Identification of the Key Residue of Calcitonin Gene Related Peptide (CGRP) 27−37 to Obtain Antagonists with Picomolar Affinity at the CGRP Receptor

Manja Lang,[†] Silvia De Pol,[‡] Carsten Baldauf,[†] Hans-Jörg Hofmann,[†] Oliver Reiser,[‡] and Annette G. Beck-Sickinger*[,†]

Institute of Biochemistry, University of Leipzig, D-04103 Leipzig, Germany, and Institute of Organic Chemistry, University of Regensburg, D-93040 Regensburg, Germany

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Calcitonin gene related peptide (CGRP) plays an important role in the CNS and in the cardiovascular system. To identify high-affinity antagonists in competitive binding studies, we identified a novel radioactive tracer, [^{3}H-propionyl-K^{24}]-hαCGRP 8−37, which was labeled in solution by a recently developed strategy using photolabile protecting groups at reactive side chains. This tracer was shown to be as potent as commercially available ^{125}I-tracers for the determination of agonists and to have increased sensitivity for antagonists. We applied it to investigate the predicted turn structures centered at Pro^{29} and Pro^{34}. The substitution at positions 29 and 34 by turn-inducing amino acid mimetica showed that these turns are highly diverse. At position 29, a hydrophobic residue is preferred that constricts the secondary structure, whereas position 34 is required to stabilize the conformation of the backbone. All high-affinity analogues showed antagonistic properties with potency similar to CGRP 8−37.

Introduction

Calcitonin gene related peptide (CGRP) is a 37 amino acid neuropeptide with a characteristic sequence. A ring structure is formed at the N-terminus by a disulfide bond between Cys^2 and Cys^7, which is followed by an amphiphilic helix (residues 8−18)[1,2] and an amidated C-terminus (Figure 1A). CGRP exists in humans in two isoforms (α and β) that derive from two different genes and are distinguished by residues at positions 3, 22, and 25 (Figure 1B).[3,4] Both isoforms are widely distributed in the central and peripheral nervous system where they exhibit several physiological functions.[5] The most widely described effects of CGRP are associated with the cardiovascular system owing to the vasodilatatory, inotropic, and chronotropic activity.[6] Furthermore, it has been hypothesized that high concentrations of CGRP produce dilatation of cranial vessels and activate the trigeminal vascular system leading to migraine headache. Therefore, inhibition of CGRP binding by selective antagonists has become relevant in drug development,[7,8] and CGRP antagonists, such as the small molecule BIBN4069BS, are in clinical trials.[9]

The target of CGRP is a family B G-protein coupled receptor (GPCR) with a very unusual structure, because only coexpression of the transmembrane heptahelical calcitonin receptor like receptor (CRLR) with the transmembrane single helical receptor activity modifying protein 1 (RAMP1) forms the CGRP receptor.[10] An additional intracellular accessory protein, the receptor component protein (RCP), seems to be required to obtain cAMP-mediated signaling.[11] Several segments of the receptor have been identified to contribute to ligand affinity and activity. The N-terminal domains of CRLR and RAMP1 form the binding site for agonists and antagonists,[12,13] but receptor activity seems to be promoted only by the interaction of the agonists with residues 91−103 of RAMP1.[14]

Also for the natural ligand CGRP, different regions have been identified to be responsible for affinity and activity, respectively.

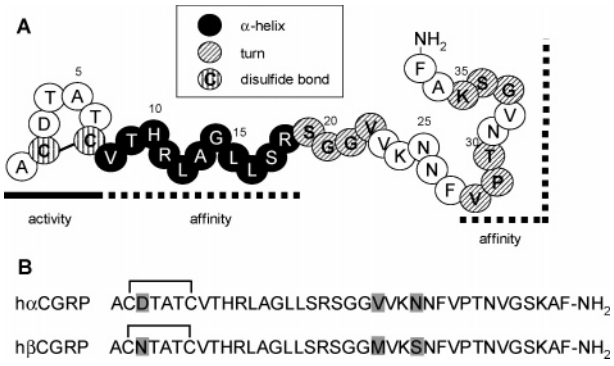

Figure 1. (A) Secondary structure elements and regions of hαCGRP that are important for the interaction with the receptor and (B) amino acid sequence of human CGRP. Deviations between α and β CGRP are highlighted in gray.

Structure−activity relationship studies of reduced size CGRP analogues have shown that the C-terminal segment of the peptide promotes affinity whereas the N-terminal ring structure is responsible for activity. Therefore N-terminally truncated analogues have been identified as antagonists. The most potent peptide was shown to be hαCGRP 8−37, which lacks only the N-terminal ring structure.[15] Further truncation of the N-terminus up to [Y^0]-CGRP 28−37 is possible; however, the affinity drops to micromolar concentration.[16,17] To increase the affinity, we have recently optimized the sequence of the C-terminal undecapeptide of CGRP, which led to the high-affinity antagonist [D^{31},P^{34},F^{35}]-CGRP 27−37.[18] In this analogue, aspartate at position 31 instead of asparagine led to much more potent analogues as found especially in animal studies, probably because of the improved solubility.[19] Furthermore, we demonstrated that position 34 is sensitive to changes that influence the 3D structure.[19] Recently, NMR spectroscopy showed that a β-turn is centered at Pro^{34} and an additional one centered at Pro^{29} is present in the 11-mer CGRP analogue.[20] Information on the biologically active conformation obtained by constriction of the turns may help to design novel small non-peptide ligands that can be used for drug development and identify the binding mode of CGRP at this unusual receptor complex.

* To whom correspondence should be addressed. Mailing address: Institute of Biochemistry, Bruederstrasse 34, D-04103 Leipzig, Germany. Tel: ++49 341 97 36901. Fax: ++49 341 97 36909. E-mail: beck-sickinger@uni-leipzig.de.

[†] University of Leipzig.

[‡] University of Regensburg.

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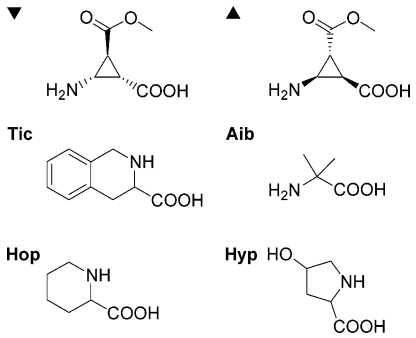

Figure 2. Formulas of the turn-inducing amino acid derivatives that have been introduced into the sequence of CGRP 27−37.

Table 1. Analytical Data of N^{α}-Nvoc-[K^{35}-Nvoc]-hαCGRP 8−37 as Precursor for the ^{3}H-Labeling Reaction and of [Propionyl-K^{24}]-hαCGRP 8−37 as Nonradioactive Reference Peptide

		mass (Da)			
no.	peptide	calcd	exptl	t_{ret}[c] (min)	purity (%)
1	N^{α}-Nvoc-[K^{35}-Nvoc]-hαCGRP 8−37	3604.4	3605.9 ± 2.4[a]	24.0	96
2	[propionyl-K^{24}]-hαCGRP 8−37	3183.7	3182.8[b]	21.7	95

[a] Molecular mass obtained by ESI-MS. [b] Molecular mass obtained by MALDI-MS. [c] Retention times from analytical HPLC.

