor without activating it[30−32] and CGRP 8-37 was found to be a highly potent antagonist (predominantly at the $CGRP_1$ receptor).[7−12] N-Terminal fragments like CGRP 1-12, CGRP 1-15, and CGRP 1-22 bind to the receptors in an agonistic manner, although very high concentrations are needed to induce vasodilatation.[33] These observations led to the speculation that agonists and antagonists of this hormone may be obtained by opti-

* Address correspondence to this author at Department of Pharmacy, ETH Zürich, Winterthurer Strasse 190, CH 8057 Zürich, Switzerland. Phone: +41-1-635 6063. Fax: +41-1-635 6884. e-mail: beck-sickinger@pharma.ethz.ch.

† Dedicated to Prof. Dr. G. Jung (University Tübingen) on the occasion of his 60th birthday.

‡ ETH Zürich.

§ Dr. Karl Thomae GmbH.

⊗ Abstract published in *Advance ACS Abstracts,* December 15, 1997.

S0022-2623(97)00533-5 CCC: $15.00 © 1998 American Chemical Society
Published on Web 01/01/1998

mizing the N- and C-terminal structures, respectively, or by using spacers to connect N- and C-terminus.[34]

In our study we focused on optimizing the structurally poorly defined C-terminal part of CGRP. We describe a rational approach to identify the primary structure which is necessary for high $CGRP_1$ receptor affinity. The C-terminal segment CGRP 28-37 has been modified systematically by its sequential truncation starting from the C- and N-terminus, respectively. Furthermore socalled scans have been performed which help to understand the role of single amino acids within a given sequence.

Ala scans which contain single Ala residues instead of the natural amino acids and which require as many analogs as the length of the special segment give information on the role of a specific side chain, because the methyl group of the side chain is able to induce secondary structure but lacks the specific properties of many trifunctional amino acids.

Phe scans, where phenylalanine is used to replace single amino acids, indicate the role of hydrophobicity at specific parts of the peptide, whereas Pro and D-amino acid scans indicate segments, which are sensitive to structural changes.

In addition to the scans single amino acids have been replaced by others with similar side chains in order to detect the effect of minimal changes. The combined replacement of those exchanges led to the most active analogs yielded in the segment [D^{31},P^{34},F^{35}]CGRP 27-37 which exhibits a 100-fold increased affinity compared to that of the native one. Accordingly, it is the smallest peptide which shows high-binding capacity at the $hCGRP_1$ known up to now.

Results

All the peptides (shown in Table 1) were obtained by multiple automatic solid-phase peptide synthesis using the N_α-protection Fmoc strategy. The sections of analogs are derived from r-α-CGRP Y^0-28-37 and r-α-CGRP 27-37, respectively. The peptides were characterized by analytical HPLC and electrospray mass spectrometry. $CGRP_1$ receptor affinity of each product was investigated on human neuroblastoma cells SK-N-MC, which selectively express the human $CGRP_1$ receptor.

The modified C-terminal fragment r-α-CGRP Y^0-28-37 (no. 3) exhibits the same affinity (IC_{50} = 3000 nM) to the $hCGRP_1$ receptor as the C-terminal segment r-α-CGRP 27-37 (no. 1). Tyr and Phe at the position 27 are, therefore, equally potent in C-terminal ligand segments. N-Terminal acetylation, which characterizes the importance of the N-terminal amino group of r-α-CGRP 27-37 (no. 5), does not lead to a significant change in $hCGRP_1$ receptor affinity. Therefore, for all further modification the C-terminal undecapeptides were used in a nonacetylated form.

In contrast to the sequence of r-α-CGRP, which contains glutamic acid, the basic amino acid Lys is found in position 35 in the r-β-CGRP as well as in the h-α- and h-β-CGRP. Surprisingly Lys in position 35 of r-α-CGRP 27-37 has no influence on binding (no. 2), but for [K^{35}]r-α-CGRP Y^0-28-37 (no. 4), slightly reduced receptor affinity is observed. First, we focused on truncation and modification of the potent analog r-α-CGRP Y^0-28-37 (no. 3), whose antagonism with respect to the $CGRP_1$ receptor has been described already.[31,32] The sequential shortening of this peptide, starting form the C-terminus (no. 6-13) does not lead to a compound that exhibits any binding capacity. In the next series of analogs in which we preserved the Tyr at the N-terminus, followed by a stepwise deletion of the amino acids 28-35 (no. 14-21), binding was lost as well. The only exceptions were the analogs CGRP Y^0-29-37 (no. 14) and CGRP Y^0-30-37 (no. 15) which maintained some $hCGRP_1$ receptor affinity.

As shown in Figure 1 the sequence homology of adrenomedullin with hCGRP, rCGRP, and amylin is not high. They share a six residue ring structure near the N-terminus formed by an intramolecular disulfide linkage and the C-terminal amide structure. Nevertheless, adrenomedullin binds with high affinity to the $hCGRP_1$ receptor (K_i = 0.37 nM).[35] Although there is much higher structural similarity (46%) between CGRP and amylin, the latter interacts only minimally with the $CGRP_1$ receptor.[16] To elucidate the role of a sequential combination of certain amino acids for receptor recognition, we decided to synthesize chimeras of the C-terminal part of the three peptides CGRP, amylin, and adrenomedullin. All the analogs (no. 22-29), however, showed no significant receptor affinity (IC_{50} = 10 000 nM). That means neither the presence of certain three, four, or five amino acids themselves nor the combination of such native segments seem to be responsible for receptor interaction.

