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CONSTRAINED ANALOGUES OF THE CALCITONIN GENE-RELATED PEPTIDE

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Summary: The calcitonin gene-related peptide, CGRP, has potential medicinal use for instance as a vasodilator or in the regulation of bone metabolism. In this study new analogues of CGRP based on molecular modelling of active fragments were synthesised and tested. The analogues were found to have affinities for the receptor comparable to those seen for native CGRP. Two analogues were found to be agonists. The analogues give an insight to the bioactive conformation of CGRP as they were constrained by disulphide bridges. © 1994 Academic Press, Inc.

The calcitonin gene-related peptide (CGRP) is a 37 residue peptide hormone produced by alternative splicing of the calcitonin mRNA (1) first isolated from a human medullar thyroid carcinoma (2). Physiological actions of CGRP, especially vasodilatation (3) and effects on bone metabolism (4), have motivated a search for analogues of this peptide for possible medicinal use.

Structure-activity studies suggest that CGRP contains two regions interacting independently with the receptor (5). The C-terminal region CGRP28-37 was found to bind to the receptor without activating it and CGRP8-37 is a high affinity antagonist at the CGRP receptors (6). The N-terminal fragments CGRP1-12, CGRP1-15 and CGRP1-22 induce the vasodilatation characteristic for CGRP although at higher concentrations (7). These observations lead to the possibility that agonists and antagonists of this hormone may be obtained by optimising the N- and C-terminal structures, respectively. In our previous work

Abbreviations: CGRP - calcitonin gene-related peptide, IBMX - 3-isobutyl-1-methyl-xanthine.

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sets of conformations for CGRP1-20 and CGRP30-37 were modelled in order to facilitate the design of new analogues of the peptide (Hakala and Vihinen, in preparation). In the present study our aim was to design, synthesise and test analogues of CGRP favouring some of the modelled conformations. This was achieved by constraining the analogues by disulphide bridges and by deleting residues from the N-terminal loop region. These modifications lead to two agonists reduced in size and with affinities comparable to the native sequence. Also modelling fragments of the new agonists shed light on the bioactive conformations of CGRP.

Materials and methods

Reagents: Fmoc-amino acid derivatives were purchased from DuPont de Nemours (Deutschland) GmbH, Germany and Bachem AG, Switzerland and 2,4-dimethoxybenzhydrylamine resin (RapidAmide resin) from Du Pont de Nemours. Human (2-[^{125}I]iodohistidyl^{10}) CGRP, specific activity 2000 Ci/mmol, [α-^{32}P]ATP and [8-^{3}H]cAMP were from Amersham International, England. Unlabelled rat and human CGRP were obtained from Peninsula Laboratories, Inc., Belmont, California. Bacitracin, aprotinin, GTP, ATP, cAMP and IBMX were from Sigma Chemical Company, St Louis.

Molecular modelling: The software package Sybyl (Tripos Ass, Saint Louis) was run on Evans & Sutherland ESV30 and Silicon Graphics Iris Crimson workstations. The Kollman united- atom force field (8) and a distance dependent dielectric constant were used for all calculations. All structures were minimised to an energy gradient of 0.050 kcal mol^{-1} Å^{-1} using the steepest descent, conjugate gradient and Powel minimisation algorithms. Two conformations modelled previously for the fragments CGRP1-20 and CGRP30-37 (Hakala and Vihinen, in preparation) were used for the modelling of VH193 and the C-terminal ends of the analogues SH992 and SH1092, respectively. For the creation of the N-terminal analogue ordered helical structures were chosen. For the C-terminal analogues two low energy conformations containing turns Asn-31-Ser-34 β I and Val-32-Lys-35 β II' were chosen. The structures were mutated to the corresponding analogues and minimised. Both N-terminal analogues were subjected to 200 ps and the four C-terminal analogues to 100 ps of molecular dynamics. The simulations were run at 300 K with a 1 fs time step. The structures were analysed for hydrogen bonds indicating helixes and turns.