Accordingly, we modified the predicted turn structures by replacing Pro^{29} and Pro^{34} with turn-inducing building blocks (Figure 2) and analyzed their affinity in competitive binding assays on SK-N-MC cells. To improve the identification of high-affinity antagonists, we recently developed a novel radioactive tracer by selective tritiation of hαCGRP 8−37 in solution at Lys^{24}. This new radioligand was proven to be as potent as the commercially available ^{125}I-tracer in competing hαCGRP, and additionally, the specificity for the antagonist hαCGRP 8−37 was 20-fold increased. Subsequently, the undecapeptide CGRP analogues that contain modifications of position 29 or 34 by turn-inducing building blocks were studied. All modifications at position 29 displayed higher affinity at the receptor than the known high-affinity antagonist CGRP 8−37. That position was accordingly identified as key residue for affinity. Additionally, dependence on the stereochemistry was found at position 34. All high-affinity ligands maintained their antagonistic properties up to pA_2 values of 8.2.

Results

Peptide Synthesis. All peptides (shown in Tables 1−3) were synthesized on Rink amide resin by automated solid-phase peptide synthesis using the orthogonal Fmoc/*t*Bu strategy to directly obtain peptide amides after cleavage from the resin. To selectively modify resin-bound peptides, N^{ϵ}-Dde-protected lysine building blocks were used at those positions that should be modified either by reprotection with the photolabile Nvoc-group or by acylation with propionic acid as recently described for neuropeptide Y analogues.[22] The resulting modified peptides, N^{α}-Nvoc-[K^{35}-Nvoc]-hαCGRP 8−37 (**1**) and [propionyl-K^{24}]-hαCGRP 8−37 (**2**), as well as the unmodified peptides, were cleaved from the resin by using TFA, which additionally removes all acid-labile protecting groups. The Nvoc protecting groups were not affected by this procedure. Subsequently the peptides were characterized by HPLC and mass spectrometry. Peptides were purified by preparative HPLC to obtain products with >95% purity. The analytical data of peptides **1** and **2** are summarized in Table 1.

The correct formation of the disulfide bond of hαCGRP (**4**) and the existence of the free SH-groups in reduced hαCGRP (**8**) were examined by the selective reaction of *N*-(3-maleimidopropionyl)-biocytin (MPB) with free thiol groups[21] and subsequent analysis of the reaction products by MALDI mass spectrometry. As expected there were no addition of MPB to oxidized hαCGRP (MW_{theo} 3787.4; MW_{exp} 3787.8) and an addition of two MPB molecules to the reduced hαCGRP (MW_{theo} 4836.1; MW_{exp} 4836.0).

Radioactive Labeling. [^{3}H-propionyl-K^{24}]-hαCGRP 8−37 was prepared as described previously[22] to improve the identification of high-affinity antagonists by using competitive binding assays. Selective tritiation of Lys^{24} of N^{α}-Nvoc-[K^{35}-Nvoc]-hαCGRP 8−37 (**1**) was achieved in solution by OSu-activation with *N*-hydroxysuccinimidyl-[2,3-^{3}H]-propionate. After irradiation of the photolabile protecting groups by UV light, the product was purified and identified by HPLC (Figure 3A) and β-counting (Figure 3B). The retention time of the desired product corresponded to the nonradioactive reference compound (**2**) in each synthesis. Dependent on the batch of *N*-hydroxysuccinimidyl-[2,3-^{3}H]-propionate, we obtained a tracer with specific activity of 13.5 ± 10.5 GBq/mmol.

Proof of Binding. The suitability of [^{3}H-propionyl-K^{24}]-hαCGRP 8−37 for studying hαCGRP binding was determined in competition binding assays on SK-N-MC cells, which endogenously express the human CGRP receptor complex.[23] First, we investigated the ability of unlabeled [propionyl-K^{24}]-hαCGRP 8−37 (**2**) to compete with [^{3}H-propionyl-K^{24}]-hαCGRP 8−37 to determine specific binding. The concentration required to inhibit 50% of the tritium tracer (IC_{50}) was determined to be 1.2 ± 0.7 nM. To further characterize the tracer, we performed saturation binding studies. By application of [^{3}H-propionyl-K^{24}]-hαCGRP 8−37 in concentrations of 0.15−4 nM, the specific tritiated tracer binding was demonstrated to be saturable (Figure 4A) and the dissociation constant (K_D) was calculated to be 0.9 ± 0.1 nM. B_{max} was calculated to be 702 ± 272 fmol/mg of receptor protein binding to the novel tracer on SK-N-MC cells.

Next, we investigated the displacement of the 30-mer hαCGRP 8−37 (**3**) and the 37-mer hαCGRP (**4**) to compare the resulting K_i values with those obtained previously with 2-[^{125}I-Tyr^{10}]-CGRP on SK-N-MC cells. The K_i value obtained for analogue **4** (0.29 ± 0.05 nM) was in the same range as shown before for replacing the ^{125}I-tracer (0.18 nM).[24] However, for hαCGRP 8−37 (**3**) on SK-N-MC cells, affinity was increased 5-fold compared to hαCGRP (**4**, Figure 4B) and 22-fold compared to the ^{125}I-tracer (1.3 nM).[24]

Linear hαCGRP. Agonists and antagonists are discriminated by the N-terminal ring structure formed by a disulfide bond between Cys^2 and Cys^7. To study the role of the disulfide bond, we synthesized reduced hαCGRP (**8**). Binding at the receptor was 100-fold reduced compared to the native ligand, but activation of the receptor was still possible with EC_{50} of 122 ± 79 nM compared to 21 ± 3 nM of the oxidized peptide (**4**).

Modification at Position 34. The biologically active conformation at position 34 was recently investigated;[18] however, the competitive binding experiments were performed with the ^{125}I-tracer. To enhance the comparability between the modifications at position 29 and 34, we reanalyzed the high-affinity antagonists **5**−**7**. All analogues showed increased affinities compared to the ^{125}I-tracer but to a different extent and without changing the order of potency.

To further analyze the bioactive structure of the undecapeptide of CGRP, we used the enantiomers of the conformationally

Table 2. Comparison of Binding Data Obtained with 2-[^{125}I-Tyr^{10}]-hαCGRP and [^{3}H-Propionyl-K^{24}]-hαCGRP

			mass (Da)				K_i (nM)	
no.	peptide	sequence	calcd	exptl	t_{ret} (min)	purity (%)	^{125}I-CGRP	^{3}H-CGRP 8−37
2	[propionyl-K^{24}]-hαCGRP 8−37	VTHRLAGLLSRSGGVV**K(prop)**NNFVPTNVGSKAF	3182.7	3182.9	21.7	95		0.6 ± 0.3
3	hαCGRP 8−37	VTHRLAGLLSRSGGVVKNNFVPTNVGSKAF	3125.7	3125.7	18.5	100	1.3[a]	0.06 ± 0.05
4	hαCGRP	ACDTATCVTHRLAGLLSRSGGVVKNNFVPTNVGSKAF	3787.4	3787.8	20.6	96	0.18[a]	0.29 ± 0.05
5	[D^{31},P^{34},F^{35}]-hαCGRP 27−37	FVPT**D**VG**PF**AF	1194.6	1194.3	22.3	100	14[b]	0.02 ± 0.01
6	[D^{31},Tic^{34},F^{35}]-hαCGRP 27−37	FVPT**D**VG-**Tic**-FAF[c]	1256.6	1256.6	25.7	100	70.5[b]	1.8 ± 0.1
7	[D^{31},A^{34},F^{35}]-hαCGRP 27−37	FVPT**D**VG**AF**AF	1168.6	1168.5	21.6	100	76.8[b]	13.6 ± 5.2

[a] Reference 24. [b] Reference 18. K_i calculated from IC_{50} by using K_D = 0.05 nM. [c] Tic = tetrahydroisoquinoline-3-carboxylic acid.