In order to evaluate the significance of each amino acid, and the role of their side chains in receptor interaction, we systematically exchanged each residue of r-α-CGRP (Y^0-28-37) by L-Ala (no. 30-39) and L-Phe (no. 40-48) and by the corresponding D-amino acids (no. 49-58). The most sensitive amino acids are Thr^{30}, Val^{32}, Gly^{33}, and Phe^{37} because the replacement of these residues led to a total loss of receptor interaction in all cases. Since neither Val^{32} nor Gly^{33} can be partly substituted by a hydrophobic amino acid (Ala or Phe) the region 30-33 seems to be extremely important for $CGRP_1$ receptor binding. Val in position 28 appears to be the less sensitive amino acid in this segment. Exchanges by Ala (no. 31), Phe (no. 40), and D-Val (no. 50) resulted in as much as a 6-fold decrease in receptor affinity. Even the deletion of Val^{28} (no. 14) resulted in an analog which still exhibits slight receptor interaction. One of the most remarkable analogs from the Ala scan is [A^{34}]CGRP Y^0-28-37 (no. 37). With an IC_{50} of 4000 nM, it is nearly as potent as the unmodified segment even though the polar amino acid Ser^{34} was replaced by the more hydrophobic Ala. The replacement of Ser^{34} by the very hydrophobic and space-filling amino acid Phe (no. 46) is not tolerated. Another analog which shows no significant loss in receptor affinity is [F^{35}]-CGRP Y^0-28-37 (no. 47). Surprisingly, the replacement of the polar, negatively charged Glu^{35} by an hydrophobic Phe does not disturb receptor interaction (IC_{50} = 4790 nM).

To further characterize the essential property of certain amino acids, single residues were varied in CGRP Y^0-28-37 by structurally and biophysically related ones. The replacement of Pro in position 29 by hydroxyproline (no. 59) (Figure 2) leads to a 3-fold decrease in affinity. The exchange of Thr^{30} by Ser (no. 60) which is

Table 1. Affinity of CGRP 27-37 Analogs to the CGRP$_1$ Receptor (Human Neuroblastoma Cells SK-N-MC) and Analytical Data Obtained by Electrospray Mass Spectra (Theoretical and Experimental Mass)[a]

no.	peptide	sequence	hCGRP$_1$ IC$_{50}$, [nM]	mass (th), [amu]	mass (exp), [amu]
1	CGRP 27-37	FVPTNVGSEAF	3 000	1165.6	1165.5
2	[K^{35}]CGRP 27-37	FVPTNVGS**K**AF	2 700	1164.6	1165.0
3	CGRP Y^{0}-28-37	YVPTNVGSEAF	3 000	1181.6	1182.0
4	[K^{35}]CGRP Y^{0}-28-37	YVPTNVGS**K**AF	6 500	1180.6	1181.0
5	CGRP Ac-27-37	**Ac**-FVPTNVGSEAF	4 500	1207.6	1208.0
6	CGRP Y^{0}-28-36	YVPTNVGSEA	n.d.	1034.5	1034.5
7	CGRP Y^{0}-28-35	YVPTNVGSE	n.d.	963.5	963.5
8	CGRP Y^{0}-28-34	YVPTNVGS	n.d.	834.4	834.5
9	CGRP Y^{0}-28-33	YVPTNVG	n.d.	747.4	747.0
10	CGRP Y^{0}-28-32	YVPTNV	n.d.	690.4	690.0
11	CGRP Y^{0}-28-31	YVPTN	n.d.	591.3	591.0
12	CGRP Y^{0}-28-30	YVPT	n.d.	477.3	477.0
13	CGRP Y^{0}-28-29	YVP	n.d.	376.2	376.0
14	CGRP Y^{0}-29-37	YPTNVGSEAF	24 000	1082.5	1082.5
15	CGRP Y^{0}-30-37	YTNVGSEAF	18 000	985.5	985.5
16	CGRP Y^{0}-31-37	YNVGSEAF	n.d.	884.4	884.5
17	CGRP Y^{0}-32-37	YVGSEAF	n.d.	770.4	770.0
18	CGRP Y^{0}-33-37	YGSEAF	n.d.	671.3	671.0
19	CGRP Y^{0}-34-37	YSEAF	n.d.	614.3	614.0
20	CGRP Y^{0}-35-37	YEAF	n.d.	527.3	527.0
21	CGRP Y^{0}-36-37	YAF	n.d.	389.2	398.0
22	CGRP26-34, ADR50-52	NFVPTNVGS**QGY**	10 000	1280.6	1281.0
23	ADR40-43, CGRP30-34, ADR50-52	**NVAP**TNVGS**QGY**	10 000	1204.6	1205.0
24	CGRP26-29, ADR44-52	NFVP**RSKISPQGY**	10 000	1490.8	1491.0
25	CGRP26-29, ADR44-49, CGRP35-37	NFVP**RSKISP**EAF	10 000	1489.8	1490.0
26	ADR40-49, CGRP35-37	**NVAPRSKISP**EAF	10 000	1413.8	1414.0
27	ADR40-43, CGRP30-37	**NVAP**TNVGSEAF	10 000	1203.6	1204.0
28	CGRP26-34, IAPP35-37	NFVP**TNVGS***NTY*	10 000	1310.6	1311.0
29	IAPP26-34, CGRP30-37	*ILSS***TNVGS**EAF	10 000	1222.6	1223.0
30	[A^{27}]CGRP 27-37	**A**VPTNVGSEAF	24 000	1089.6	1089.5
31	[A^{28}]CGRP Y^{0}-28-37	Y**A**PTNVGSEAF	8 000	1153.5	1153.5
32	[A^{29}]CGRP Y^{0}-28-37	YV**A**TNVGSEAF	n.d.	1155.6	1155.5
33	[A^{30}]CGRP Y^{0}-28-37	YVP**A**NVGSEAF	n.d.	1151.6	1151.5
34	[A^{31}]CGRP Y^{0}-28-37	YVPT**A**VGSEAF	>100 000	1138.6	1138.5
35	[A^{32}]CGRP Y^{0}-28-37	YVPTN**A**GSEAF	n.d.	1153.5	1153.5
36	[A^{33}]CGRP Y^{0}-28-37	YVPTNV**A**SEAF	n.d.	1195.6	1195.5
37	[A^{34}]CGRP Y^{0}-28-37	YVPTNVG**A**EAF	4 000	1165.6	1165.5
38	[A^{35}]CGRP Y^{0}-28-37	YVPTNVGS**A**AF	>10 000	1123.6	1123.5
39	[A^{37}]CGRP Y^{0}-28-37	YVPTNVGSEA**A**	n.d.	1105.5	1105.5
40	[F^{28}]CGRP Y^{0}-28-37	Y**F**PTNVGSEAF	14 000	1229.6	1229.5
41	[F^{29}]CGRP Y^{0}-28-37	YV**F**TNVGSEAF	45 000	1231.6	1231.5
42	[F^{30}]CGRP Y^{0}-28-37	YVP**F**NVGSEAF	n.d.	1227.6	1227.5
43	[F^{31}]CGRP Y^{0}-28-37	YVPT**F**VGSEAF	70 000	1214.6	1214.5
44	[F^{32}]CGRP Y^{0}-28-37	YVPTN**F**GSEAF	n.d.	1229.6	1229.5
45	[F^{33}]CGRP Y^{0}-28-37	YVPTNV**F**SEAF	n.d.	1271.6	1271.5
46	[F^{34}]CGRP Y^{0}-28-37	YVPTNVG**F**EAF	n.d.	1241.6	1241.5
47	[F^{35}]CGRP Y^{0}-28-37	YVPTNVGS**F**AF	4 790	1199.6	1199.5
48	[F^{36}]CGRP Y^{0}-28-37	YVPTNVGSE**F**F	n.d.	1257.6	1257.5
49	CGRP Y^{0}-28-37	**y**VPTNVGSEAF	80 000	1181.6	1181.5
50	[v^{28}]CGRP Y^{0}-28-37	Y**v**PTNVGSEAF	19 000	1181.6	1181.5
51	[p^{29}]CGRP Y^{0}-28-37	YV**p**TNVGSEAF	12 000	1181.6	1181.5
52	[t^{30}]CGRP Y^{0}-28-37	YVP**t**NVGSEAF	n.d.	1181.6	1181.5
53	[n^{31}]CGRP Y^{0}-28-37	YVPT**n**VGSEAF	>100 000	1181.6	1181.5
54	[v^{32}]CGRP Y^{0}-28-37	YVPTN**v**GSEAF	n.d.	1181.6	1181.5
55	[s^{34}]CGRP Y^{0}-28-37	YVPTNVG**s**EAF	>100 000	1181.6	1181.5
56	[e^{35}]CGRP Y^{0}-28-37	YVPTNVGS**e**AF	n.d.	1181.6	1181.5
57	[a^{36}]CGRP Y^{0}-28-37	YVPTNVGSE**a**F	>100 000	1181.6	1181.5
58	[f^{37}]CGRP Y^{0}-28-37	YVPTNVGSEA**f**	n.d.	1181.6	1181.5
59	[Hyp^{29}]CGRP Y^{0}-28-37	YV-**Hyp**-TNVGSEAF	9 000	1197.6	1197.5
60	[S^{30}]CGRP Y^{0}-28-37	YVP**S**NVGSEAF	>100 000	1167.6	1168.5
61	[L^{31}]CGRP Y^{0}-28-37	YVPT**L**VGSEAF	7 000	1180.6	1181.5
62	[Q^{31}]CGRP Y^{0}-28-37	YVPT**Q**VGSEAF	95 000	1195.6	1196.5
63	[D^{31}]CGRP Y^{0}-28-37	YVPT**D**VGSEAF	5 000	1181.6	1183.5
64	[H^{35}]CGRP Y^{0}-28-37	YVPTNVGS**H**AF	4 275	1198.6	1199.0
65	[Q^{35}]CGRP Y^{0}-28-37	YVPTNVGS**Q**AF	6 900	1180.6	1181.0
66	[L^{35}]CGRP Y^{0}-28-37	YVPTNVGS**L**AF	8 550	1165.6	1166.0
67	[G^{36}]CGRP Y^{0}-28-37	YVPTNVGSE**G**F	51 000	1167.6	1167.5
68	[Y^{37}]CGRP Y^{0}-28-37	YVPTNVGSEA**Y**	32 000	1197.6	1197.5

