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Bioactive β-bend structures for the antagonist hα $CGRP_{8-37}$ at the $CGRP_1$ receptor of the rat pulmonary artery

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1 The aim of this study was to determine β-bend structures and the role of the N- and C-terminus in the antagonist hα $CGRP_{8-37}$ at the rat pulmonary artery CGRP receptor mediating hα CGRP relaxation.

2 Hα $CGRP_{8-37}$ Pro^{16} (10^{-6} M), with a bend-biasing residue (proline) at position 16, did not antagonize hα CGRP responses, while a structure-conserving amino acid ($alanine^{16}$) at the same position retained antagonist activity (apparent pK_B 6.6±0.1; 10^{-6} M). Hα $CGRP_{8-37}$ Pro^{19} (10^{-6} M), with proline at position 19 was an antagonist (apparent pK_B 6.9±0.1).

3 Incorporation of a β-bend forcing residue, BTD (beta-turn dipeptide), at positions 19 and 20 in hα $CGRP_{8-37}$ (10^{-6} M) antagonized hα CGRP responses (apparent pK_B 7.2±0.2); and BTD at positions 19,20 and 33,34 within hα $CGRP_{8-37}$ was a competitive antagonist (pA_2 7.2; Schild plot slope 1.0±0.1).

4 Hα $CGRP_{8-37}$ analogues, substituted at the N-terminus by either glycine[8] or *des*-NH_2 valine[8] or proline[8] were all antagonists (apparent pK_B 6.9±0.1; (10^{-6} M), 7.0±0.1 (10^{-6} M), and pA_2 7.0 (slope 1.0±0.2), respectively); while replacements by proline[8] together with glutamic acid[10,14] in hα $CGRP_{8-37}$ (10^{-6} M) or alanine amide[37] at the C-terminus of hα $CGRP_{8-37}$ (10^{-5} M) were both inactive compounds.

5 In conclusion, possible bioactive structures of hα $CGRP_{8-37}$ include two β-bends (at 18–21 and 32–35), which were mimicked by BTD incorporation. Within hα $CGRP_{8-37}$, the N-terminus is not essential for antagonism while the C-terminus may interact directly with $CGRP_1$ receptors in the rat pulmonary artery.

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Abbreviations: BTD, beta-turn dipeptide

Introduction

Calcitonin gene-related peptide (CGRP) is a 37 residue neuropeptide with numerous biological actions (for review see Poyner *et al*., 1992; Bell & McDermott, 1996). CGRP receptors have been divided into $CGRP_1$ and $CGRP_2$ (Dennis *et al*., 1989; 1990; Mimeault *et al*., 1991; Quirion *et al*., 1992), mainly based on the antagonist hα $CGRP_{8-37}$, which has a higher affinity at $CGRP_1$ in the guinea-pig atrium than at the $CGRP_2$ receptor in the rat vas deferens. The lack of pharmacological tools has been a major problem in the further characterization of CGRP receptors. In the vasculature CGRP causes relaxation *via* endothelium-dependent and independent mechanisms (Marshall, 1992) and different CGRP receptors may be involved (Wisskirchen *et al*., 1999b).

Conformational and modelling studies (Lynch & Kaiser, 1988; Manning, 1989; Hubbard *et al*., 1991; Breeze *et al*., 1991; Hakala & Vihinen, 1994) have been undertaken to investigate the structure of CGRP and fragments, and have given evidence of structural features. These include an inducible amphipathic α-helical region between residues 8 to about 18 (Lynch & Kaiser, 1988; Manning, 1989; Breeze *et al*., 1991; Hubbard *et al*., 1991), and a tentative assignment to β-bend regions around residues 17 to 21 (Lynch & Kaiser, 1988; Breeze *et al*., 1991; Hubbard *et al*., 1991) and 29 to 35 (Hubbard *et al*., 1991; Hakala & Vihinen, 1994).

The amphipathic α-helix has been suggested to be an important feature for interaction of the peptide with its receptors, as its length and sequence is critical for both the biological activity of CGRP (Lynch & Kaiser, 1988) and the antagonist affinity of hα $CGRP_{8-37}$ (Dennis *et al*., 1989; Mimeault *et al*., 1991; 1992; Boulanger *et al*., 1996). To date, the characterization of this region has been difficult; for instance, in several structure-activity studies the sequential replacement of helical residues with alanine (which conserves structure but removes functionality) has led to equivocal results.

With respect to the predicted bend regions, recent structure-activity work suggested the presence of two β-bends in hα $CGRP_{8-37}$ (Wisskirchen *et al*., 1999a). The incorporation of BTD (beta-turn dipeptide, which forces a 4 residue β-bend formation; Nagai & Sato 1985) at positions 19,20 and 33,34 identified β-bends at 18–21 and 32–35 as relevant structures for antagonism of hα $CGRP_{8-37}$ at the $CGRP_2$ receptor in the rat vas deferens.