Table 3. Observed Masses of hαCGRP Analogues Obtained by MALDI-MS and Their Binding Affinities for the CGRP Receptor Complex (Human Neuroblastoma SK-N-MC Cells)[a]

			mass (Da)				
no.	peptide	sequence	calcd	exptl	t_{ret} (min)	purity (%)	K_i (nM)
4	hαCGRP	ACDTATCVTHRLAGLLSRSGGVVKNNFVPTNVGSKAF	3787.4	3787.8	20.6	96	0.29 ± 0.05
8	hαCGRP (reduced)	ACDTATCVTHRLAGLLSRSGGVVKNNFVPTNVGSKAF	3789.4	3789.1	19.6	95	10.2 ± 7.5
9	[▼^{34},F^{35}]-hαCGRP 27−37	FVPTNVG**▼F**AF	1237.7	1237.9	19.9	95	>10 000
10	[▲^{34},F^{35}]-hαCGRP 27−37	FVPTNVG**▲F**AF	1237.7	1237.9	21.0	97	>10 000
11	[D^{31},▼^{34},F^{35}]-hαCGRP 27−37	FVPT**D**VG**▼F**AF	1238.7	1238.9	18.9	97	>10 000
12	[D^{31},▲^{34},F^{35}]-hαCGRP 27−37	FVPT**D**VG**▲F**AF	1238.7	1238.9	20.3	>99	>10 000
13	[▼^{34},F^{35}]-hαCGRP 8−37	VTHRLAGLLSRSGGVVKNNFVPTNVG**▼F**AF	3198.1	3198.4	20.6	95	>10 000
14	[▲^{34},F^{35}]-hαCGRP 8−37	VTHRLAGLLSRSGGVVKNNFVPTNVG**▲F**AF	3198.1	3197.8	21.2	95	>10 000
15	[D^{31},▼^{34},F^{35}]-hαCGRP 8−37	VTHRLAGLLSRSGGVVKNNFVPT**D**VG**▼F**AF	3199.1	3198.7	19.6	96	31.0 ± 19.2
16	[D^{31},▲^{34},F^{35}]-hαCGRP 8−37	VTHRLAGLLSRSGGVVKNNFVPT**D**VG**▲F**AF	3199.1	3199.2	20.8	>99	65.3 ± 62.1
17	[Hop^{29},D^{31},P^{34},F^{35}]-hαCGRP 27−37	FV-**Hop**-T**D**VG**PF**AF	1208.5	1208.3	20.9	>99	>10 000
18	[Tic^{29},D^{31},P^{34},F^{35}]-hαCGRP 27−37	FV-**Tic**-T**D**VG**PF**AF	1256.6	1256.7	20.9	>99	0.02 ± 0.01
19	[Hyp^{29},D^{31},P^{34},F^{35}]-hαCGRP 27−37	FV-**Hyp**-T**D**VG**PF**AF	1209.5	1209.4	18.8	95	0.006 ± 0.001
20	[Aib^{29},D^{31},P^{34},F^{35}]-hαCGRP 27−37	FV-**Aib**-T**D**VG**PF**AF	1183.6	1183.2	20.5	>99	0.002 ± 0.001
21	[A^{29},D^{31},P^{34},F^{35}]-hαCGRP 27−37	FV**A**T**D**VG**PF**AF	1168.6	1168.9	20.1	>99	8.4 ± 8.0
22	[▼^{29},D^{31},P^{34},F^{35}]-hαCGRP 27−37	FV**▼**T**D**VG**PF**AF	1238.7	1238.9	20.1	>99	>10 000
23	[▲^{29},D^{31},P^{34},F^{35}]-hαCGRP 27−37	FV**▲**T**D**VG**PF**AF	1238.7	1238.4	21.0	>99	0.7 ± 0.6

[a] ▼ = (1*S*,2*S*,3*S*)-β-aminocyclopropane carboxylic acid; ▲ = (1*R*,2*R*,3*R*)-β-aminocyclopropane carboxylic acid; Aib = aminoisobutyric acid; Hop = L-homoproline; Hyp = L-4-hydroxyproline; Tic = tetrahydroisoquinoline-3-carboxylic acid.

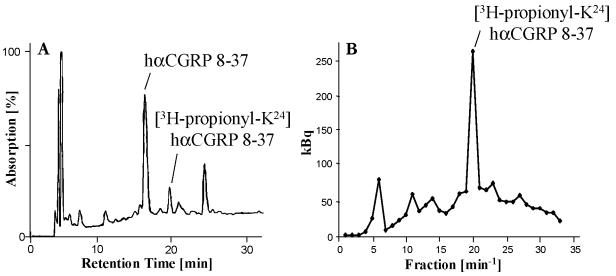

Figure 3. (A) HPLC chromatogram of the labeling reaction mixture after tritiation and UV irradiation. The final product was eluted with a retention time of 19.2 min, which corresponds to the retention time of the nonradioactive [propionyl-K^{24}]-CGRP 8−37. (B) The radioactivity profile displays the radioactivity content of the collected fraction (min^{-1}). The radioactive peptide was obtained from fraction 20, which corresponds to the HPLC peak with the retention time of 19.2 min.

constrained β-aminocyclopropane carboxylic acid (β-ACC) (Figure 2), which have been recently shown to stabilize peptide structures depending on their configuration.[25−27] In contrast to these investigations, the absolute configuration of the β-ACC moiety had no influence on binding affinity of all analogues (**9**−**16**). The 11-mer hαCGRP analogues (**9**−**12**) are devoid of any CGRP receptor binding (K_i > 10 000 nM). Therefore we investigated the ability of the elongated peptide chain to stabilize the biologically active conformation. The analogues [▼^{34},F^{35}]-hαCGRP 8−37 (**13**) and [▲^{34}F^{35}]-hαCGRP 8−37 (**14**) are devoid of any receptor affinity, but the additional exchange of Asn^{31} by Asp^{31} (**15**, **16**) led to analogues with some affinity at the receptor (K_i = 31.0 ± 19.2 and 65.3 ± 62.1 nM).

