ELSEVIER

Regulatory Peptides 79 (1999) 153–158

REGULATORY PEPTIDES

CGRP 27–37 analogues with high affinity to the $CGRP_1$ receptor show antagonistic properties in a rat blood flow assay

Beate Rist[a], J. Silvain Lacroix[b], Michael Entzeroth[c], Henry N. Doods[c], Annette G. Beck-Sickinger[a,*]

[a]*Department of Pharmacy, ETH Zürich, Winterthurer Str. 190, CH 8057 Zürich, Switzerland*
[b]*Department of Otorhinolaryngology, Hopital Cantonal Universitaire de Geneve, Geneva, Switzerland*
[c]*Department of Biochemical Research, Dr. Karl Thomae GmbH, Biberach, Germany*

Received 5 May 1998; received in revised form 6 November 1998; accepted 6 November 1998

Abstract

CGRP Y^0-28–37 is known as a selective $CGRP_1$ receptor antagonist. We succeeded in optimising the $CGRP_1$ receptor affinity of this fragment by multiple amino acid replacement. The analogues [P^{34}, F^{35}]CGRP 27–37 and [D^{31}, P^{34}, F^{35}]CGRP 27–37 exhibit a 100-fold increased affinity compared to the unmodified segment. Receptor binding studies were performed with human neuroblastoma cells SK-N-MC, which selectively express the $hCGRP_1$ receptor. Blood flow, which is increased by exogenous CGRP, was measured in the right femoral artery. Preincubation of the rats with [P^{34}, F^{35}]CGRP 27–37 and [D^{31}, P^{34}, F^{35}]CGRP 27–37 led to a significant decrease in CGRP induced increase in vascular conductance indicating the antagonistic properties of these compounds. Interestingly, an exchange of the amino acid Asn^{31} to Asp^{31} in [P^{34}, F^{35}]CGRP 27–37 shortened the period of the antagonistic effect significantly, suggestive of a different rate of metabolism for the two ligands. Secondary structure investigations obtained by circular dichroism measurements revealed that an increase in ordered structure correlates with high binding affinity.

Keywords: $CGRP_1$ receptor antagonist; Neuroblastoma cells; Vascular conductance; Circular dichroism; Synthetic peptides; Peptide mimetics

1. 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 [1,2]. In contrast to calcitonin 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]. In fact, CGRP is co-localised 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 dilatation, an increase in heart rate, regulation of calcium metabolism and 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 $CGRP_2$) [6,7] which are both G-protein coupled. The $hCGRP_1$ receptor has been cloned recently [8].

Structure–activity studies of reduced size CGRP analogues showed that the C- and N-terminal region of the hormone interact independently with their receptors. Since we were interested in antagonistic ligands at the $hCGRP_1$ receptor, we focused on the optimisation of the structurally poorly defined C-terminal part of the hormone. The segment CGRP Y^0-28–37 which is reported as a selective $CGRP_1$ receptor antagonist [9,10], however with low

*Corresponding author. Tel.: +41-1-635-6063; fax: +41-1-635-6884; e-mail: beck-sickinger@pharma.ethz.ch

affinity. No activity has been found up to the micromolar concentration [11] which is in agreement with our results. In order to improve binding affinity, multiple amino acid replacement within this segment has been performed [12] and resulted in a series of high affinity ligands, including [P^{34}, F^{35}]CGRP 27–37 and [D^{31}, P^{34}, F^{35}]CGRP 27–37. Within the present study we could confirm the suggested antagonistic properties of C-terminal analogues of CGRP Y^0-28–37 using a rat in vivo assay. Furthermore, we were able to identify a portion of the peptide sequence which appears to be sensitive to proteolytic cleavage but does not affect receptor binding affinity.

Circular dichroism studies have been performed in order to elucidate the difference in secondary structure between peptidic ligands exhibiting poor and high receptor binding affinities, respectively.

2. Methods

2.1. Peptide synthesis

The linear peptides were synthesised 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. 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-hydroxy-benzotriazole activation, sevenfold excess and a coupling time of 40 min.

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

The peptides were purified to homogeneity higher than 94% by reversed-phase HPLC on a nucleosil C-18 column, 6 μm, 25 × 300 mm (Waters), using acetonitrile/water mixed with 0.1% trifluoroacetic acid as eluent. Isocratic conditions between 20–30% acetonitrile were applied, depending on the polarity of the peptide. Analysis of the peptides was performed on a nucleosil C-18 column, 5 μm, 3 × 125 mm (Merck-Hitachi) by a gradient of 15–60% acetonitrile over 30 min.

Correct mass was identified by ion-spray mass spectrometry (API III, Sciex, Toronto).