The present study was undertaken to investigate the putative β-bends and the role of the N- and C-terminus of hα $CGRP_{8-37}$ at the $CGRP_1$ receptor in the rat pulmonary artery (Wisskirchen *et al*., 1998), by assaying the effect of structurally modified $CGRP_{8-37}$ analogues against hα CGRP responses. The putative β-bends were examined, using structure-conserving (alanine), bend-biasing (proline) and bend-forcing (BTD) residues incorporated in hα $CGRP_{8-37}$. The N-terminal region and the C-terminus of hα $CGRP_{8-37}$

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were investigated, using substitutions in position 8 (glycine or proline or *des*-NH_2 valine), in the helical region (proline[8] and glutamic acid[10,14]), and at the C-terminus (alanine amide[37]).

Methods

Male Sprague Dawley rats (320–480 g) were stunned and killed by cervical dislocation. The pulmonary artery was isolated, cut into rings (2–3 mm in length), suspended on tungsten wires (0.125 mm diameter) under 0.5 g resting tension in Krebs solution containing (mM): Na^+ 143, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 128, HCO_3^- 25, HPO_4^{2-} 1.2, SO_4^{2-} 1.2 and glucose 11 at 37°C, oxygenated with 95% O_2/5% CO_2, and allowed to equilibrate for 100 min. The isometric tone was recorded with Grass FT.03 transducers, which were connected to a Grass 7D polygraph.

Contractile tone was evoked by phenylephrine (3×10^{-8} M), whose stability was assessed over 10 min, and acetylcholine (10^{-6} M) was added. Tissues showing less than 80% relaxation of the spasmogen-induced tone to acetylcholine were discarded as having partially damaged endothelium. A cumulative concentration response curve to hα CGRP was constructed on the phenylephrine-induced tone, in endothelium-intact rings (100 min later). The effect of hα CGRP$_{8-37}$ analogues (10^{-6} M; 20 min pretreatment) was tested on second curves to hα CGRP, after another 100 min. Hα CGRP$_{8-37}$ analogues were substituted in position 8 by glycine (hα CGRP$_{8-37}$Gly[8]), *des*-NH_2 valine (hα CGRP$_{8-37}$ *des*-NH_2 Val[8]), or proline (hα CGRP$_{8-37}$Pro[8]), in position 8, 10 and 14 by proline and glutamic acid (hα CGRP$_{8-37}$Pro[8], Glu[10,14]), in position 16 by alanine (hα CGRP$_{8-37}$Ala[16]), in position 16 or 19 by proline (hα CGRP$_{8-37}$Pro[16], hα CGRP$_{8-37}$Pro[19]), in positions 19,20 and 33,34 by BTD (hα CGRP$_{8-37}$BTD[19,20]; hα CGRP$_{8-37}$ BTD[19,20 and 33,34]), and in position 37 by alanine amide (hα CGRP$_{8-37}$Ala[37]). The effect of hα CGRP$_{8-37}$ BTD[19,20 and 33,34] was tested at 10^{-7}–10^{-6} M, of hα CGRP$_{8-37}$Pro[16] and hα CGRP$_{8-37}$Ala[37] at 10^{-5} M, and of hα CGRP$_{8-37}$ Pro[8] at 3×10^{-7}–3×10^{-6} M (20 min pretreatment of a single concentration per tissue), on hα CGRP responses. CGRP fragments were tested on basal tone and on the spasmogen-induced tone.

Chemicals

Phenylephrine hydrochloride was obtained from Sigma, U.K, and was prepared daily in distilled water (10^{-3} M). The peptides hα CGRP, hα CGRP$_{8-37}$ and its analogues were donated by GlaxoWellcome Research Laboratories (Beckenham, Kent), having been synthesized and purified as previously described (Wisskirchen *et al*., 1999a). Apart from hα CGRP$_{8-37}$BTD[19,20 and 33,34], being diluted in DMSO, all other peptides were diluted in distilled water to form a 10^{-2} M stock solution and stored at −20°C.