Dose−response experiments of **15** and **16** showed no change of the cellular cAMP concentration. Consequently, we investigated their potency to inhibit the cAMP accumulation evoked by hαCGRP in 10 and 100 nM concentrations. For comparison of the antagonist potency, we analyzed the known antagonist hαCGRP 8−37. Because all peptides shifted the dose-response curve of hαCGRP parallel to the right, pA_2 values were calculated. The (▲)-configured analogue (**16**) was less potent

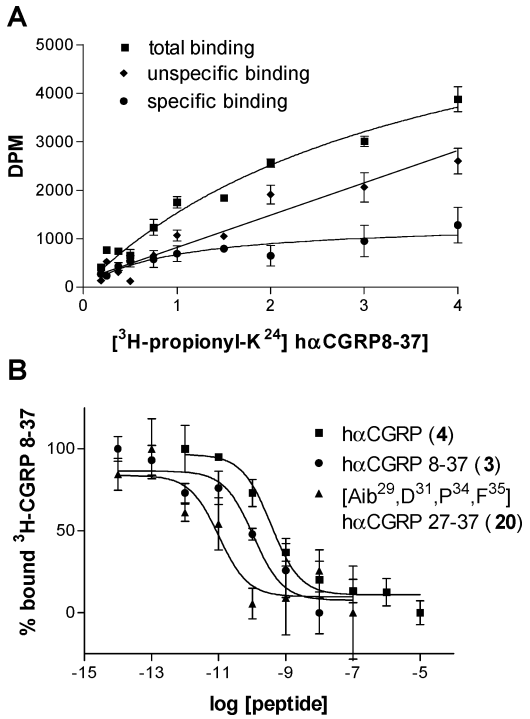

Figure 4. (A) Saturation isotherm analysis using [^{3}H-propionyl-K^{24}]-hαCGRP 8−37 on SK-N-MC cells. Ligand concentration ranged from 0.15 to 4 nM. Total binding (■) and unspecific binding (◆) increased (1 μM hαCGRP 8−37), while specific binding (●) reaches a plateau. The dissociation constant (K_D) was determined to be 0.9 ± 0.1 nM, and B_{max} was 702 ± 272 fmol/mg of protein. (B) Displacement of [^{3}H-propionyl-K^{24}]-hαCGRP 8−37 by unlabeled hαCGRP (**4**, ■), hαCGRP 8−37 (**3**, ●), and [Aib^{29},D^{31},P^{34},F^{35}]-hαCGRP 27−37 (**20**, ▲) at the CGRP receptor complex expressed in SK-N-MC cells. Data are means ± SEM.

(pA_2 = 7.0 ± 0.4) than the (▼)-configured (**15**, pA_2 = 7.9 ± 0.2), which was in the same range as hαCGRP 8−37 (**3**, pA_2 = 8.3 ± 0.2).

Modification at Position 29. A set of [D^{31},P^{34},F^{35}]-CGRP 27−37 analogues (**17**−**23**) was synthesized with modification of Pro^{29} to constrain the predicted β-turn at this position. After incorporation of Hop (**17**), which extends the side chain by one carbon atom, no affinity was measured. The same affinity (K_i = 0.02 ± 0.01 nM) as for peptide **5** was observed for the analogue containing Tic^{29} (**18**), which is characterized by a large hydrophobic side chain. The introduction of the Hyp residue (**19**) led to a 3-fold increase in affinity. The highest affinity (K_i = 0.002 ± 0.001 nM) was observed for the Aib analogue (**20**), which was found to have a 145-fold increased affinity compared to the native ligand hαCGRP and a 30-fold increased affinity compared to the antagonist hαCGRP 8−37 (Figure 4B). To investigate whether the hydrophobic interaction or stabilization of the secondary structure contributes to the increased affinity, we exchanged Pro^{29} with Ala^{29} (**21**). This n/a resulted in a 420-fold decrease of affinity. To further analyze the configuration, we incorporated the β-ACC building blocks. Analyzing the analogues **22** and **23** revealed that in contrast to position 34 the absolute configuration of the β-ACC residue had a strong effect on binding. Only the analogue with the (▲)-configured moiety (**23**) completely inhibited binding of the radioactive tracer and displayed an affinity (K_i) of 0.7 ± 0.6 nM for the CGRP receptor. In contrast, the analogue with the (▼)-configured β-ACC residue (**22**) was devoid of any CGRP receptor binding (K_i > 10 000 nM).

High-affinity analogues (**18**−**21** and **23**) were studied for their agonistic activity at the receptor by analyzing the dose-dependent change of the cellular cAMP concentration. Because none of them showed any direct effect, the antagonistic properties were investigated. All peptides shifted the dose−response curve of the agonist parallelling to the right (for example, see Figure 5), except **23**, which did not alter the response of the agonist. Calculation of antagonist potency led to pA_2 values between 7.0 and 8.2 (Table 4).

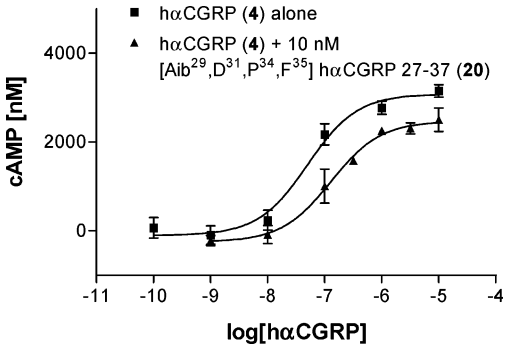

Figure 5. Dose−response curve of cAMP accumulation assays in SK-N-MC cells for hαCGRP alone (**4**, ■) and in the presence of 10 nM [Aib^{29},D^{31},P^{34},F^{35}]-hαCGRP 27−37 (**20**, ▲). Data are means ± SEM.

Table 4. Antagonist Activities of hαCGRP 8−27 and CGRP 27−37 Analogues on hαCGRP-Induced cAMP Accumulation at Human Neuroblastoma SK-N-MC Cells

no.	peptide	pA_2[a]
3	hαCGRP 8−37	8.3 ± 0.2
15	[D^{31},▼^{34},F^{35}]-hαCGRP 8−37	7.9 ± 0.2
16	[D^{31},▲^{34},F^{35}]-hαCGRP 8−37	7.0 ± 0.4
18	[Tic^{29},D^{31},P^{34},F^{35}]-hαCGRP 27−37	8.2 ± 0.2
19	[Hyp^{29},D^{31},P^{34},F^{35}]-hαCGRP 27−37	7.5 ± 0.2
20	[Aib^{29},D^{31},P^{34},F^{35}]-hαCGRP 27−37	8.0 ± 0.4
21	[A^{29},D^{31},P^{34},F^{35}]-hαCGRP 27−37	7.6 ± 0.3

[a] pA_2 values were obtained from concentration ratios using 10 and 100 nM antagonists.

To investigate whether changes of the secondary structure are responsible for the different biological activity, circular dichroism studies were performed. However, no significant difference among the circular dichroism spectra was observed in phosphate buffer at neutral pH supplemented with 30% TFE in concentrations of 40 μM (data not shown).

Stimulated by two contradictory experimental structure analyses that suggest β-turn or, alternatively, polyproline helix torsion angles between the positions 28 and 31, we investigated the capacity of our amino acid constituents to adopt such conformations after their substitution for proline at position 29. For this purpose, quantum chemical studies employing the methods of ab initio MO theory were performed on blocked dipeptide model compounds with alanine, proline, Aib, Hop, (▲)-β-ACC, and (▼)-β-ACC at the i + 1 position and alanine at the i + 2 position. As can be seen in Table 5, all α-amino acid constituents enable the β-turn formation with a preference for the βI turn, which is typical for β-turns with proline at the position i + 1 (Figure 6). For Aib, the stabilities of the βI and βII′ turns are comparable. It is interesting that the two β-ACC moieties, which represent β-amino acid constituents, are also able to form turn-like structures as part of an α-amino acid sequence. The most stable turn with a (▼)-configured β-ACC constituent is characterized by an 11-membered hydrogen-bonded pseudocycle (Figure 7). The corresponding turn structure is also possible with the (▲)-configured β-ACC moiety, but a turn with a nine-membered pseudocycle connecting the peptidic NH group of residue i + 1 with the peptidic CO group of residue 3 is more stable as shown in Table 5. The cyclopropane ring of

Table 5. Turn Structures in Blocked Dimers Me-CO-Xaa-L-Ala-NHMe at the HF/6-31G* Level of ab Initio MO Theory