2.2. Circular dichroism

Circular dichroism spectra were measured over 250–190 nm on a Jasco J720 spectrapolarimeter at room temperature in nitrogen atmosphere. The peptides were dissolved in 2,2,2-trifluoroethanol/phosphate buffer (pH = 7, 10 mM) in the ratio 3/7, at concentrations of 0.20–0.22 mM. All measurements were performed three times using a sample cell with path length of 0.2 mm. Response time was set to 2 s at a scan speed of 20 nm/min, a sensitivity range of 10 mdeg, a band width of 2.0 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 spectra of pure solvents were subtracted from the peptide solution to eliminate interference from cell, solvent and optical equipment. The circular dichroism data are given as mean residue ellipticities $[\Theta]_m$.

2.3. 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, supplemented with 0.02% EDTA. Resuspended in 20 ml 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 homogenised 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.

2.4. 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. Then, 230 μl of the homogenate were 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 polyethylene imine (0.1%)-treated GF/B glass fibre filters using a cell harvester. The protein-bound radioactivity is determined in a gamma-counter. The non-specific 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]iodohistidyl-calcitonin gene-related peptide of three separate experiments each performed in triplicate is given as the K_i value (Table 1) ±S.E.M. (Fig. 1). Data analysis was performed with GraphPad.

2.5. Blood flow

Experiments were carried out in adult female Wistar rats weighing between 250 and 280 g anaesthetized with sodium pentobarbitone (Nembutal, Boehringer-Ingelheim; 60 mg/kg, intraperitoneal). Intravenous supplements of pentobarbitone were given to maintain anaesthesia. The trachea was cannulated and connected to a positive pressure rodent ventilator (Ugo Basile 6025). The left femoral artery was cannulated for continuous recording of arterial blood pressure via a Gould–Statham physiological pressure transducer (P23XL) which was connected to one channel of a pen recorder (Graphtec WR7500). Temperature was continuously monitored via a rectal probe (Digitron Model 1808) and kept in the range 36±1°C. The electrocardiogram was recorded through subcutaneous needle electrodes and displayed on a storage oscilloscope. The electrocardiogram was used to obtain beat-by-beat pulse interval (PI) after processing with Neurolog modules (Digitimer, UK, NL200,304,600). Triggering was checked with a counter. PI was preferred to heart rate because of the linear relation between PI and frequency of vagal stimulation. Both vagus nerves were cut high in the neck. This was done to eliminate vagally-mediated reflex effects on the heart which occur when blood pressure is decreased by CGRP. The left femoral vein was cannulated for administration of CGRP and analogues of CGRP, as well as further doses of anaesthetic. The right femoral artery was exposed and separated from the right femoral vein and saphenous nerve, and the perivascular portion of the flow probe was placed around the femoral artery. Blood flow was measured in the right femoral artery using a perivascular flow probe (Model 0.5VB152 Transonic systems). The cavity created for the probe was filled with K-Y lubricating jelly (Johnson & Johnson) to provide acoustic coupling to the probe. The flowprobe was connected to an ultrasonic volume flowmeter (Model T106 Transonic Systems) which in turn was connected to a polygraph (Grass Model 79) for data analysis.

Doses of CGRP (0.5 nmol/kg) were administered intravenously to obtain a control response. This dose was selected because it decreased the resting blood pressure by an average 40 mmHg. When controls were obtained a bolus injection of the CGRP analogue, 0.17 μmol/kg or 0.068 μmol/kg was administered followed by a dose of CGRP within 10 min. Additional doses were given if the response to CGRP was attenuated. Experiments were performed in quadruplicate and data are shown±S.E.M. (Fig. 3). Data were analyzed using Student's *t*-test and $P < 0.05$ was found for significant values.

2.6. 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, 2,2,2-trifluoroethanol, 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 albumin, bacitracin were purchased from Sigma, polyethylenimine from Serva, 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]Iodohistidyl-calcitonin gene related peptide was ordered from Amersham.

3. Results

3.1. Receptor binding studies

The peptides that are listed in Table 1 have been obtained by multiple automatic solid phase peptide synthesis. They have been characterised by analytical HPLC and electrospray mass spectrometry. $CGRP_1$ receptor affinity was investigated at human neuroblastoma cells SK-N-MC (Fig. 1). The most potent peptidic compounds are derived from CGRP 27–37 and [D^{31}]CGRP 27–37 which themselves only have a minor potency (K_i 728 and 2390 nM, respectively). The introduction of a proline in position 34 (no. 2, 5) leads in both segments to a 6- to 10-fold increased affinity. Within these modified segments, the replacement of Glu in position 35 by Phe (no. 3, 6) furthermore improves binding by one order of magnitude, thus leading to analogues with K_i values of 19 and 14 nM.