Data analysis

Responses to drugs are expressed as a percentage relaxation of the phenylephrine-induced tone. All values are expressed as mean±s.e.mean. One-way ANOVA, followed by Dunnett's test (multiple comparison) and Students *t*-tests (for paired and unpaired groups) were used where appropriate to assess the significance of difference between means, with $P<0.05$ being taken as statistically significant. The EC_{50} values (molar concentration of the agonist that produced 50% of the maximal response) were calculated by non-linear regression curve fitting, using Graphpad Prism 2.0 (Graphpad Software, U.S.A.), and were used to determine the pEC_{50} values ($-\log EC_{50}$). The Hill slope of each non-linear regression curve was determined, using Graphpad Prism. In the presence of an antagonist with a single concentration, an apparent pK_B value was calculated given by the equation: $pK_B=\log(CR-1)-\log[B]$, where CR is the concentration ratio of the molar EC_{50} values in the presence and absence of the antagonist and [B] is the molar concentration of the antagonist. Apparent pK_B values were only calculated if the Hill slopes of agonist and antagonist curves were not significantly different from one another. Where multiple concentrations of antagonist were used, a Schild plot of $\log(CR-1)$ against $\log[B]$ was plotted, and the pA_2 and Schild plot slope determined by linear regression, using Graphpad Prism. The pA_2 and apparent pK_B values were calculated from the individual control concentration response curves and the respective curves obtained in the presence of hα CGRP$_{8-37}$ analogues.

Results

In the rat endothelium-intact pulmonary artery, phenylephrine (3×10^{-8} M)-induced tone (0.19±0.02 g; $n=4$) was relaxed by hα CGRP with a pEC_{50} 8.4±0.1 (Hill slope 1.0±0.1; $n=4$) and 100% maximum relaxation.

Effect of proline and alanine replacement around the predicted central bend region of hα CGRP$_{8-37}$

The fragments hα CGRP$_{8-37}$Pro[16], hα CGRP$_{8-37}$Ala[16] and hα CGRP$_{8-37}$Pro[19] (up to 10^{-6} M) did not alter basal tone or the spasmogen-evoked tone. Preliminary experiments indicated that 10^{-5} M of hα CGRP$_{8-37}$Ala[16] or hα CGRP$_{8-37}$Pro[19] relaxed the phenylephrine-induced tone (20–60%), while this was not observed with hα CGRP$_{8-37}$Pro[16] (10^{-5} M). Therefore, the former two analogues were investigated at 10^{-6} M, while the latter was tested at 10^{-5} M.

Hα CGRP$_{8-37}$Pro[16] (10^{-5} M), with a bend-biasing residue (proline) in position 16, did not modify hα CGRP responses (Figure 1a; Table 1), while hα CGRP$_{8-37}$Ala[16] (10^{-6} M), with a structure-conserving residue (alanine) at the same position, produced a parallel rightward shift of the agonist curve to hα CGRP, without reducing the maximum response (Figure 1b; Table 1). Hα CGRP$_{8-37}$Pro[19] (10^{-6} M), with proline at position 19, antagonized hα CGRP responses with no depression in the maximum effect (Figure 1c). The apparent pK_B value for hα CGRP$_{8-37}$Pro[19] was similar to the affinity of hα CGRP$_{8-37}$, obtained previously (Table 1), indicating that a bend-biasing structure at position 19 retained antagonism of hα CGRP$_{8-37}$.

Effect of BTD replacement in the predicted bend regions of hα CGRP$_{8-37}$

Hα CGRP$_{8-37}$BTD[19,20] and hα CGRP$_{8-37}$BTD[19,20 and 33,34] (up to 10^{-6} M) did not modify either basal or phenylephrine-evoked tone. Preliminary results indicated that hα CGRP$_{8-37}$ BTD[19,20] at 10^{-5} M produced relaxation of the phenylephrine-evoked tone (30–60% relaxation). Therefore, results with the BTD analogues were obtained at concentrations no higher than 10^{-6} M.

Hα CGRP$_{8-37}$ BTD[19,20] (10^{-6} M), with a bend-forcing residue (BTD) at positions 19,20, antagonized hα CGRP responses, and shifted the agonist curve to the right with no reduction in the maximum response (Figure 2; Table 1). Hα CGRP$_{8-37}$ BTD[19,20 and 33,34] (10^{-7}–10^{-6} M), with BTDs at