Xaa	turn	φ_{i+1}[a]	ψ_{i+1}[a]	φ_{i+2}[a]	ψ_{i+2}[a]	ΔE[b]
Ala	βI	−72.8	−18.8	−98.8	7.1	0.0[c]
	βI′	62.7	32.1	65.0	22.7	15.6
	βII	−60.3	133.7	66.8	19.7	4.7
	βII′	54.4	−132.1	−94.9	8.2	6.4
Aib	βI	−63.1	−30.0	−96.1	5.5	0.0[d]
	βI′	62.0	31.6	64.6	23.6	10.2
	βII	−52.1	133.5	66.6	18.9	11.1
	βII′	52.1	−132.3	−95.3	9.5	0.2
Hop	βI	−56.4	−36.1	−97.4	11.8	0.0[e]
	βII	−52.3	135.0	68.1	14.8	7.1
Pro	βI	−66.0	−22.9	−96.4	6.6	0.0[f]
	βII	−57.3	131.6	67.2	17.9	3.2
β-ACC (▼)	C_{11}[g]	63.0	−131.4	−105.5	14.6	*h*
β-ACC (▲)	C_{11}[i]	52.0	−126.8	−113.0	23.2	19.7
	C_9[j]	150.6	−49.1	−65.1	147.3	0.0[k]

[a] Xaa φ_{i+1} and ψ_{i+1}; L-Ala φ_{i+2} and ψ_{i+2}. [b] In kJ/mol. [c] E_T = −738.713 228 au. [d] E_T = −777.746 999 au. [e] E_T = −854.642 471 au. [f] E_T = −815.613 263 au. [g] Eleven-membered hydrogen-bonded cycle; torsion angle of cyclopropane bond, θ = 0.6°. [h] E_T = −815.590 394 au. [i] Eleven-membered hydrogen-bonded cycle; torsion angle of cyclopropane bond, θ = −1.8°. [j] Nine-membered hydrogen-bonded cycle; torsion angle of cyclopropane bond, θ = 4.8°. [k] E_T = −815.585 716 au.

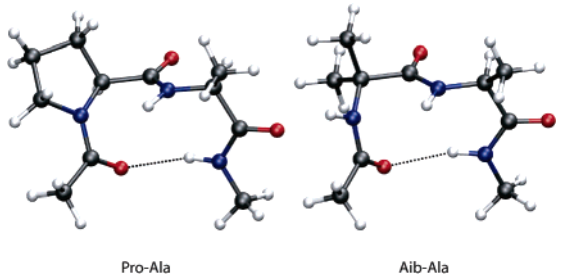

Figure 6. βI-turns in blocked Pro-Ala and Aib-Ala dipeptides.

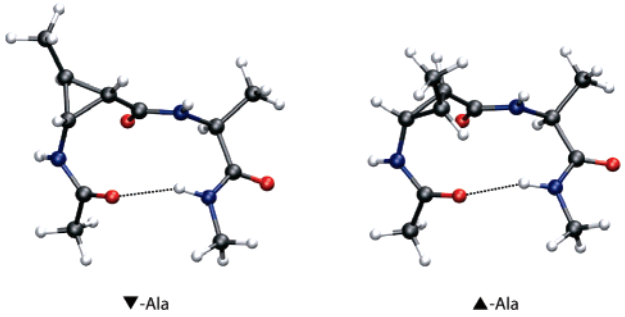

Figure 7. Turn structures with 11-membered hydrogen-bonded pseudocycles in blocked (▼)-β-ACC−Ala (labeled as ▼-Ala) and (▲)-β-ACC−Ala (labeled as ▲-Ala) dipeptides, which are comparable with β-turns in native peptides.

(▲)-configured β-ACC is in a pseudoaxial orientation referred to the turn plane, whereas the cyclopropane ring of the (▼)-configured β-ACC alternative is in a pseudoequatorial orientation. It is remarkable that the values of the torsion angle φ and ψ in the C_{11} turns closely correspond to those of a βII′ turn despite the additional CC single bond of the cyclopropane ring in the backbone, whose torsion angle θ is approximately kept fixed at about 0°.

The investigation of the possibility of polyproline helix conformations for all modified derivatives reveals that the corresponding torsion angles of about −60° for φ_{i+1} and about 140° for ψ_{i+1} are only kept for the amino acids proline, Hop, and Aib, whereas the other amino acid residues prefer different torsion angle values.

Discussion

The aim of this study was to extend the knowledge on the bioactive conformation of hαCGRP analogues by changing the predicted turn structures by introducing constricted unnatural amino acids. To increase the sensitivity of competitive binding assays for antagonists, we applied the novel radioactive tracer, [^{3}H-propionyl-K^{24}]-hαCGRP 8−37. This was obtained by using a labeling strategy that combines the advantages of solid-phase peptide synthesis with the flexibility of labeling in solution.[22] The advantage of this strategy is that very small amounts of peptide (nanogram) can be labeled. In contrast, direct incorporation of radioactive amino acids or radioactive labeling of selectively deprotected residues still bound to the polymer would require huge amounts of radioactivity because milligram amounts are the smallest scales to be handled by solid-phase synthesis. For labeling, the antagonist hαCGRP 8−37 was selected because of its high affinity at the CGRP receptor complex.[28] Modification of the side chain at position 24 was chosen as minimal loss of activity, and no agonistic effects were expected.

Competition binding experiments of the unlabeled [propionyl-K^{24}]-hαCGRP 8−37 on SK-N-MC cells demonstrated, with a K_i of 0.6 ± 0.3 nM, that competitor and tracer are indistinguishable in binding at the receptor and therefore no additional modification beside Lys^{24} has taken place. A decrease of affinity could have been observed if the side chain of Lys^{35} was modified, because this residue is located next to positions that are essential for affinity.[18,28] Alternatively, modification of the N-terminus could have been made;[48] however the close vicinity of the active site let us to go for position 24. In saturation experiments, it was further demonstrated that [^{3}H-propionyl-K^{24}]-hαCGRP 8−37 binding on SK-N-MC cells was saturable with a single high-affinity binding site. This was also observed previously by investigations with the agonist tracer [^{125}I-Tyr^{10}]-hαCGRP,[29,30] as well as with the antagonist tracer [^{3}H]-BIBN4096BS.[31] However, the K_D value (0.9 ± 0.1 nM) was increased by about 20-fold compared to other radioactive CGRP tracers. The reason remains unclear because acylation of Lys^{24} was shown to be tolerated without changing affinity of hαCGRP.[32] So far no difference was observed between the affinity of the agonist tracer ([^{125}I-Tyr^{10}]-hαCGRP; K_D = 50 pM),[24] antagonist tracer ([^{125}I-Tyr^{10}]-hαCGRP 8−37; K_D = 160 pM),[30] and non-peptide antagonist tracer ([^{3}H]-BIBN4096BS; K_D = 160 pM).[31] The quantity of binding sites detected in SK-N-MC cells (702 ± 272 fmol/mg of protein) is in accordance with earlier data reported by using the [^{3}H]-BIBN4096BS tracer (402 fmol/mg of protein).[31] Comparison of maximal binding capacities of the agonist ligand [^{125}I-Tyr^{10}]-hαCGRP (15−52 fmol/mg of protein)[24,29,33] reveals that antagonists recognize significantly higher quantities of binding sites. This is as expected, because agonists recognize preferentially the high-affinity state of the receptor.[34] Another reason may be the different interaction of agonists and antagonists with RAMP1.[14]