Table 1 (Continued)

no.	peptide	sequence	hCGRP$_1$ IC$_{50}$, [nM]	mass (th), [amu]	mass (exp), [amu]
69	[P^{28}]CGRP 27-37	F**P**PTNVGSEAF	3 400	1163.6	1164.0
70	[P^{30}]CGRP 27-37	FVP**P**NVGSEAF	>10 000	1161.6	1162.0
71	[P^{31}]CGRP 27-37	FVPT**P**VGSEAF	4 500	1148.6	1149.0
72	[P^{32}]CGRP 27-37	FVPTN**P**GSEAF	>10 000	1163.6	1164.0
73	[P^{33}]CGRP 27-37	FVPTNV**P**SEAF	>10 000	1205.6	1206.0
74	[P^{34}]CGRP 27-37	FVPTNVG**P**EAF	280	1175.6	1176.0
75	[P^{35}]CGRP 27-37	FVPTNVGS**P**AF	>10 000	1133.6	1134.0
76	[P^{36}]CGRP 27-37	FVPTNVGSE**P**F	>10 000	1191.6	1192.0
77	[D^{31}]CGRP 27-37	FVPT**D**VGSEAF	>3 000	1166.6	1166.5
78	[D^{31},Q^{35}]CGRP 27-37	FVPT**D**VGS**Q**AF	1 200	1165.6	1166.0
79	[D^{31},P^{34}]CGRP 27-37	FVPT**D**VG**P**EAF	470	1176.6	1176.5
80	[D^{31},A^{34},H^{35}]CGRP 27-37	FVPT**D**VG**AH**AF	360	1158.6	1159.0
81	[D^{31},A^{34},F^{35}]CGRP 27-37	FVPT**D**VG**AF**AF	159	1168.6	1196.0
82	[D^{31},P^{34},F^{35}]CGRP 27-37	FVPT**D**VG**PF**AF	29	1194.6	1195.0
83	[D^{31},A^{34},F^{35}]CGRP Ac-27-37	**Ac**-FVPT**D**VG**AF**AF	205	1210.6	1211.0
84	[D^{31},A^{34},F^{35}]CGRP Ac-28-37	**Ac**-VPT**D**VG**AF**AF	300	1063.5	1064.0
85	[D^{31},A^{34},F^{35}]CGRP 28-37	VPT**D**VG**AF**AF	200	1021.5	1022.0
86	[D^{31},A^{34},F^{35}]CGRP Ac-29-37	**Ac**-PT**D**VG**AF**AF	490	964.5	964.5
87	[D^{31},A^{34},F^{35}]CGRP 29-37	PT**D**VG**AF**AF	220	922.5	922.5
88	[D^{31},Acp^{34},F^{35}]CGRP 27-37	FVPT**D**VG-**Acp**-**F**AF	>3 000	1194.6	1194.5
89	[D^{31},Pac^{34},F^{35}]CGRP 27-37	FVPT**D**VG-**Pac**-**F**AF	>3 000	1270.6	1270.5
90	[D^{31},Tic^{34},F^{35}]CGRP 27-37	FVPT**D**VG-**Tic**-**F**AF	146	1256.6	1256.0
91	[D^{31},Hyp^{34},F^{35}]CGRP 27-37	FVPT**D**VG-**Hyp**-**F**AF	620	1210.6	1210.5
92	[D^{31},Hop^{34},F^{35}]CGRP 27-37	FVPT**D**VG-**Hop**-**F**AF	910	1208.6	1208.5
93	[D^{31},Aib^{34},F^{35}]CGRP 27-37	FVPT**D**VG-**Aib**-**F**AF	170	1182.6	1183.0