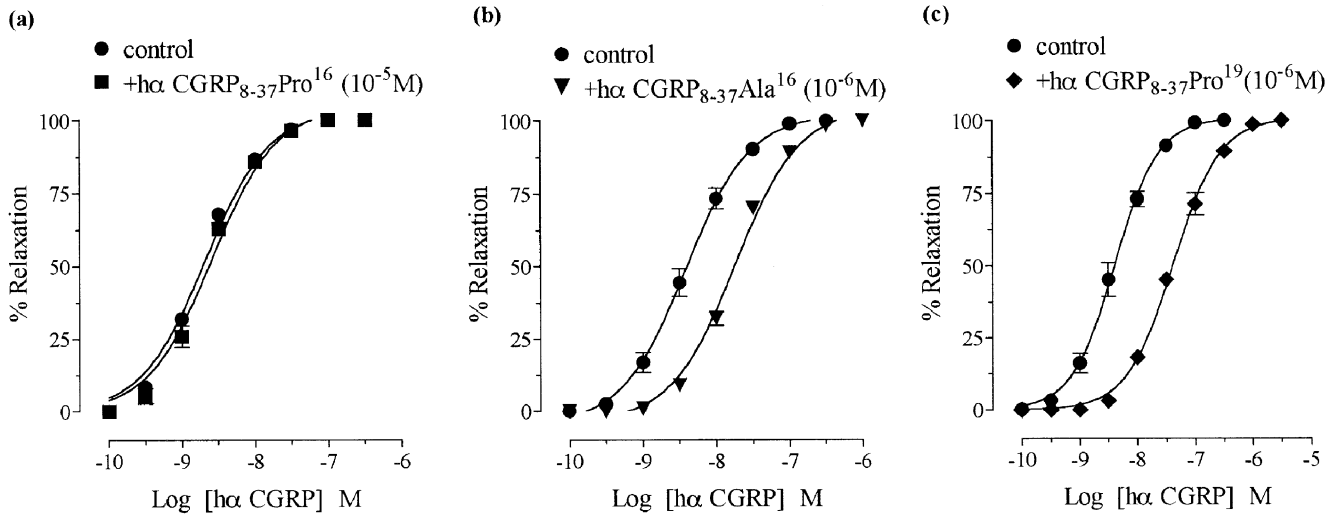

Figure 1 Effect of replacement by either proline[16], alanine[16] or proline[19] in hα CGRP$_{8-37}$ on hα CGRP relaxation in rat preconstricted pulmonary artery. Concentration response curves to hα CGRP on phenylephrine-induced tone, and in the presence of (a) hα CGRP$_{8-37}$Pro^{16} (10^{-5} M), (b) hα CGRP$_{8-37}$Ala^{16} (10^{-6} M) and (c) hα CGRP$_{8-37}$Pro^{19} (10^{-6} M). Results are expressed as percentage relaxation of the spasmogen-induced tone. Points and error bars represent the mean±s.e. mean of four or five separate experiments.

Table 1 Antagonist affinities of hα CGRP$_{8-37}$ and analogues against hα CGRP relaxation on phenylephrine-induced tone in the rat pulmonary artery

Antagonist	*pA_2/pK_B* value*	*Schild slope*
hα CGRP$_{8-37}$	6.9	1.2±0.1
hα CGRP$_{8-37}$Pro^{16}	<5*	–
hα CGRP$_{8-37}$Ala^{16}	6.6±0.1*	–
hα CGRP$_{8-37}$Pro^{19}	6.9±0.1*	–
hα CGRP$_{8-37}$BTD^{19,20}	7.2±0.2*	–
hα CGRP$_{8-37}$BTD$^{19,20\text{ and }33,34}$	7.2	1.0±0.1
hα CGRP$_{8-37}$Gly^{8}	6.9±0.1*	–
hα CGRP$_{8-37}$ *des*-NH$_2$ Val^{8}	7.0±0.1*	–
hα CGRP$_{8-37}$Pro^{8}	7.0	1.0±0.1
hα CGRP$_{8-37}$Pro^{8}Glu^{10}Glu^{14}	<6*	–

Apparent pK_B values (*) were obtained from concentration ratios using 10^{-6} M of the antagonists (or 10^{-5} M of hα CGRP$_{8-37}$Pro^{16}), and are expressed as the mean±s.e.mean. PA_2 values were obtained from a Schild plot by linear regression of various concentrations of the CGRP antagonists, and the Schild plot slope was expressed as mean± s.e.mean. Values were obtained from at least four separate experiments. Data for hα CGRP$_{8-37}$ are quoted from previous studies (Wisskirchen *et al.*, 1998).

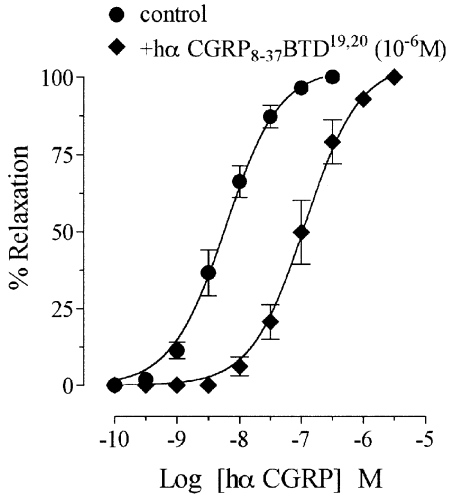

Figure 2 Effect of replacement by BTD^{19,20} in hα CGRP$_{8-37}$ on hα CGRP relaxation in rat preconstricted pulmonary artery. Concentration response curves to hα CGRP on phenylephrine-evoked tone, and in the presence of hα CGRP$_{8-37}$BTD^{19,20} (10^{-6} M). Results are expressed as percentage relaxation of the spasmogen-induced tone. Points and error bars represent the mean±s.e. mean of five separate experiments.

positions 19,20 and 33,34, concentration-dependently inhibited hα CGRP responses, in a manner consistent with competitive antagonism (Figure 3). The pA_2 value for the double BTD compound was close to that of the parent compound, hα CGRP$_{8-37}$ (Table 1), illustrating that enforcement of a bend at these positions preserved antagonism.