Peptide synthesis: Synthesis of CGRP fragments and analogues exept P70 and P79, were carried out by solid-phase methods (9,10) using Fmoc/tBu chemistry on a multiple peptide synthesis system (RaMPS, DuPont). Peptides were de protected and cleaved from the resin with 95 % TFA / 4.5 % aq. phenol / 0.5 % 1,2-ethanedithiol. Deprotection of Cys(tBu) and formation of the intramolecular disulphide bond was accomplished in one step by treating the linear peptide with methyltrichlorosilane in the presence of diphenylsulfoxide and anisole (11). The crude cyclisised peptide was subjected to gel filtration on Sephadex G-25 using 20 % acetic acid as the eluent. Fractions containing the peptide were combined and lyophilised. Peptides P70 and P79 were synthesised using an automated peptide synthesiser (Model 431A, Applied Biosystems, San Diego, CA) and Fmoc-chemistry. The purification was carried out by by preparative reversed phase HPLC. The purity of all peptides was checked by gradient reversed phase HPLC using a µBondpack C-18 column (particle size 10 µ, 3.9 * 300 mm). Solvents A: 0.1 % TFA in acetonitrile/water (10/90), and B: 0.1 % TFA in acetonitrile/water (40/60) were used for the gradient.

Binding assay: The rabbit lung tissue was homogenised by a glass-glass homogeniser in 20 vol. of ice-cold buffer: 10 mM Tris-HCl, pH 7.4, 0.32 M sucrose, and centrifuged at 1000 g for 10 minutes at +4°C. The supernatant was further centrifuged at 48000 g for 30 minutes and the resulting pellet was washed by resuspension and centrifugation. The membranes were

stored at -80 °C until used. In the assay 0.4 mg (w/w.) of membranes were incubated in the incubation buffer: 50 mM Tris-HCl, pH 7.4, 1% BSA, 0.1 mg/ml bacitrasin and 0.05 mg/ml aprotinin, with the test substances or with 0.5 μM rat CGRP (non-specific binding) for 10 min on ice. Then radiolabeled human CGRP was added (final conc. 0.08 nM). The incubation was finished after 30 minutes (at +37 °C) or 2 hours (on ice) by centrifugation for 3 minutes at 11500 g at +4 °C. The pellets were counted with a Wallac Compugamma counter and analysed by the nonlinear least squares curve-fitting program Ligand.

Adenylate cyclase activation: The adenylate cyclase activation was measured using a modified method based on Salomon (12,13). In this method the formation of [^{32}P]cAMP from [α-^{32}P]ATP is measured. The rabbit lung tissue was homogenised in 2 mM Tris-maleate buffer, 2mM EDTA, pH 7.4. 1 mg lung tissue / tube was used. 80 mM Tris-HCl buffer, pH 7.4, 2 mM $MgSO_4$ and 0.2 mM EDTA, was used as an incubation buffer. GTP and IBMX (final concentrations 0.1 mM and 0.24 mM, respectively), a possible agonist and tissue homogenate were added to the incubation buffer. The reaction was started by adding 1 μCi [^{32}P]ATP and ATP (final concentration 1 mM) and was continued for 10 minutes at 37 °C. The assay was stopped by adding 15% TCA with 0.5 mM cAMP. [^{3}H]cAMP (approximately 5000 cpm) was added, centrifuged at 11500 g for 3 minutes and the supernatants were removed and cAMP was separated chromatographically and measured by a liquid scintillation counter.

Results and discussion

The structures and affinities of the synthesised analogues and their fragments are given in Table 1. The affinity of the analogue SH892 containing the constrained disulphide loop in the N-terminal region was of the same order as that of CGRP. The N-terminal fragment VH193 of this molecule had also about the same affinity as CGRP1-18. The shorter analogue SH992 containing a disulphide bridge in the C-terminal region had an affinity one order of magnitude less than CGRP. This was however one order of magnitude better than that found for the analogue P79 which did not contain any cysteines and SH1092 which had a different disulphide bridge. Figure 1. shows the activation of adenylate cyclase of the analogues SH892 and SH992 compared to that of CGRP. No adenylate cyclase activation was found with SH1092 possibly because of the lower affinity. This was also true for P79 which did not contain any disulphide bridge.