[a] a, e, f, g, n, p, s, t, v, y = D-amino acids, corresponding to the standard one letter code; Ac = acetyl; Acp = 2-(aminomethyl)cyclopropanoic acid; ADR = adrenomedullin; Aib = aminoisobutyric acid; Bpa = L-*p*-benzoylphenylalanine; BSS = balanced salts solution; CGRP = calcitonin gene-related peptide; CT = calcitonin; Hop = L-homoproline; Hyp = L-4-hydroxyproline; IAPP = islet amyloid polypeptide, amylin; n.d. = no displacement; Pac = 1-phenyl-2-(aminomethyl)cyclopropanoic acid; PBS = phosphate buffered saline; TFE = 2,2,2-trifluoroethanol; L-Tic = tetrahydroisochinoline-3-carboxylic acid.

h-α-CGRP		ACDTATCVTHRLAGLLSRSGGVVKN	NFVPTNVGSKA-F-NH2
h-β-CGRP		ACNTATCVTHRLAGLLSRSGGMVKS	NFVPTNVGSKA-F-NH2
r-α-CGRP		SCNTATCVTHRLAGLLSRSGGVVKD	NFVPTNVGSEA-F-NH2
r-β-CGRP		SCNTATCVTHRLAGLLSRSGGVVKD	NFVPTNVGSKA-F-NH2
h-Amylin		KCNTATCATQRLANFLVHSSNNFGA	ILSSTNVGSNT-Y-NH2
r-Amylin		KCNTATCATQRLANFLVRSSNNLGP	VLPPTNVGSNT-Y-NH2
h-Calcitonin		CGNLSTCMLGTYTQDFNKFHT----	-FPQTAIGVGA-P-NH2
r-Calcitonin		CGNLSTCMLGTYTQDLNKFHT----	-FPQTSIGVGA-P-NH2
Adrenomedullin	YRQSMNNFQGLRSF	GCRFGTCTVQKLAHQIYQFTDKDKD	NVAPRSKISPQGY-NH2

Figure 1. Comparison of the amino acid sequence of human/rat αCGRP, βCGRP, amylin, calcitonin, and human adrenomedullin.

one methyl group shorter destroyed receptor affinity. Asn^{31} was replaced by Leu (no. 61), Gln (no. 62) and Asp (no. 63), while Asp^{31} (IC$_{50}$ 5000 nM) and Leu^{31}(IC$_{50}$ = 7000 nM) were still well tolerated, [Q^{31}]CGRP Y^0-28-37 (IC$_{50}$ = 95 000 nM) shows no receptor affinity. The replacement of Glu^{35} by His (no. 64), Gln (no. 65), and Leu (no. 66) has no dramatic effects, affinity decreases maximally 3-fold. [G^{36}]CGRP Y^0-28-37 (no. 67) and [Y^{37}]-CGRP Y^0-28-37 (no. 68) show only weak binding, although the amino acids were substituted by similar ones, Ala by Gly and Phe by Tyr. No single amino acid exchange leads to an analog exhibiting higher CGRP$_1$ receptor affinity than the unmodified segment CGRP Y^0-28-37.

Proline is known as a turn-inducing amino acid, or at least as a residue which often affects the secondary structure of a peptide. In order to get information on which position of the C-terminal fragment is very sensitive to conformational changes, we performed a Pro scan based on the segment CGRP 27-37 (no. 69-76). For amino acids which already have been detected to be sensitive to modification like Thr^{30}, Val^{32}, Gly^{33}, and Ala^{36}, an exchange by proline (no. 70, 72, 73, 76) led to a significant loss in receptor affinity (IC$_{50}$ > 10 000 nM). Furthermore, [P^{35}]CGRP 27-37 showed reduced affinity of the same range. The analogs [P^{28}]- (no. 69) and [P^{31}]-CGRP 27-37 (no. 71) are as potent as the unmodified segment. Interestingly, [P^{34}]CGRP 27-37 (no. 74) exhibits a 10-fold increase in binding capacity. Since [A^{34}]-CGRP Y^0-28-37 (no. 37) binds in the same range as CGRP 27-37, we speculate that the excellent binding of [P^{34}]CGRP 27-37 is not a result of direct interaction between P^{34} and the receptor. Possibly the Pro in position 34 serves to stabilize a conformation of the C-terminal segment which is needed for receptor interaction.

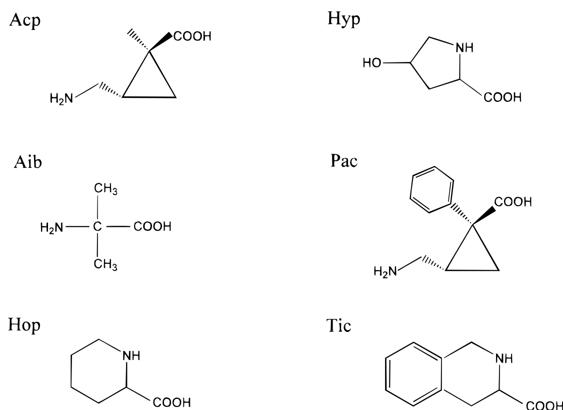

Figure 2. Formula of the turn-inducing amino acid derivatives which have been introduced in sequence of CGRP 27-37 at position 34.