Effect of structural modifications at the N-terminal region of hα CGRP$_{8-37}$

Analogues which were replaced at the N-terminus of hα CGRP$_{8-37}$ by either glycine (hα CGRP$_{8-37}$Gly^{8}) or *des*-NH$_2$ valine (hα CGRP$_{8-37}$ *des*-NH$_2$ Val^{8}) had no effect on basal or phenylephrine-evoked tone up to 10^{-6} M, while preliminary experiments indicated that higher concentrations (10^{-5} M) produced contractile responses. Hα CGRP$_{8-37}$Pro^{8}, with proline at the N-terminus, did not alter basal or spasmogen-evoked tone up to 3×10^{-7} M, but in some tissues 10^{-6} M produced contractions on the basal (0.07±0.03 g tension; $n=4$) and on top of the spasmogen-induced tone (25–35% increase in tone). Hα CGRP$_{8-37}$Pro^{8}Glu^{10}Glu^{14}, with proline^{8} and glutamic acid^{10,14} incorporated, did not alter basal or phenylephrine-evoked tone up to 10^{-6} M. Subsequent experiments with hα CGRP$_{8-37}$Gly^{8}, hα CGRP$_{8-37}$ *des*-NH$_2$ Val^{8} and hα CGRP$_{8-37}$Pro^{8}Glu^{10}Glu^{14} were performed at 10^{-6} M, while hα CGRP$_{8-37}$Pro^{8} was investigated at 3×10^{-7} M and 10^{-6} M, using only those tissues where 10^{-6} M of the fragment did not affect basal tone or contractions to phenylephrine (four out of eight tissues).

Hα CGRP$_{8-37}$Gly^{8}, hα CGRP$_{8-37}$ *des*-NH$_2$ Val^{8} and hα CGRP$_{8-37}$Pro^{8} antagonized hα CGRP responses with similar affinities (Figure 4; Table 1). The analogues hα CGRP$_{8-37}$Gly^{8} and hα CGRP$_{8-37}$ *des*-NH$_2$ Val^{8} (10^{-6} M each) produced a

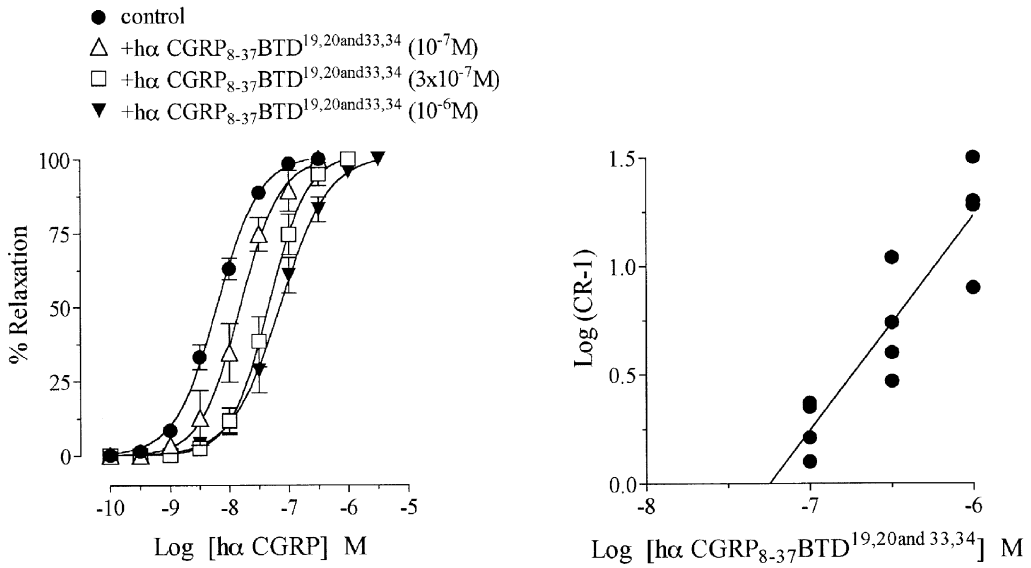

Figure 3 Effect of replacement by BTD^{19,20} and BTD^{33,34} in hα CGRP$_{8-37}$ on hα CGRP relaxation in rat preconstricted pulmonary artery. Graph (left) showing a concentration response curve to hα CGRP on phenylephrine-evoked tone, and in the presence of hα CGRP$_{8-37}$BTD$^{19,20\text{ and }33,34}$ (10^{-7} M, 3×10^{-7} M and 10^{-6} M). Results are expressed as percentage relaxation of the spasmogen-induced tone. Points and error bars represent the mean ± s.e. mean of four to twelve separate experiments. The Schild plot (right) for hα CGRP$_{8-37}$BTD$^{19,20\text{ and }33,34}$ against hα CGRP, where points represent individual data of twelve separate experiments.