Molecular modelling suggested that the removal of Ala-1 and Asn-3 would stabilise the helical conformation of the N-terminal part of the molecule. This was seen as a stable

Table 1. The structures and affinities of analogues, fragments and the whole CGRP

Name	Structure	IC_{50} (nM)	
		4°C	37°C
hCGRP-α	ACNTATCVTHRLAGLLSRSGGVVKNNFVPTNVGSKAF	1.4	0.6
SH892	CVATCVTHRLAGLLSRSGGVVKNNFVPTNVGSKAF	1.3	1.1
P79	TPPTHRLAGLLSRSGGVVKNNFVPTNVGSKAF	37	370
SH992	TPPTHRLAGLLSRSGGVVKNNFVPTCVGSKCF	3.5	6.7
SH1092	TPPTHRLAGLLSRSGGVVKNNFVPCNVGSCAF	140	290
CGRP1-18	ACNTATCVTHRLAGLLSR	780	3400
VH193	CTATCVTHRLAGLLSR	1800	~10000
P70	TPPTHRLAGLLSR	>10000	>10000

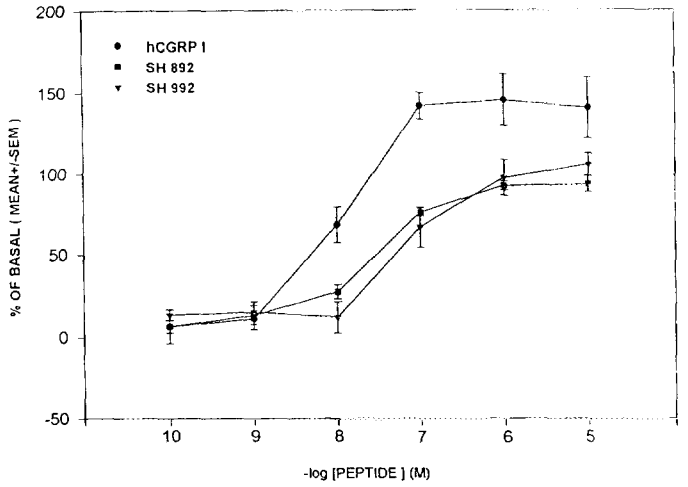

Fig. 1. The effects of CGRP and CGRP analogues SH892 and SH992 on adenylate cyclase activity in rabbit lung tissue. The stimulation is expressed as percent of basal level. Each point indicates the mean and SEM of at least three experiments.

ordered α-helix during the simulation. The stabilising effect was due to the constrained conformation of the loop region which favoured a hydrogen bond between Ala-5 inside the loop and Thr-9 in the helical region (Table 2.). It has been shown by circular dichroism spectroscopy that the loop region is essential for the stability of the helical region (14). The importance of hydrogen bonds between the loop and the helical region was also suggested by our previous molecular modelling results. It may be concluded that Ala-1 and Asn-3 is not needed for the specific binding of the activating end of CGRP to the receptor and that this fragment is able to obtain the bioactive conformation needed for receptor activation. As this compound contains only three residues inside the disulphide loop there is a more stringent limitation of conformations for this compound than for CGRP. A model obtained by 200 ps molecular dynamics followed by minimisations is shown in figure 2.