We decided to carry out a multiple amino acid replacement based on the C-terminal segment [D^{31}]-CGRP 27-37 (no. 77). Amino acids which have been well tolerated in certain positions were combined in single analogs. In the segment [D^{31},Q^{35}]CGRP 27-37 (no. 78) the exchanged amino acids are structurally very similar to the original ones; however, the functional groups of the side chains were arranged in the opposite manner. While single amino acid replacement of Asn^{31} by Asp^{31} (no. 63) and Glu^{35} by Gln^{35} (no. 65) in CGRP Y^0-27-37 does not improve binding capacity, the combined replacement in CGRP 27-37 yielded an analog which shows a 3-fold increase in affinity. Besides Gln (no. 65), His (no. 64) and Phe (no. 47) have been well tolerated in CGRP Y^0-27-37 at position 35. Additionally Ala in position 34 (no. 37) does not disturb receptor interaction of the latter analog. These findings lead to the two compounds [D^{31},A^{34},H^{35}]- (no. 80) and [D^{31},A^{34},F^{35}]CGRP 27-37 (no. 81), which both show a significant increased receptor affinity. [D^{31},A^{34},F^{35}]CGRP 27-37 is at least 20 times more potent than the unmodified segment. Since Pro in position 34 was most effective in improving binding capacity, we synthesized [D^{31},P^{34},F^{35}]CGRP 27-37 (no. 82). With an IC_{50} of 29 nM [D^{31},P^{34},F^{35}]CGRP 27-37 is the one exhibiting highest $hCGRP_1$ receptor affinity.

Further investigations were performed to find out the relevance of the N-terminal amino acids in the segment [D^{31},A^{34},F^{35}]CGRP 27-37 (no. 81). A sequential deletion of its N-terminal, two amino acids shows no dramatic effect. Shortening the segment by one or two amino acids (no. 85, 87) led to a slight decrease in affinity represented by IC_{50} values of 200 and 220 nM, respectively, compared to an IC_{50} of 159 nM found for [D^{31},A^{34},F^{35}]CGRP 27-37 (no. 81). N-Terminal acetylation resulted for all three analogs (no. 83, 84, 86) in a loss of affinity. The shorter the analog, the bigger the effect.

In order to further characterize the role of Pro^{34} for receptor interaction, we synthesized several analogs of [D^{31},Pro^{34},F^{35}]CGRP 27-37 (no. 82) in which Pro^{34} was replaced by other turn-inducing amino acids (Figure 2). By incorporation of cyclopropane derivatives in position 34 (no. 88, 89) a more than 100-fold decrease in affinity was found. 4-Hydroxyproline (no. 91) and homoproline (no. 92) instead of Pro^{34} led to analogs exhibiting a 20- and 30-fold weaker binding. In the series, Aib^{34} (no. 93) and Tic^{34} (no. 90) were the most tolerated amino acids. With IC_{50} values of 170 and 146 nM they bind as well as [D^{31},A^{34},F^{35}]CGRP 27-37 (no. 81), but 6- and 5-fold less than [D^{31},P^{34},F^{35}]CGRP 27-37 (no. 82).

Discussion

The C-terminal F-NH_2 of CGRP is known to be essential for binding to its specific receptors.[28,36,37] This could be confirmed by our studies, since C-terminal deletion and the replacement by D-configurated F-NH_2 as well as by A-NH_2 totally destroys receptor recognition of the segment CGRP 27-37. Even the substitution of F-NH_2 by Y-NH_2, which has only one additive hydroxy group, is not tolerated, supporting the idea that F-NH_2 is directly involved in receptor interaction. We could show that some highly conserved amino acids of amylin and CGRP like Thr^{30}, Val^{32}, and Gly^{33} are possibly involved in direct receptor interaction, additionally. They cannot be substituted by similar amino acids without loss of receptor affinity. Whether they are important because of a direct interaction of the backbone and/or because their side chains interact with the receptor or because they determine a certain bioactive secondary structure in the C-terminal region is not clear. Although Pro^{29} is highly conserved too, it is not essential for binding. Similarly, the side chains of Phe^{27} and Val^{28} seemed not to be involved in direct receptor interactions. In the segment CGRP 27-37, F^{27} could be replaced by Tyr without any loss of affinity. Val in position 28 could be replaced by Pro and Ala, even the change of its configuration is tolerated. These results correspond to the assumption of Hakala et al.[33] where the C-terminal fragment CGRP 30-37 is modeled and two different type of turns a γ-turn or a βII′-turn are suggested.

Single amino acid replacements in position 31 and 35 showed that these positions are not very sensitive themselves. Nevertheless, by performing multiple amino acid replacement it became evident that they influence receptor recognition. If Asn^{31} is replaced by Asp, a positively charged or a hydrophobic amino acid in position 35 is favored. Therefore we speculate that there might exist a direct interaction between their side chains.

Several research groups predicted a probable turn in the C-terminal region of CGRP. On the basis of NMR investigations of the analog [But-Cys^{18}]-h-β-CGRP-19-37, Sagoo et al.[39] suggested a type βI-turn in the region Asn^{31}-Val^{32}-Gly^{33}. Provided that in the analog [D^{31},P^{34},F^{35}]CGRP 27-37 the turn-inducing amino acid proline is located at position 34, a βI-turn in the region 31-33 cannot be supported by our studies. Performing molecular modeling either the presence of a Val^{32}-Ser^{34} γ-turn or a Val^{32}-Lys^{35} βII′-turn has been suggested.[38] In order to stabilize the latter one, they designed the analog [P^7,P^8,C^{31},C^{36}]h-α-CGRP 6-37] with a disulfide bridge Cys^{31}-Cys^{36} in the C-terminal region.[40] The relevance of this bridge for inducing a bioactive conformation in the C-terminal part of the hormone could not be confirmed by our studies. N-Terminal shortening of the analog by synthesizing [C^{31},C^{36}]h-α-CGRP 20-37 and [C^{31},C^{36}]h-α-CGRP 27-37 decreases $hCGRP_1$ receptor affinity by 2 orders of magnitude (IC_{50} > 10 000 nM, preliminary results). We agree that a turn structure

could be possible in the C-terminal region of CGRP. However, as we identified a strongly increased affinity of analogs containing proline in position 34, a βI-turn with proline in the $i + 1$ position should range from Gly[33] to Ala[36].[41,42] Alternatively, a βIV-turn from Val[32]-Lys[35] containing a cis peptide bond is in agreement with our structure–affinity relationship studies.[42,43]

It is obvious that the C-terminal segment V[32]GPFAF[37] represents a hydrophobic and sensitive binding domain of the ligand [D[31],P[34],F[35]]CGRP 27-37. The replacement of P[34] by the large and hydrophobic Tic led to a better binder than the incorporation of the smaller homoproline, or at least of cyclopropane derivatives. This makes evident that position 34 is sensitive to changes which influence the 3D-structure of this specific C-terminal part. In addition, it shows that there is space for further hydrophobic groups and therefore sterical hindrance by large side chains plays a minor role for decreased binding capacities. Ala in position 35 can not be substituted by Gly which furthermore indicates that the binding side of the receptor is probably located at its surface and that the ligand interacts with the receptor by this hydrophobic segment.