parallel rightward shift of the agonist curve without reducing the maximum response (Figure 4a,b). Hα CGRP$_{8-37}$Pro^8 (3×10^{-7}–10^{-6} M) concentration-dependently antagonized hα CGRP responses, and the Schild plot gave a pA_2 with a slope of unity, consistent with competitive antagonism as established for hα CGRP$_{8-37}$ (Figure 4c; Table 1). (In those tissues where hα CGRP$_{8-37}$Pro^8 (10^{-6} M) produced contractions on phenylephrine-induced tone it still antagonized hα CGRP relaxation (estimated apparent pK_B 6.9 ± 0.1; $n=4$) although hα CGRP (up to 10^{-5} M) did not relax the contractile component of hα CGRP$_{8-37}$Pro^8). Hα CGRP$_{8-37}$Pro^{8}Glu^{10}Glu^{14} (10^{-6} M) did not alter hα CGRP responses (the pEC_{50} values for hα CGRP were 8.3 ± 0.1 and 8.1 ± 0.1 ($n=4$ each) in the absence and presence of CGRP$_{8-37}$Pro^{8}Glu^{10}Glu^{14}, respectively), i.e. these substitutions abolished antagonism of hα CGRP$_{8-37}$ (Table 1).

Effect of an alanine replacement at the C-terminus of hα CGRP$_{8-37}$

Hα CGRP$_{8-37}$Ala^{37}, with alanine amide^{37} at the C-terminus, did not affect the basal tone or the spasmogen-evoked tone up to 10^{-5} M. The fragment (10^{-5} M) was not active against hα CGRP responses, i.e. incorporation of an amidated alanine^{37} abolished antagonism (the pEC_{50} values for hα CGRP were 8.4 ± 0.1 and 8.2 ± 0.1 ($n=4$ each) before and after treatment with hα CGRP$_{8-37}$Ala^{37}; 10^{-5} M).

Discussion

The present study provides evidence for two biologically relevant β-bend structures in hα CGRP$_{8-37}$ (18–21 and 32–35), as the incorporation of BTD retained antagonism at the CGRP receptor in the rat pulmonary artery. Further, while the N-terminus is not important for antagonism, this is the opposite with the C-terminus.

As predicted from conformational studies on CGRP, a β-bend between residues 18–21 was suggested to terminate the α-helix (Lynch & Kaiser, 1988; Breeze *et al*., 1991). The present investigation on hα CGRP$_{8-37}$ supports this view. Firstly, replacement by a bend-biasing residue (proline^{19}) within region 18–21 retained antagonism, indicating that residue 19 (serine) is not essential for receptor interaction. Secondly, a bend-biasing residue outside the predicted bend region (proline^{16}) abolished antagonism, which could be due to disruption of the helical folding, since a structure-conserving residue (alanine) at the same position retained antagonism, indicating that residue 16 (leucine) is not involved in receptor binding. Thirdly, the incorporation of BTD, a β-bend mimetic (Nagai & Sato, 1985), at positions 19,20 produced an antagonist, indicating that serine^{19} and glycine^{20} are not essential for receptor interaction. Therefore, a 18–21 β-bend has been identified as a relevant bioactive structure for hα CGRP$_{8-37}$ antagonism at the rat pulmonary artery receptor.

Modelling studies and structure activity work on CGRP predicted another turn region around residues 32–35 (Hakala & Vihinen, 1994; Hakala *et al*., 1994), with a possible β- or γ-turn type formation. The present work supports a 32–35 β-bend type in hα CGRP$_{8-37}$, as incorporation of two BTDs at 19,20 and 33,34 (i.e. within β-bend regions 18–21 and 32–35) retained antagonism, indicating that glycine^{33} and serine^{34} residues are not essential for receptor recognition. Therefore, two β-bend regions at 18–21 and 32–35 are compatible with high affinity binding of hα CGRP$_{8-37}$ at the rat pulmonary artery receptor. Whether hα CGRP$_{8-37}$ may adopt alternative conformations in these regions (Hakala & Vihinen, 1994, Rist *et al*., 1998), however, cannot be excluded from these data.