Additionally, we investigated the peptides **3**−**7** in competitive binding experiments for comparison with affinities obtained with the commercially available [^{125}I-Tyr^{10}]-hαCGRP. The K_i values determined with the native ligand hαCGRP (**4**) correlate well with the K_i values obtained in studies using the ^{125}I-tracer,[24] but an increase in affinity was observed applying the previously investigated antagonists (**3** and **5**−**7**).[10,18] The extent was different, which suggests different binding sites at the unusual CGRP receptor complex. As shown recently the N-terminal domains of both CRLR and RAMP1 are involved in ligand recognition.[12,13] The increase in affinity of the antagonists by

applying [^{3}H-propionyl-K^{24}]-hαCGRP 8−37 as radioactive competitor indicates that the agonist tracer might not fully be competitive because agonists and antagonists might occupy slightly different binding sites. Furthermore, the antagonist tracer is not internalized in contrast to agonistic tracers that also might account for the differences found in competition binding assays because incubation time of 90 min already is in the same time schedule as receptor internalization. In all competition binding experiments, the observed binding curve was monophasic, indicating that the tracer labels a single binding site in neuroblastoma cells, which is in agreement with earlier investigations.[29]

For agonist activity, it is known that the N-terminal segment composed of the disulfide bond between Cys^{2}and Cys^7 is essential. However, controversial discussion is reported on the relevance of this bond with respect to agonistic and antagonistic properties. By using our systematic approach to study the bioactive conformation of CGRP antagonists, we found that affinity and activity of the reduced CGRP was decreased, but it acted still as an agonist. Thus, the constriction of the N-terminal segment seems to be required for an efficient interaction with the receptor but not in general for receptor activation. This finding is in agreement with previously performed substitutions of both cysteines either by penicillamine (Pen)[35] or by Cys(Et).[33] We suggest therefore that only the surrounding amino acids interact with the receptor for generation of activity and that the disulfide bond constrains the bioactive conformation that provides the right orientation of the required residues. However, we cannot exclude that oxidation of the reduced CGRP takes place during the binding assay although in vitro this was not found under similar conditions.

We have previously identified the high-affinity antagonist [D^{31},P^{34},F^{35}]-hαCGRP 27−37 by optimizing the C-terminal segment of CGRP.[18] This analogue was recently investigated by NMR spectroscopy resulting in the prediction of two turn structures located at positions 29 and 34.[20] To improve the antagonist potency and to obtain information about the bioactive conformation of the 11-mer analogue, we further constrained the secondary structure by introducing turn-inducing unnatural amino acids at position 29 and 34. Extension of the side chain was tolerated only by an additional increase of hydrophobicity. In general, the exchange with hydrophobic building blocks was extremely efficient to obtain high-affinity analogues. This was not expected because previous replacements with hydrophobic building blocks led to decreased activity.[18] However, in that study [Y^0]-rαCGRP 28−37 was investigated, which may not be able to form a turn in the N-terminal region. Accordingly, there are two possibilities that may contribute to ligand−receptor interaction: constriction of the secondary structure and direct interaction by hydrophobic residues. To prove the first assumption, we introduced β-ACC building blocks at position 29. We found a strong influence on the conformation to achieve affinity, but the loss of hydrophobicity was accompanied by decreased affinity. In agreement with recent investigations,[25] the active moiety was the (▲)-configured β-ACC but not the (▼)-configured residue, which has a high propensity to helix formation when it occurs at position $i + 1$ in α,β-peptides.[26] Obviously the conformation of the residues 28 and 29 and a hydrophobic moiety are important for receptor recognition. However, by investigating the secondary structure of all analogues with substitutions at position 29 by CD spectroscopy, we observed no significant difference. This was unexpected because of the opposite configured β-ACC moieties and the recently identified trans configuration of the Val^{28}−Pro^{29} peptide bond.[20] Thus, all analogues are highly flexible in solution despite the constriction of the backbone, and accordingly bioactive conformation is induced by the receptor/RAMP 1 complex. The use of more constrained building blocks might be helpful in the future to obtain constrained analogues also in solution. Since a β-turn is suggested between Val^{28} and Asp^{31} with proline in position 29,[20] we performed a systematic theoretical study on model compounds with the various amino acid constituents. According to the results, this could well be a βI turn, which is indeed favored in most cases. Obviously, all amino acids including the β-ACC analogues are able to adopt β-turn-like structures. Thus, an explanation of the affinity differences arising from modifications in this region might be more related to differences in the interaction behavior than to different secondary structures. This is in agreement with the results obtained by CD spectroscopy. Some attention deserves the suggestion of a polyproline II helix conformation in this region, which comes from another NMR study.[1,2] We have estimated such a possibility on peptide tetramers with the various amino acid analogues at the position $i + 1$. A polyproline II helix can be adopted for the residues proline, Aib, and Hop, whereas a comparable structure is not kept for other residues. Indeed, the modifications with proline and Aib at position 29 exhibit high affinity, whereas the affinity of the other modified derivatives is decreased. Unfortunately, the latter is also the case for Hop, for which a good affinity should be expected in case of a polyproline II helix as the biologically active conformation. Accordingly, the existence of a polyproline II helix cannot be decided with the included analogues.

We showed recently that high-affinity and low-affinity analogues with different residues at position 34 can be distinguished by their secondary structure.[19] This conformation does not seem to be imitated with β-ACC building blocks. We assume, therefore, the existence of a different turn in the biologically active conformation of the C-terminal segment of CGRP at this position. Several research groups have analyzed the biological active structure of this segment. Constriction of the C-terminal region by disulfide bonds and molecular modeling of these structures led to the suggestion of a βII′-turn with Gly^{33} at the position $i + 1$.[36] This assumption was confirmed by the restriction of positions 33 and 34 by the β-turn dipeptide (BTD).[37] Owing to the replacement of Ser^{34} by proline in another study also a βI-turn with Pro in the $i + 1$ position or a βIV-turn with Pro in the $i + 2$ position are possible.[18] In a recent NMR study, Carpenter et al.[20] showed the Gly^{33}−Pro^{34} peptide bond to adopt the cis and the trans configuration to a similar extent. This is in agreement with our results on β-ACC-containing CGRP 8−37 analogues that showed no preference for a definite configuration. Therefore, we assume that either a βII′-turn or a β-VIa1-turn with cis configured Pro^{34} at the $i + 2$ position is possible. Alternatively, a proline in trans configuration should be placed at the $i + 1$ position to form a βI- or βVIII-turn.[38,39]

The extension of the amino acid sequence to the 30-mer β-ACC^{34} CGRP analogues showed that the helix of CGRP is important for the stabilization of the bioactive configuration of peptide ligands. This might be due to their amphipathic character as was shown by the tolerance toward the replacement with various amphipathic sequences.[1] Furthermore the two positively charged arginine residues at positions 11 and 18 have been suggested to align the helix with the hydrophobic face in contact with the receptor by either stabilization of the peptide structure or direct interaction with the receptor.[28,40]