Conclusion

We succeeded in optimizing the sequence of the reported segment CGRP Y[0]-28-37. Our studies yielded analogs which exhibit $hCGRP_1$ receptor affinity in the nM range. With a series of systematic approaches we were able to describe the structural requirements of CGRP 27-37 which are essential for receptor interaction. By performing multiple amino acid replacements within one segment, we could improve binding capacity by 2 orders of magnitude. Highest $hCGRP_1$ receptor affinity, with an IC_{50} of 29 nM, was exhibited by [D[31],P[34],F[35]]-CGRP 27-37. We speculate that in this analog proline in position 34 forces a turn structure. This supports the idea that the bioactive conformation of the entire CGRP molecule is characterized by a folding back of the C-terminus of the hormone.

Experimental Section

Materials. Fmoc-protected amino acids were obtained from Alexis (Läufelfingen, Switzerland), 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin from NovaBiochem (Läufelfingen, Switzerland), diisopropylcarbodiimide and thiocresol from Aldrich, 1-hydroxybenzotriazole, diisopropylethylamine, trifluoroacetic acid, thioanisole and piperidine from Fluka, and dimethylformamide (p.a. grade), diethyl ether, acetonitrile, and *tert*-butyl alcohol from Merck. Hepes, Tris, bovine serum albumine, and bacitracin were purchased from Sigma, polyethylenimine from Serva, and Dulbecco's modified Eagle medium and PBS-buffer (041-04190 M) from Gibco. EDTA and all salts for buffer preparation were either from Fluka or from Merck. (^{125}I-Iodotyrosyl)calcitonin gene-related peptide was ordered from Amersham. Acp and Pac have been kindly provided by Prof. W. Reissig (University of Dresden) and N_α-protected with Fmoc-OSu as described previously.[44]

Peptide Synthesis. The linear peptides were synthesized by automated multiple solid phase peptide synthesis using a robot system (Syro, MultiSynTech, Bochum). In order to obtain a peptide amide, 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin was used.[45] The polymer matrix was polystyrene–1% divinylbenzene (30 mg, 15 μmol). The side chain protection was chosen as follows: Tyr(*tert*-butyl), Glu(*tert*-butyl), His(trityl), Gln(trityl), Asn(trityl), Thr(*tert*-butyl), and Lys(*tert*-butyloxy-carbonyl), Ser(trityl). Double coupling procedures were performed with diisopropylcarbodiimide/1-hydroxybenzotriazole activation, 7-fold excess, and a coupling time of 40 min.

The linear peptide amides were cleaved with trifluoroacetic acid/thioanisole/thiocresol (90:5:5% v/v) within 3 h and precipitated from cold diethyl ether. The products were collected by centrifugation and resuspended twice in diethyl ether. Finally they were lyophilized from water/*tert*-butyl alcohol (1:2).

Peptide Purification and Characterization. The peptides were purified to homogeneity higher than 94% by reversed-phase HPLC on a Nucleolsil C-18 column, 6 μm, 25 × 300 mm (Waters) by using isocratic conditions between 20 and 30% acetonitrile, depending on the polarity of the peptide. Analysis of the peptides was performed on a Nucleolsil C-18 column, 5 μm, 3 × 125 mm (Merck-Hitachi) by the following gradients of acetonitrile/water mixed with 0.1% trifluoroacetic: 15–60% and 0–50% acetonitrile in 30 min.

Correct mass was identified by ion-spray mass spectrometry (API III, Sciex, Toronto). (Analytical data are shown in Table 1.)

Membrane Preparation. SK-N-MC cells were cultivated in Dulbecco's modified Eagle Medium. The medium of confluent culture was removed, and the cells were washed twice with PBS buffer. The cells were detached by the addition PBS buffer and supplemented with 0.02% EDTA. Resuspended in 20 mL of Balanced Salts Solution (BSS (in mM): NaCl 120, KCl 5.4, $NaHCO_3$ 16.2, $MgSO_4$ 0.8, NaH_2PO_4 1.0, $CaCl_2$ 1.8, D-glucose 5.5, HEPES 30, pH 7.40) the cells were centrifuged twice at 100*g* and resuspended in BSS. After determination of their number, the cells are homogenized using an Ultra-Turrax and centrifuged for 10 min at 3000*g* (4 °C). The supernatant was discarded, and the pellet was resuspended in Tris buffer (10 mM Tris, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, pH 7.40) supplemented with 1% bovine serum albumin and 0.1% bacitracin, recentrifuged, and resuspended (1 mL/1 000 000 cells). The homogenate was frozen at −80 °C. The membrane preparations are stable for more than 6 weeks.

Receptor Binding. After thawing the homogenate was diluted 1:10 with assay buffer (50 mM Tris, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, pH 7.40) and treated for 30 s with an Ultra-Turrax. The homogenate (230 μL) was incubated for 180 min at room temperature with 50 pM (^{125}I-iodotyrosyl)-calcitonin gene-related peptide and increasing concentrations of the test compound in a total volume of 250 μL. The incubation is terminated by either centrifugation for 10 min at 2900*g* or filtration through polyethyleneimine (0.1%)-treated GF/B glass fiber filters using a cell harvester. The protein-bound radioactivity is determined in a gamma-counter. The nonspecific binding is defined as radioactivity bound in the presence of 1 μM human $CGRP_1$ during the incubation period. Half-maximal inhibition of the specific binding of (^{125}I-iodotyrosyl)calcitonin gene-related peptide of two to three separate experiments each performed in triplicate is given as the IC_{50} value (Table 1).

Acknowledgment. The technical assistance of U. Schmid and H. Steiner is gratefully acknowledged.

References

(1) Amara, S. G.; Jonas, V.; Rosenfeld, M. G.; Ong, E. S.; Eavens, R. M. Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptides products. *Nature* **1982**, *298*, 240–244.