3.2. Circular dichroism investigations

In Fig. 2a the circular dichroism spectra of the ligands with low affinity (no. 1, 4) are compared with those in which proline in position 34 was introduced (no. 2, 5). The secondary structure in solution does not seem to be significantly affected by the exchange of S^{34} to P^{34}, because the circular dichroism spectra of CGRP 27–37 and [D^{31}]CGRP 27–37 are very similar to the ones of [P^{34}]CGRP 27–37 and [D^{31}, P^{34}]CGRP 27–37, respective-

Table 1
Sequences of CGRP 27–37 analogs and their affinity to the $CGRP_1$ receptor, investigated on human neuroblastoma SK-N-MC cells

No.	Peptide	Sequence	$hCGRP_1$ K_i (nM)
1	CGRP 27–37	FVPTNVGSEAF	728
2	[P^{34}]CGRP 27–37	FVPTNVGPEAF	123
3	[P^{34}, F^{35}]CGRP 27–37	FVPTNVGPFAF	19
4	[D^{31}]CGRP 27–37	FVPTDVGSEAF	2390
5	[D^{31}, P^{34}]CGRP 27–37	FVPTDVGPEAF	226
6	[D^{31}, P^{34}, F^{35}]CGRP 27–37	FVPTDVGPFAF	14

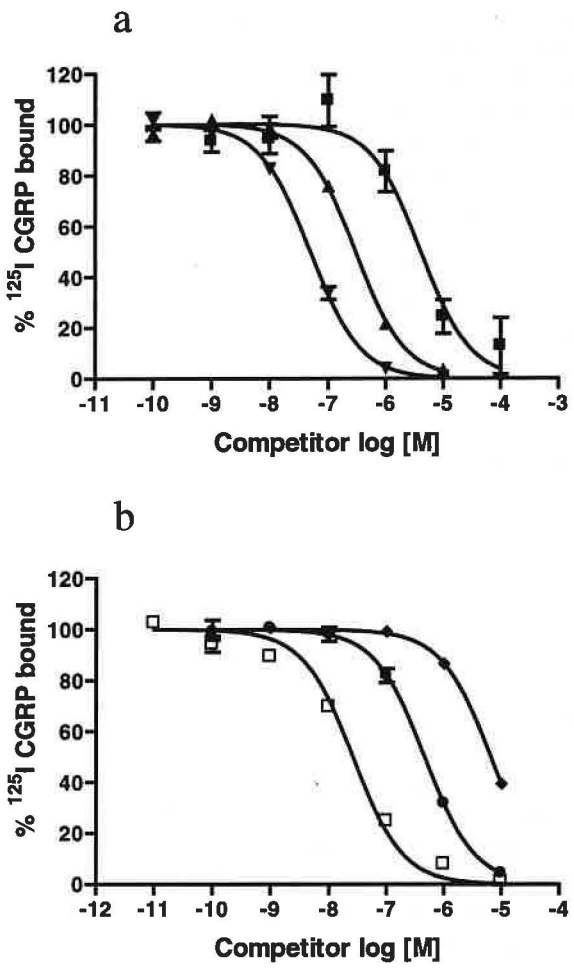

Fig. 1. Inhibition of specific [^{125}I]CGRP binding to SK-N-MC cell membranes by peptide no. 1 (■), no. 2 (▲), no. 3 (▼) (a) and by peptide no. 4 (♦), no. 5 (○) and no. 6 (□) (b) as described in Section 2. Data represent the mean±S.E.M. of three determinations.