The two disulphide bridges in the C-terminal binding domain were designed to favour two different β-turns indicated by our previous modelling results. The bridges Cys-31, Cys-36 in SH992 and Cys-30, Cys-35 in SH1092 were designed to stabilise βII´ and βI turns with Gly-33 in the position i+1 and i+2, respectively. These conformations had previously been

Table 2. Hydrogen bonding pattern of the models of VH193

	Time	Energy	Residue										
	ps	kcal/mol	V-8	T-9	H-10	R-11	L-12	A-13	G-14	L-15	L-16	S-17	R-18
Model 1	0	-113.2	T-4	A-5	A-5	C-7	C-7	V-8	T-9	R-11	L-12	A-12	L-15
	100	-123.0	T-4	A-5	T-6	C-7	V-8	T-9	T-9	R-11	L-12	L-16	G-14
	200	-121.0	T-4	A-5	T-6	C-7	V-8	T-9	H-10	R-11	L-12	L-16	G-14
Model 2	0	-101.3	T-4	T-4	T-6	-	V-8	T-9	H-10	R-11	L-12	A-13	G-14
	100	-117.6	A-5	A-5	T-6	C-7	V-8	T-9	H-10	R-11	L-12	A-13	G-14
	200	-120.3	A-5	A-5	T-6	C-7	V-8	T-9	H-10	R-11	L-12	A-13	G-14

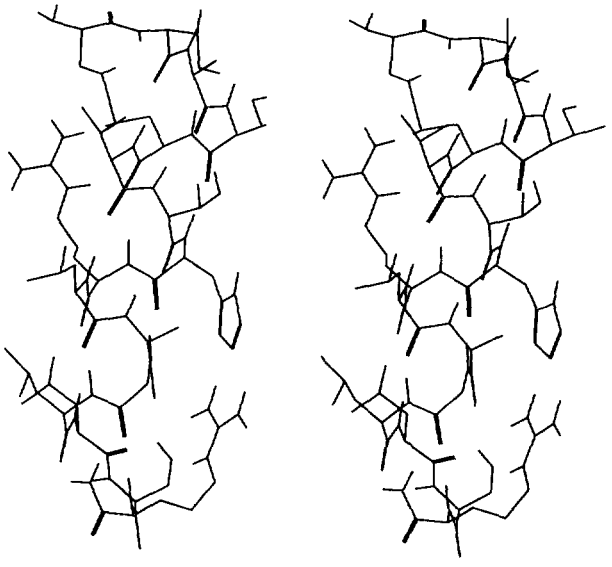

Fig 2. Stereo draving of the conformation of VH193 obtained by 200 ps of molecular dynamics followed by minimisation.

suggested to be possible bioactive conformations for the fragment CGRP30-37 (Hakala and Vihinen, in preparation). The conformations obtained by molecular dynamics simulations (Table 3.) shows that a βII′ turn between valine and lysine is a stable low energy conformation of the C-terminal fragment of SH992. The model 1A obtained by minimisation after 100 ps of molecular dynamics is shown in Figure 3A. The low energy conformation 3B obtained for SH1092 is shown in Figure 2B. As SH992 has relatively good affinity for the receptor and is able to activate adenylate cyclase, the βII′ turn conformation favoured by the disulphide bridge may be the bioactive conformation for the C-terminal binding region. SH1092 is probably also able to adopt a receptor binding conformation although this may be energetically unfavourable as this analogue has an inferior affinity for the receptor. The change in the affinity due to the change in temperature is smaller in both SH992 and SH1092 constrained by a disulphide bridge than in the unconstrained P79. This may be due to the reduction in the entropy term due to the constraints.