The BTD mimic of the bioactive hα CGRP$_{8-37}$ conformation is consistent with that found in the rat vas deferens (Wisskirchen *et al*., 1999a), suggesting that this architecture of the hα CGRP$_{8-37}$ molecule does not change upon interaction with either the $CGRP_2$ receptor in the rat vas deferens or the $CGRP_1$ receptor in the rat pulmonary artery. The antagonist affinity for hα CGRP$_{8-37}$ differs 10 fold between these tissues (Wisskirchen *et al*., 1998), a difference reflected in the relative affinity of hα CGRP$_{8-37}$BTD$^{19,20\text{ and }33,34}$ in the two rat tissues.

Investigations on the N-terminus (valine^8) of hα CGRP$_{8-37}$ have suggested that valine^8 does not play an important role for

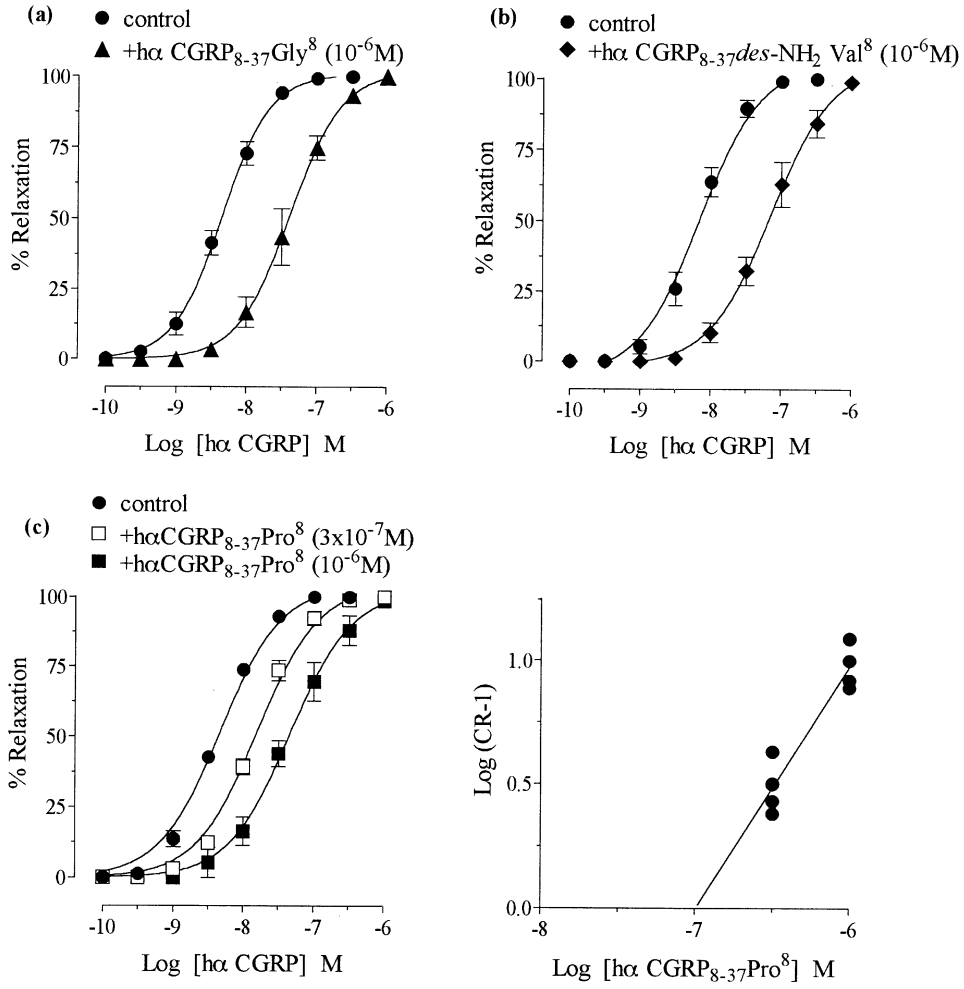

Figure 4 Effect of replacement by either glycine[8], *des*-NH_2 valine[8] or proline[8] in hα $CGRP_{8-37}$ on hα CGRP relaxation in rat preconstricted pulmonary artery. Concentration response curves to hα CGRP on phenylephrine-induced tone, and in the presence of (a) hα $CGRP_{8-37}Gly^8$ (10^{-6} M), (b) hα $CGRP_{8-37}$*des*-NH_2 Val^8 (10^{-6} M), and (c) hα $CGRP_{8-37}Pro^8$ (3×10^{-7} M, 10^{-6} M). A Schild plot (bottom, right) for hα $CGRP_{8-37}Pro^8$ against hα CGRP. Results on the graphs are expressed as percentage relaxation of the spasmogen-evoked tone. Points and error bars represent the mean±s.e. mean of four to eight separate experiments. On the Schild plot, points represent individual data of eight separate experiments.

either the helical structure, the antagonist activity (Mimeault *et al*., 1991; 1992), or the binding of hα $CGRP_{8-37}$ at its receptor (Rist *et al*., 1998). The present results agree with the proposal that valine[8] is not essential for receptor interaction, as deletion of both the N-terminal charge (*des*-NH_2 valine) and the isopropyl side chain (glycine) retained antagonism. However, whether the N-terminus has an influence on the helicity cannot be determined by the current data.