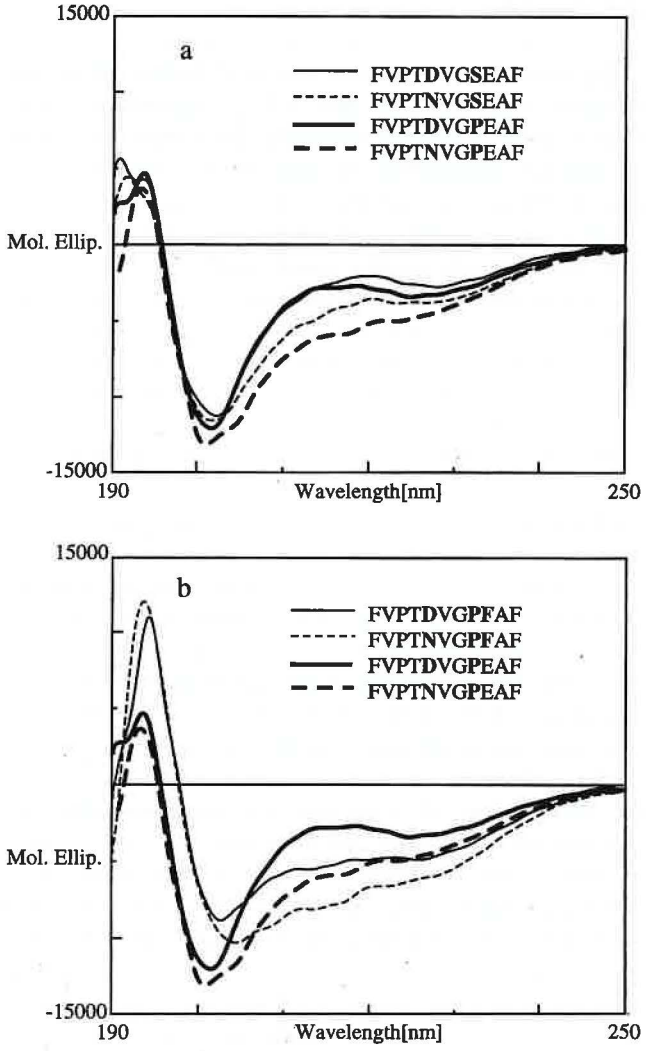

Fig. 2. Comparative circular dichroism spectra of the CGRP 27–37 analogs.

ly. Therefore we conclude that the proline in position 34 does not induce an unusual three dimensional arrangement, moreover it serves to stabilise a turn which is already present in the unmodified segment.

Fig. 2b shows the effect on secondary structure of the [P^{34}] peptides, when Glu in position 35 is replaced by Phe, additionally. In this case, a significant effect on the secondary structure could be observed, which is indicated by a change of the symmetry of the obtained curves. The characteristic wavelength at which the molar ellipticity of zero is detected, has shifted to a higher wavelength in the [P^{34}, F^{35}] peptides compared to [P^{34}] analogues. Furthermore, a shift of the maximum and minimum of the circular dichroism spectra has been detected. The intensity of the positive Cotton effect in the range of 192–196 nm is increased considerably. [P^{34}, F^{35}]- and [D^{31}, P^{34}, F^{35}]CGRP 27–37 exhibit the most ordered structures within the whole set of peptides. The increase in binding affinity which is gained by the introduction of Phe in position 35 correlates with a particular change in secondary structure.

3.3. Blood flow measurements

In order to investigate the functional role of the CGRP segments, the changes in vascular conductance in the femoral artery (Vcfa) of rat have been measured. A dose of CGRP which decreased the resting blood pressure by 40 mmHg was applied. CGRP led to an increase in Vcfa of 85 to 90% compared to control animals without CGRP administration (Fig. 3a and b, grey bars). The effect of CGRP is reduced to less than 20% increase in Vcfa after 8 min. For testing the CGRP analogues, the peptides were pre-injected to the application of CGRP. The increase of Vcfa was significantly reduced compared to the effect of CGRP without pre-injection of the peptides (65% in the case of 170 nmol/kg [D^{31}, P^{34}, F^{35}]CGRP 27–37, 58% in