Conclusion

We have shown that [^{3}H-propionyl-K^{24}]-hαCGRP is a novel high-affinity tracer for the investigation of CGRP receptor pharmacology, in particular for the identification of antagonists. By using this new tracer, we investigated the predicted turn structures of hαCGRP 27−37 by replacing Pro^{29} and Pro^{34} with turn-inducing amino acid mimetics. The replacements of residue 34 required building blocks that force the backbone into a distinct conformation. At residue 29, hydrophobic building blocks that constrict the secondary structure showed highest affinity. Because all analogues with modification of position 29 displayed higher affinity than the same building blocks at position 34, this position has been identified as the key position. Highest affinity at the CGRP receptor complex was exhibited by [Aib^{29},D^{31},P^{34},F^{35}]-hαCGRP 27−37 with a K_i of 0.002 ± 0.001 nM. All high-affinity analogues are shown to be antagonists with similar potency as hαCGRP 8−37. This was surprising owing to the increased affinities of the 11-mer analogues. Possibly these analogues are too small to completely cover all sites at the receptor responsible for affinity and activity of the agonist hαCGRP or slightly bind to a different binding pocket. Another explanation might be differences in their stability in the cell culture systems owing to different sensitivities to amino peptidases. Additionally we could show that the disulfide bond is not required for maintaining agonist activity of hαCGRP. The identification of structural requirements to obtain CGRP receptor agonists and antagonists can be used for the development of new therapeutics in the field of migraine and cardiovascular diseases that provide higher efficiency and increased bioavailability compared with BIBN4096BS.

Experimental Section

Materials. Peptide synthesis used N$^\alpha$-Fmoc-protected amino acids, N$^\alpha$-Boc-protected valine, 1-hydroxybenzotriazole (HOBt), 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU), and the 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy (Rink Amide) resin purchased from Novabiochem (Germany, Schwalbach). The side chain protecting groups for the amino acids were *tert*-butyl (*t*Bu) for serine and threonine, *tert*-butoxy (*t*BuO) for aspartate, trityl (Trt) for asparagine, cystine, and histidine, 2,2,4,6,7-pentamethyldihydrobenzofurane-5-sulfonyl (Pbf) for arginine, and either *tert*-butoxycarbonyl (Boc) or 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde) for lysine according to the synthesis strategy. *N*,*N*′-Diisopropylcarbodiimide (DIC), iodine, and 6-nitroveratryloxycarbonyl chloride (Nvoc) were obtained from Sigma-Aldrich (Germany, Taufkirchen). Trifluoroacetic acid (TFA), 1-methyl-2-pyrrolidone, diisopropylethylamine (DIEA), thioanisole, *p*-thiocresol, 1,2-ethanedithiol, hydrazine hydrate solution, propionic acid anhydride, trimethylsilylbromide, triethylammonium phosphate, *tert*-butyl alcohol, and piperidine were purchased from Fluka (Germany, Taufkirchen). Acetonitrile (ACN, for HPLC) was obtained from Merck (Germany, Darmstadt). Diethyl ether, dichloromethane, and *N*,*N*′-dimethylformamide (DMF, peptide synthesis grade) were obtained from Biosolve (Netherlands, Valkenswaard). *N*-Succinimidyl-[2,3-^{3}H]-propionate was purchased from Amersham (Germany, Freiburg). The scintillation cocktail Wallac HiSafe3 was from PerkinElmer (Germany, Rodgau-Jügesheim). *N*-(3-Maleimidopropionyl)-biocytin (MPB) was synthesized according to ref 20.

For cell culturing, the following media and supplements were used: modified Eagle medium (MEM), phosphate-buffered saline (PBS), PBS−EDTA, sodium pyruvate, nonessential amino acids, fetal calf serum (FCS), and glutamine obtained from Gibco Life Technologies (Germany, Karlsruhe); bovine serum albumin obtained from Sigma-Aldrich; HEPES, Tris, glucose, Pefabloc SC, and 3-isobutyl-1-methylxanthine (IBMX) purchased from Fluka.

Peptide Synthesis. Synthesis of CGRP analogues was performed as described previously by automated multiple solid-phase peptide synthesis with automated Fmoc/*t*Bu coupling on the Rink amide resin (30 mg, resin loading 0.6 mmol/g) to directly obtain the peptide amide.[41] The enantiomers ((▼)- or (▲)-configured) of β-aminocyclopropane carboxylic acids (β-ACC) were coupled with Fmoc-L-Val to the diastereomeric building blocks (Fmoc-Val-βACC or with Gly to the enantiomeric building blocks (Fmoc-Gly-βACC) to the general procedure described recently[42,43] and coupled manually into the growing peptide chain as dipeptide building block, as described by Koglin et al.[25]

Disulfide bond formation of hαCGRP was performed on the resin for 4 h in a solution of iodine (1.15 equiv) in methanol/$CHCl_3$ (1:1; v/v).[44]

For radioactive labeling, hαCGRP 8−37 was synthesized with Fmoc-Lys(Dde)-OH at position 35. After complete synthesis of the peptide sequence, the Dde protecting group was selectively removed by applying 2% hydrazine in DMF.[45] Subsequently the free primary amino groups (Lys^{35}, N-terminus) were reprotected with the photolabile Nvoc-group.[22] Therefore the preswollen resin was incubated overnight in a solution of 5 equiv of Nvoc-Cl, 5 equiv of HOBt, and 10 equiv of DIEA in DMF. Completeness of the reaction was examined by a ninhydrin assay.[46]

The nonradioactive propionylated reference substance, [propionyl-K^{24}]-hαCGRP 8−37, was synthesized by using Lys(Dde) protection at position 24 and N$^\alpha$-Boc protection for the N-terminal valine. After removal of the Dde group, the unprotected Lys^{24} was acylated by applying propionic anhydride (10 equiv) twice in the presence of 10 equiv of DIEA for 10 min.

After complete modification all peptides were cleaved from the resin by using a mixture of TFA/thioanisole/thiocresol (90:5:5, v/v) for 3 h, except for reduced CGRP 8−37, which was cleaved by using a mixture of TFA/thioanisole/ethanedithiol (90:7:3, v/v). Then the peptides were precipitated from ice-cold diethyl ether, collected by centrifugation, washed four times, dissolved in *tert*-butyl alcohol/water (1:3, w/w) and lyophilized.

Purification of the peptides was achieved by preparative HPLC on a RP C-18 column (Vydac, 250 mm × 25 mm, 10 μm) with a gradient of 25−65% B in A (A = 0.1% TFA in water; B = 0.08% TFA in acetonitrile) over 60 min and a flow rate of 10 mL/min.

The peptides were analyzed by matrix-assisted laser desorption ionization (MALDI) mass spectrometry on a Voyager-DE RP workstation (Applied Biosystems, Darmstadt, Germany) and by analytical reversed-phase HPLC on a Vydac RP18 column (4.6 mm × 250 mm, 5 μm, 300 Å) using two different linear gradients of 10−60% and 20−50% B in A over 30 min and a flow rate of 0.6 mL/min. N$^\alpha$-Nvoc-[K^{35}-Nvoc]-hαCGRP 8−37 was additionally analyzed by ESI-MS on an API 3000 PE Sciex (Toronto, Canada). Found masses were in full agreement with calculated masses, and purity of all peptides was >95% according to analytical HPLC.

Completeness of oxidation was investigated by MALDI mass spectrometry after incubation with *N*-(3-maleimidopropionyl)-biocytin (MPB). MBP (5 μg) was dissolved in 70 μL of water/*tert*-butyl alcohol (1:3, v/v) and subsequently added to 5 μg of reduced and oxidized CGRP, respectively,. After 2 h of shaking at room temperature, the product was analyzed by MALDI mass spectrometry.