Structure activity studies suggested that the amphipathic α-helix is an important feature for antagonism by hα $CGRP_{8-37}$ (Dennis *et al*., 1989; Mimeault *et al*., 1991; 1992). The present attempt to stabilize the helix by helix-initiating (proline[8]) and hydrophilic residues (glutamic acid[10,14]) resulted in loss of binding affinity. While this gives no direct evidence about the effect of the triple substitutions one explanation might be that the multiple substitutions disrupted the amphipathic nature of the helical region. However, what remains unclear is whether this region does adopt a stable α-helix (Boulanger *et al*., 1996) and if so whether it purely promotes the orientation of hα $CGRP_{8-37}$ at its receptor or not.

The C-terminus of CGRP, phenylalanine amide[37] (Phe-NH_2), has been suggested to be essential for receptor binding (Poyner *et al*., 1992; O'Connell *et al*., 1993). The current observation that hα $CGRP_{8-37}Ala^{37}$ exhibits no antagonist properties could suggest that the aromatic ring of Phe is directly involved in the interaction with the receptor binding sites. Alternatively, the phenyl group might be involved in an intramolecular interaction and deletion could be responsible for a structural change. The latter seems unlikely, since conformational studies have demonstrated no structural difference between (*des*-Phe-NH_2)[37] CGRP and CGRP (O'Connell *et al*., 1993). Therefore, the current data would agree with the view that the C-terminus of hα $CGRP_{8-37}$ interacts directly with CGRP receptor binding sites and suggests further that the aromatic ring is directly involved.

In this study, a number of hα $CGRP_{8-37}$ analogues have been identified as useful tools to determine structural features in hα $CGRP_{8-37}$. The findings that the antagonists had the same affinity as hα $CGRP_{8-37}$ supports the characterization of a $CGRP_1$ receptor in the rat pulmonary artery. However, some problems have occurred with these analogues. For instance, analogues with substitutions around the bend regions (hα $CGRP_{8-37}Pro^{19}$; hα $CGRP_{8-37}Ala^{16}$, hα $CGRP_{8-37}BTD^{19,20}$) produced vasorelaxation (at high concentrations) in the pulmonary artery, consistent with the action of hα $CGRP_{8-37}$ (Wisskirchen *et al*., 1998). One possibility may be that this reflects partial agonism at the CGRP receptor. For instance, the observation that hα $CGRP_{8-37}Pro^{16}$ was inactive (up to

10^{-5} M) and did not alter tone could support this hypothesis. Alternatively, the vasodilator component may be produced by release of endogenous CGRP from within the artery. For instance, Nuki *et al*. (1994a) suggested that hα CGRP$_{8-37}$ can block presynaptic CGRP receptors and thus inhibit a negative feed back mechanism, in the rat mesenteric vasculature. Yet, another possibility would be that the vasodilator effect is caused by interaction with a non-CGRP receptor, in the rat pulmonary artery.

Analogues substituted at the N-terminus (hα CGRP$_{8-37}$ Gly^8, hα CGRP$_{8-37}$ *des*-NH$_2$ Val^8, hα CGRP$_{8-37}$Pro^8) produced vasoconstriction in the pulmonary artery, at high concentrations. This action could be associated with CGRP receptors, but the observation that hα CGRP$_{8-37}$Pro^8 was an antagonist makes this unlikely. Alternatively, the contractile tone may be mediated *via* a non-CGRP receptor. It is noteworthy that for hα CGRP$_{8-37}$, both vasodilator and vasoconstrictor effects have been reported. For instance, a vasodilator component was noted in rabbit skin, *in vivo* (Hughes & Brain, 1991) and cat cerebral vasculature (Wahl *et al*., 1994), while vasoconstriction was observed in rat perfused mesenteric artery (Han *et al*., 1990; Nuki *et al*., 1994b), perfused kidney (Chin *et al*., 1994) and the rat vasculature, *in vivo* (Gardiner *et al*., 1990). Therefore, the present findings illustrate that not only hα CGRP$_{8-37}$ but also its analogues have limitations as antagonists, underlining the need for more selective compounds.

In conclusion, two bioactive β-bend regions at 18–21 and 32–35 are possible common features in the conformation of hα CGRP$_{8-37}$ at its receptors. Further, the N-terminus is not essential for antagonism, while the C-terminus interacts with CGRP receptor binding sites.

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