Table 3. Energies and conformations for the models of the C-terminal fragments of SH992 (1A and 2A) and SH1092 (1B and 2B)

Model	Time	Energy	Conformation
1A	0	-62.3	Val-Lys, βII′
	100	-77.5	Val-Lys, βII′
1B	0	-71.8	Val-Lys, βII′
	100	-87.1	Val-Ser, γ
2A	0	-81.3	Val-Ser, inverse γ
	100	-75.2	Val-Ser, inverse γ
2B	0	-78.5	Asn-Ser, βI
	100	-89.4	Val-Ser, inverse γ

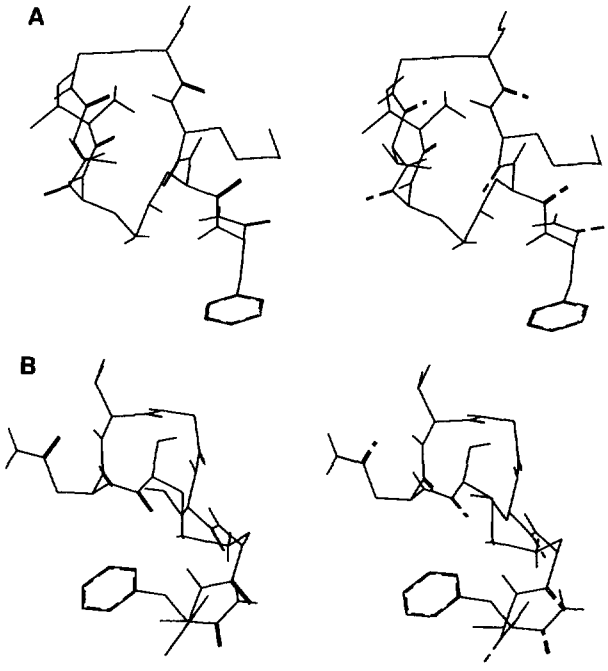

Fig. 3. The low energy conformations of the C-terminal sequences TCVGSKCF of SH992 (A) and CNVGSCAF of SH1092 (B) obtained by 100 ps molecular dynamics and minimisation.

The new analogues of CGRP are interesting as such as the reduced size and high affinity of the compounds is desirable, but they are more valuable as probes of the bioactive conformations of the peptide hormones. The insight obtained by design of constrained analogues may lead to the development of small molecule mimics for medicinal use.

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References

1. Amara, S.G., Jonas, V., Rosenfeld, M.G., Ong, E. S., and Evans, R. M. (1982) Nature 298:240-244
2. Morris, H.R., Panico, M., Etienne, T., Tippins, J., Girgis, S.I. and MacIntyre, I. (1984) Nature 308:746-748
3. Gennari, C. and Fischer, J.A. (1985). Calcif. Tissue Int. 37:581-584
4. D'Souza, S.M., MacIntyre, I., Girgis, S.I. and Mundy, G.R. (1986) Endocrinology 119:58-61
5. Watanabe, T., Takada, M., Hiroshima, O., and Tachibana, S. (1989) Peptide Chemistry 235-238 Ed. M. Uski. Protein research Foundation, Osaka
6. Maton, P.N., Pradhan, T., Zhou, Z.-C., Gardner, J.D., and Jensen, R.T. (1990) Peptides 11:485-489

7. Maggi, C.A., Rovero, P., Giuliani, S., Evangelista, S., Regoli, D., and Meli, A. (1990) Eur. J. Pharmacology 179:217-219
8 Weiner, S., Kollman, P., Case, D., Singh, U. Chandra, G., Caterina, A., Giuliano, P. and Weiner, P. (1984) J. Am. Chem. Soc. 106:765-784
9. Atherton, E., and Sheppard, R.C. (1989) Solid Phase Peptide Synthesis - A Practical Approach. IRL Press, Oxford
10. Fields, G.B., and Noble, R.L. (1990) Int. J. Peptide Protein Res. 35:161-214
11 Akaji, K., Tatsumi, T., Yoshida, M., Kimura, T., Fujiwara, Y., and Kiso, Y. (1992) J. Am. Chem. Soc. 114:4137-4143
12 Salomon, Y. (1979) Adv. Cyclic Nucleotide Res. 10:35-55
13 Osborne, N. and Barnett, N. (1991) Exp. Eye Res. 53:131-133
14. Mimeault, M., St-Pierre, S., and Fournier, A. (1993) Eur. J. Biochem. 213:927-934