Radioactive Labeling. For radioactive labeling, 100 μL of a solution of *N*-succinimidyl-[2,3-^{3}H]-propionate (1 nmol) in toluene with a radioactive concentration of 37 MBq/mL and a specific activity of 3.15−3.59 TBq/mmol was used. The toluene was removed with a gentle nitrogen stream, and 10 μL of N$^\alpha$-Nvoc-[K^{35}-Nvoc]-hαCGRP 8−37 (2 nmol dissolved in water/ACN/boric acid (pH 8, 50 mM) in the ratio 5/3/2) was added. The reaction was terminated after 2 h by addition of 10 μL of 40% B in A (A = 0.1% TFA in water; B = 0.08% TFA in acetonitrile). Then the Nvoc group was removed by irradiation with UV light (Atlas Fluotest forte, 366 nm, 180 W) for 1 h at 4 °C. The selectively radiolabeled and fully deprotected peptide was purified by HPLC on an Impaq RP18 column (4.6 mm × 250 mm, 5 μm, 300 Å) using a linear gradient of 20−50% B in A over 45 min and a flow rate of 0.6 mL/min. Ten microliter aliquots were taken from

fractions collected every minute, mixed with scintillation cocktail, and measured by β-counting.

Fractions corresponding to the desired product according to the retention time of the nonradioactive propionylated reference and the radioactivity profile were pooled. Then solvents were removed in a vacuum. For storage at 4 °C, 60 μL of a buffer of 33% ACN in triethylammonium phosphate (pH 3.4, 0.15 mm) was added. Specific activity was 15.5 ± 10.5 GBq/mmol.

Circular Dichroism Spectroscopy. Circular dichroism spectra were measured over 250−190 nm on a JASCO J715 spectropolarimeter at 20 °C in nitrogen atmosphere. The peptides were dissolved in 2,2,2-trifluoroethanol/phosphate buffer (pH 7.4, 10 mM) in the ratio 3/7 at concentrations of 0.04 mM. The concentrations of the CGRP analogues were determined by measuring the absorption at 209 nm ($\epsilon = 120$ mol cm^{-1} L^{-1}). All measurements were performed three times using a sample cell with path length of 2 mm. Response time was set to 2 s at a scan speed of 20 nm/min, a sensitivity range of 10 mdeg, a bandwidth of 2 nm, and a step resolution of 0.1 nm. High-frequency noise was reduced by means of a low-path Fourier transform filter. The circular dichroism spectrum of the pure solvent was subtracted from the peptide solution to eliminate interference from cell, solvent, and optical equipment.

Cell Culture. Human neuroblastoma SK-N-MC cells were purchased from ATTC and cultured in DMEM supplemented with 10% (v/v) fetal calf serum, 4 mM glutamine, 1 mM sodium pyruvate, and 0.2 mM nonessential amino acids. Cells were grown to confluence in 75 cm^2 culture flasks (Techno Plastic-Products AG, Trasadingen, Switzerland) at 37 °C in a humidified atmosphere (95%) containing 5% CO_2. For subculture and biological assays, the cells were washed twice with PBS (Gibco Life Technologies), treated with 3 mL of PBS−EDTA for 3 min, and detached after removal of the PBS−EDTA solution.

Determination of Binding Affinity. The cells were resuspended in 10 mL of assay buffer (50 mM TRIS (pH 7.4), 100 mM sodium chloride, 5 mM magnesium chloride, 50 μM Pefabloc SC, and 1% BSA), isolated by centrifugation (450 × *g*, 5 min), and adjusted with assay buffer to a concentration of 3 million cells/mL. A volume of 200 μL of the cell suspension was incubated at room temperature for 90 min with 25 μL of [^{3}H-propionyl-K^{24}]-CGRP 8−37 and 25 μL of displacing compound. The incubation was terminated by centrifugation at 2000 × *g* at 4 °C for 5 min. Cell pellets were then washed once with 400 μL of cold assay buffer, centrifuged, resuspended in 100 μL of assay buffer, and mixed with scintillation cocktail. The receptor-bound radioactivity was determined by using a β-counter (Wallac). For saturation analysis, increasing concentrations of radioligand (0.15−4 nM) were used. The nonspecific binding was determined as radioactivity in the presence of 1 μM unlabeled CGRP 8−37 and the specific binding in the presence of 1% BSA in water (w/v).

For competitive binding experiments, a single concentration of the radioligand (1 nM) was displaced by increasing concentrations of the investigated peptide (10^{-5}−10^{-14} M). The nonspecific binding was determined as radioactivity in the presence of 1% BSA in water (w/v).

All experiments were performed independently three times as triplicate and analyzed by nonlinear regression using the Prism program (GraphPad software Inc., San Diego, CA) to calculate the dissociation constant (K_D) and the IC_{50} values. The mean of IC_{50} values (expressed in terms of molarity) were converted to K_i values by using the equation of Cheng and Prusoff: $K_i = IC_{50}/(1 + ([R]/K_D))$,[47] where [R] is the molar concentration of the radioligand. The results are given in Tables 2 and 3 as mean ± SEM.

Measurement of cAMP. The detached cells were washed twice with wash buffer (Hank's supplemented with 20 mM HEPES, 5.5 mM glucose, pH 7.4) and adjusted with incubation buffer (wash buffer supplemented with 1 mM IBMX, 1% BSA) to a concentration of about 2 million cells/mL. After preincubation of 250 μL at 37 °C for 15 min, 10 μL of increasing concentrations (10^{-5}−10^{-12} M) of agonist alone and in the presence of 10 μL of a fixed concentration of antagonist solution was added to continue the incubation for a further 15 min. Intracellular cAMP was then extracted by the addition of 20 μL of 1 M HCl and centrifugation (2000 × *g* at 4 °C for 15 min). An aliquot (200 μL) of the supernatant was lyophilized and subsequently dissolved with 250 μL of detection buffer (5 mM HEPES, 0.3% TWEEN-20, 0.1% BSA). The cAMP contents were determined according to the instructions of the commercial AlphaScreen technology (PerkinElmer, Italy, Monza).

The cAMP accumulation assays were performed as three independent experiments in triplicate. Concentration−response curves were obtained by subtraction of the cAMP concentration measured in the presence of ligand from those obtained in absence of ligand (basal activity). The data were analyzed by nonlinear regression using the Prism program to obtain EC_{50} values of the agonist in the presence (10 or 100 nM) and absence of antagonists. Apparent pA_2 values were calculated by using the equation pA_2 = log((CR − 1)/[B]), where [B] is the molar concentration of the antagonist and CR is the concentration ratio of the EC_{50} values in the presence and absence of the antagonist. The resulting values are given in Table 4 as mean ± SEM.

Theoretical Calculations. The calculations of the β-turns of the blocked dipeptides in Table 5 were performed employing the Gaussian 03 program package (Gaussian, Inc., Pittsburgh, PA). Starting from idealized turn geometries,[38,39] the geometry optimizations were realized at the HF/6-31G* level of ab initio MO theory. In the same way, the maintenance of the polyproline II helix angles was examined on blocked tetrapeptides varying the position $i + 1$. The turn-like structures of the β-ACC-Ala dipeptides are based on a conformational search for hydrogen-bonded secondary structures in this sequence by a systematic variation of the torsion angles, selection of potential candidates on the basis of general geometry criteria of hydrogen bonds, and reoptimization.

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