(2) Rosenfeld, M. G.; Mermod, J. J.; Amara, S. G.; Swanson, L. W.; Sawchenko, P. E.; Rivier, J.; Vale, W. W.; Evans, R. M. Production of a novel neuropeptide encoded by the calcitonin gene via tissue-specific RNA processing. *Nature* **1983**, *304*, 129–135.

(3) Amara, S. G.; Arriza, J. L.; Swanson, L. W.; Eavens, R. M.; Rosenfeld, M. G. Expression in brain of a messenger RNA encoding a novel neuropeptide homologous to calcitonin gene related peptide. *Science* (*Washington, D.C.*) **1985**, *229*, 1094–1097.

(4) Steenbergh, P. H.; Hoppener, J. W. M.; Zandberg, J.; Van de Ven, W. J. M.; Jansz, H. S.; Lips, C. J. M. A second calcitonin /CGRP gene. *FEBS Lett.* **1985**, *183*, 403–407.

(5) Tache, Y.; Holzer, P.; Rosenfeld M. G., Eds. Calcitonin Gene Related Peptide. *Ann. N.Y. Acad. Sci.* **1992**, *657*, 1–558.
(6) Quirion, R.; Van Rossum, D.; Dumont, Y.; St.-Pierre, S.; Fournier, A. Characterization of $CGRP_1$ and $CGRP_2$ receptor subtypes. *Ann. N.Y. Acad. Sci.* **1992**, *657*, 88–105.
(7) Dennis, T.; Fournier, A.; St.-Pierre, S.; Quirion, R. Structure-activity profile of calcitonin gene-related peptide in peripheral and brain tissue. Evidence for receptor multiplicity. *J. Pharmacol. Exp. Ther.* **1989**, *251*, 781–725.
(8) Chiba, T.; Yamaguchi, A.; Yamatani, T.; Nakamura, A.; Morishita, T.; Inui, T.; Fukase, M.; Noda, T.; Fujita, T. CGRP receptor antagonist hCGRP (8-37). *Am. J. Physiol.* **1989**, *256*, E331–E335.
(9) Dennis, T.; Fournier A.; Cadieux, A.; Pomerleau, F.; Jolicoeur, F. B.; St-Pierre, S.; Quirion, R. hCGRP 8-37 a calcitonin gene-related peptide antagonist revealing CGRP receptor heterogeneity in brain and periphery. *J. Pharmacol. Exp. Ther.* **1990**, *254*, 123–128.
(10) Donoso, M. V.; Fournier A.; St. Pierre, S.; Huidobro-Toro, J. P. Pharmacological characterization of $CGRP_1$ receptor subtype in the vascular system of the rat: studies with hCGRP fragments and analogs. *Peptides* **1990**, *11*, 885–889.
(11) Maggi, C. A.; Chiba, T.; Giuliani, S. Human α-calcitonin gene-related peptide (8-37) as an antagonist of exogenous and endogenous calcitonin gene-related peptide. *Eur. J. Pharmacol.* **1991**, *192*, 85–88.
(12) Mimeault, M.; Fournier, A.; Dumont, Y.; St Pierre, S.; Quirion, R. Comparative affinities and antagonistic potencies of various human CGRP fragments on CGRP receptors in brain and periphery. *J. Pharmacol. Exp. Ther.* **1991**, *258*, 1084–1090.
(13) Tippens, J. R.; DiManzo, V.; Panico, M.; Morris, H. R.; MacIntyre, I. Investigation of the structure/activity relationship of human calcitonin gene-related peptide (CGRP) *Biochem. Biophys. Res. Commun.* **1986**, *134*, 1306–1311.
(14) Kapas, S.; Clark, A. J. L. Identification of the orphan receptor gene as a Type 1 calcitonin gene related peptide receptor. *Biochem. Biophys. Res. Commun.* **1995**, *217*, 832–838.
(15) Zaidi, M.; Brain, S. D.; Tippins, J. R.; Di Marzo, V.; Moonga, B. S.; Chambers, T. J.; Morris, H. R.; MacIntyre, I. Structure-activity relationship of human calcitonin-gene-related peptide. *Biochem. J.* **1990**, *269*, 775–780.
(16) Muff, R.; Stangl, D.; Born, W.; Fischer, J. A. Comparison of a calcitonin gene-related peptide receptor in a human neuroblastoma cell line (SK-N-MC) and a calcitonin receptor in a human breast carcinoma cell line (T47D). *Ann. N.Y. Acad. Sci.* **1992**, *657*, 106–116.
(17) Veale, P. R.; Bhogal, R.; Morgan, D. G. A.; Smith, D. M.; Bloom, S. R. The presence of islet amyloid polypeptide/calcitonin gene-related peptide/salmon calcitonin binding sites in the rat nucleus accumbens. *Eur. J. Pharmacol.* **1994**, *262*, 133–141.
(18) Bhogal, R.; Smith, D. M.; Purkiss, P.; Bloom, S. R. Molecular identification of binding sites for calcitonin gene related peptide (CGRP) and islet amyloid polypeptide (IAPP) in mammalian lung: species variation and binding of truncated CGRP and IAPP. *Endocrinology* **1993**, *133*, 2351–2361.
(19) Pittner, R. A.; Albrandt, K.; Beaumont, K.; Gaeta, L. S. L.; Koda, J. E.; Moore, C. X.; Rittenhouse, J.; Rink, T. J. Molecular Physiology of Amylin. *J. Cell. Biochem.* **1994**, *55S*, 19–28.
(20) Kapas, S.; Catt, K. J.; Clark, A. J. L. Cloning and expression of cDNA encoding a rat adrenomedullin receptor. *J. Biol. Chem.* **1995**, *270*, 25344–25347.
(21) Nuki, C.; Kawasaki, H.; Kitamura, K.; Takenaga, M.; Kangawa, K.; Eto, T.; Wada, A. Vasodilator effect of adrenomedullin and calcitonin gene-related peptide receptors in rat mesenteric vascular beds. *Biochem. Biophys. Res. Commun.* **1993**, *196*, 245–251.
(22) Muff, R.; Born, W.; Fischer J. A. Calcitonin, calcitonin gene-relatd peptide, adrenomedullin and amylin: homologous peptides, separate receptors and overlapping biological actions. *Eur. J. Endocrinol.* **1995**, *133*, 17–20.