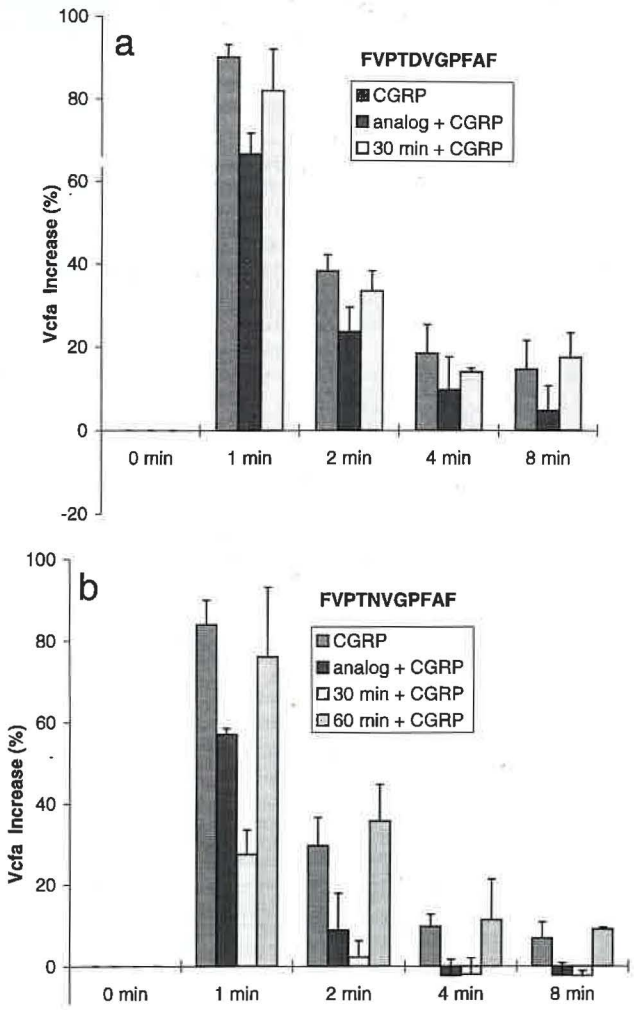

Fig. 3. Effects of i.v. injection of endogenous CGRP (0.5 nmol/kg) on the vascular conductance in the femoral artery (Vcfa) of the rat in vivo before and after i.v. pretreatment with (a) [D^{31}, P^{34}, F^{35}]CGRP 27–37 (170 nmol/kg) and (b) [P^{34}, F^{35}]CGRP 27–37 (170 nmol/kg).

the case of 170 nmol/kg [P^{34}, F^{35}]CGRP 27–37, compared to 90% and 85%, respectively). At a dose of 68 nmol/kg the effect was less pronounced but still significant. Even after 8 min, there was still a difference between the pre-injection of CGRP segments and without peptides (Fig. 3, dark bars). After 30 min CGRP was injected again. In the case of [D^{31}, P^{34}, F^{35}]CGRP 27–37 no significant difference neither in immediate Vcfa increase nor after 8 min was found compared to the application of CGRP without pre-injection of the CGRP segment (Fig. 3a, white bars). However, in the case of [P^{34}, F^{35}]CGRP 27–37, the blocking effect of the CGRP segment was further increased after 30 min to an increase of Vcfa to only 33% (Fig. 3b white bars) (compared to 85%). Only a third application of CGRP after 60 min showed the same Vcfa increase as before the injections of the CGRP segment (Fig. 3b light grey bars). Injection of the CGRP segments [D^{31}, P^{34}, F^{35}]CGRP 27–37 and [P^{34}, F^{35}]CGRP 27–37 showed no change in Vcfa per se.

4. Discussion and conclusion

Comparing the corresponding analogues [P^{34}, F^{35}]CGRP 27–37 and [D^{31}, P^{34}, F^{35}]CGRP 27–37, which only differ in position 31, it became evident that Asp^{31} instead of Asn^{31} has no significant effect on both receptor recognition and secondary structure. In contrast, however, significant differences were found in the rat blood pressure in vivo assays. Both analogues show no agonistic effect per se, but inhibit the effect of exogenous CGRP. Thus, both peptides act as antagonists. However, although the two peptides do not distinguish in their primarily antagonistic effect at the tested concentrations (estimated pA_2 values were 6.60 and 6.64, respectively), they show significant differences in their half life.

Whereas the inhibition of CGRP by [D^{31}, P^{34}, F^{35}]CGRP 27–37 lasts for maximal 30 min, [P^{34}, F^{35}]CGRP 27–37 shows a strong effect up to an hour. Further investigations revealed that the extent as well as the duration of the effect which is caused by [D^{31}, P^{34}, F^{35}]CGRP 27–37 is very similar to that of the known antagonist CGRP 8–37 [13–15], whereas for [P^{34}, F^{35}]CGRP 27–37 the duration is at least twofold increased.

Differences in the noncompetitive, irreversible receptor binding of the two analogues could be speculated, however no differences in the background of the binding assays were found.

Since both ligands exhibit receptor binding affinities in the same range (K_i = 19/14 nM), we suggested that the analogue with Asp^{31} is faster metabolised, perhaps because of an increased susceptibility for proteases. This effect could be confirmed by preliminary results with other analogues (no. 2, 5) containing either Asp or Asn in position 31. In this case as well, the effect of the Asp-containing peptides was significantly increased.

Thus we conclude that circular dichroism measurements allow discrimination between the analogues which exhibit high receptor affinity (no. 3, 6) and those with low binding affinity (no. 1, 4). We could show that binding of the analogues CGRP 27–37 and [D^{31}]CGRP 27–37 is very sensitive to changes in position 34, 35. Asp^{31} instead of Asn^{31} does not effect affinity, but has major effect upon the half life of the analogues in vivo. Asn^{31} seems to be necessary to prevent rapid degradation of [P^{34}, F^{35}]CGRP 27–37 and may therefore result in longer lasting antagonistic effects.

Acknowledgements

The present study was supported by grants from the

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.