(23) Taylor, G. M.; Meeran, K.; Oshea, D.; Smith, D. M.; Ghatei, M. A.; Bloom, S. R. Adrenomedullin inhibits feeding in the rat by mechanism involving calcitonin gene-related peptide receptors. *Endocrinology* **1996**, *137*, 3260–3264.
(24) Owji, A. A.; Gardiner J. V.; Upton, P. D.; Mahmoodi, M.; Ghatei, M. A.; Bloom, S. R.; Smith, D. M. Characterisation and molecular identification of adrenomedullin binding sites in the rat spinal cord - a comparison with calcitonin gene-related peptide receptors. *J. Neurochem.* **1996**, *67*, 2172–2179.
(25) Lynch, B. and Kaiser, E. T. Biological properties of two models of calcitonin gene related peptide with idealized amphiphilic α-helices of different lengths. *Biochemistry* **1988**, *27*, 7600–7607.
(26) Hubbard, J. A.; Martin, S. R.; Chaplin, L. C.; Bose, C.; Kelly, S. M.; Price, N. C. Solution structures of calcitonin-gene-related-peptide analogues of calcitonin-gene-related peptide and amylin. *Biochem. J.* **1991**, *275*, 785–788.
(27) Breeze, A. L.; Harvey, T. S.; Bazzo, R.; Campbell, I. D. Solution structure of human calcitonin gene-related peptide by ^{1}H NMR and distance geometry with restrained molecular dynamics. *Biochemistry* **1991**, *30*, 575–582.
(28) O'Connell, J. P.; Kelly, S. M.; Raleigh, D. P.; Hubbard, J. A. M.; Price, N. C.; Dobson, C. M.; Smith, B. J. On the role of the C-terminus of α-calcitonin-gene-related peptide (α CGRP). The structure of des-phenylalaninamide^{37}-α CGRP and its interaction with the CGRP receptor. *Biochem. J.* **1993**, *219*, 205–210.
(29) Manning, M. C. Conformation of the alpha form of human calcitonin gene-related peptide (CGRP) in a aqueous solution as determined by circular dichroism spectroscopy. *Biochem. Biophys. Res. Commun.* **1991**, *160*, 388–392.
(30) Rovero, P.; Giuliani, S.; Maggi, C. A. CGRP antagonist activity of short C-terminal fragments of human α CGRP, CGRP(23-37) and CGRP(19-37). *Peptides* **1992**, *13*, 1025–1027.
(31) Maton, P. N.; Pradhan, T.; Zhou, Z. C.; Gardner, J. D.; Jensen, R. T. Activities of calcitonin gene-related peptide (CGRP) and related peptides at the CGRP receptor. *Peptides* **1990**, *11*, 485–489.
(32) Chakder, S. and Rattan, S. [Tyr^0]-Calcitonin gene-related peptide 28-37 (rat) as a putaive antagonist of calcitonin gene-related peptide response on oposum internal anal sphincter smooth muscle. *J. Pharmacol. Exp. Ther.* **1990**, *253*, 200–206.
(33) Maggi, C.; Rovero, P.; Giuliani, S.; Evangelista, S.; Regoli, D.; Meli, A. Biological activity of N-terminal fragments of calcitonin gene-related peptide. *Eur. J. Pharm.* **1990**, *179*, 217–219.
(34) Koskinen, A. M. P.; Valo, T.; Vihavainen, S.; Hakala, J. M. L. Synthesis of α-helix substituted analogs of calcitonin gene-related peptide. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 573–578.
(35) Entzeroth, M.; Doods, H. N.; Wieland, H. A.; Wienen, W. Adrenomedullin mediates vasodilation via $CGRP_1$ receptors. *Life Sci.* **1994**, *56*, 19–25.
(36) Poyner, D. R.; Andrew, D. P.; Brown, D.; Bose, C.; Hanley M. R. Pharmacological characterization of a receptor for calcitonin gene-related peptide on rat, L6 myocytes. *Br. J. Pharmacol.* **1992**, *105*, 441–447.
(37) Smith, D. D.; Li, J.; Wang, Q.; Murphy, R. F.; Adrian, T. E.; Elias, Y.; Bockman, C. S.; Abel, P. W. Synthesis and biological activity of C-terminally truncated fragments of human α-calcitonin gene-related peptide. *J. Med. Chem.* **1993**, *36*, 2536–2541.
(38) Hakala, J. M. L. and Vihinen, M. Modelling the structure of the calcitonin genen-related peptide. *Protein Engineering* **1994**, *7*, 1069–1075.
(39) Sagoo, J. K.; Bose, C.; Beeley, N. R. A.; Tendler, S: J. B. Structural studies on the [Bu (t)-Cys^{18}] (19-37) -fragment of human β-calcitonin-gene-related peptide. *Biochem. J.* **1991**, *280*, 147–150.
(40) Hakala, J. M. L.; Valo, T.; Vihavainen, S.; Hermonen, J.; Heino, P.; Halme, M.; Koskinen, A. M. P. Constrained analogues of the calcitonin gene-related peptide. *Biochem. Biophys. Res. Commun.* **1994**, *202*, 497–503.
(41) Wilmot, C. M. and Thornton, J. M. Analysis and Prediction of the Differernt Types of β-Turn in Proteins. *J. Mol. Biol.* **1988**, *203*, 221–232.
(42) Richardson, J. S. The anatomy and taxonomy of protein structure. *Adv. Protein Chem.* **1981**, *34*, 167–339.
(43) Jakubke, H.-D. Peptid-Design. In *Peptide*, 1st ed.; Spektrum Akad. Verl.: Heidelberg, Berlin, Oxford, 1996; pp 268–282.
(44) Beck-Sickinger, A. G.; Hoffmann, E.; Paulini, K.; Reissig, H.-U.; Willim, K.-D.; Wieland, H. A.; Jung, G. High affinity analogues of neuropeptide Y containing conformationally restricted non-proteinogenic amino acids. *Biochem. Soc. Trans.* **1994**, *22*, 145–149.
(45) Rink H. Solid-phase synthesis of protected peptide fragments using a trialkoxy-diphenyl-methylester resin. *Tetrahedron Lett.* **1987**, *28*, 3787–3790.

JM970533R