British Journal of Pharmacology (2003) 138, 325–332

www.nature.com/bjp

The role of the 8-18 helix of $CGRP_{8-37}$ in mediating high affinity binding to CGRP receptors; coulombic and steric interactions

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1 The role of individual residues in the 8-18 helix of $CGRP_{8-37}$ in promoting high-affinity binding to $CGRP_1$ receptors expressed on rat L6 and human SK-N-MC cells has been examined. The relative potencies of various derivatives were estimated from their ability to inhibit the human αCGRP-mediated increase in cyclic AMP production and the binding of [^{125}I]-human αCGRP.

3 Arg^{11} and Arg^{18} were replaced by serines to give [$Ser^{11,18}$]$CGRP_{8-37}$. These bound with *pKi* values <6 to SK-N-MC cells and had apparent pA_2 values of 5.81 ± 0.04 and 5.31 ± 0.11 on SK-N-MC and L6 cells. $CGRP_{8-37}$ had a *pKi* of 8.22 on SK-N-MC cells and pK_b values on the above cell lines of 8.95 ± 0.04 and 8.76 ± 0.04.

3 The arginines were replaced with glutamic acid residues. [Glu^{11}]$CGRP_{8-37}$ had a pK_b of 7.14 ± 0.14 on SK-N-MC cells ($pKi = 7.05 \pm 0.05$) and 6.99 ± 0.08 on L6 cells. [Glu^{18}]$CGRP_{8-37}$ had a pK_b of $7.10 \pm 0.0.08$ on SK-N-MC cells ($pKi = 6.91 \pm 0.23$) and 7.12 ± 0.09 on L6 cells.

4 Leu^{12}, Leu^{15} and Leu^{16} were replaced by benzoyl-phenylalanine (bpa) residues. On SK-N-MC cells, the apparent pA_2 values of [bpa^{12}]-, [bpa^{15}]- and [bpa^{16}]$CGRP_{8-37}$ were respectively 7.43 ± 0.23, 8.34 ± 0.11 and 5.66 ± 0.16 (*pKi* values of 7.14 ± 0.17, 7.66 ± 0.21 and <6): on L6 cells they were 7.96 ± 0.36, 8.28 ± 0.21 and 6.09 ± 0.04 (all $n = 3$).

5 It is concluded that the Arg^{11} and Arg^{18} are involved in specific electrostatic interactions with other residues, either on the $CGRP_1$ receptors or elsewhere on $CGRP_{8-37}$. Leu^{16} is in a conformationally restricted site when $CGRP_{8-37}$ binds to $CGRP_1$ receptors, unlike Leu^{12} and Leu^{15}.

British Journal of Pharmacology (2003) **138,** 325–332. doi:10.1038/sj.bjp.0705040

Keywords: CGRP; $CGRP_{8-37}$; $CGRP_1$ receptor; L6 cells; SK-N-MC cells; structure-activity studies; bpa-$CGRP_{8-37}$

Abbreviations: bpa, benzoyl-phenylalanine; CGRP, calcitonin gene-related peptide; CRLR, calcitonin receptor-like receptor; h, human; RAMP1, receptor activity modifying protein 1

Introduction

Calcitonin gene related peptide (CGRP) is an abundant 37 amino acid neuropeptide. It forms a family with calcitonin, amylin and adrenomedullin (Poyner *et al*., 2002). Whilst these have only modest sequence homology, they share a number of structural features in common; a N-terminal disulphide bonded ring required for receptor activation, an amphipathic α-helix and a C-terminal amide (Poyner *et al*., 2002). Removal of the N-terminus of CGRP gives $CGRP_{8-37}$, an antagonist (Chiba *et al*., 1989). This discriminates between different CGRP-activated receptors (Quirion *et al*., 1992). The best characterized of these is the $CGRP_1$ receptor, formed of a complex between the heptahelical calcitonin receptor- like receptor (CRLR) and receptor activity modifying protein 1 (RAMP1), a protein with a single transmembrane helix (McLatchie *et al*., 1998). The $CGRP_1$ receptor has a high affinity for $CGRP_{8-37}$ i.e. $pK_b > 7$ (Quirion *et al*., 1992; Poyner *et al*., 2002). By contrast, the $CGRP_2$ receptor as identified by Quirion and co-workers has a pK_b about an order of magnitude lower. The molecular nature of the $CGRP_2$ receptor is unclear and it is possible that several distinct entities may give CGRP-responsive receptors that have low affinity for $CGRP_{8-37}$ (Poyner *et al*., 2002). However, the nomenclature is useful in indicating that there is heterogeneity within receptors that are activated by CGRP.

Within CGRP, the characteristic amphipathic α-helix stretches between residues 8 and 18; thus it is present in both the full-length peptide and $CGRP_{8-37}$. The importance of this structure in CGRP binding was first demonstrated by Lynch & Kaiser (1988). Deletion of residues 8-18 leads to a 50–100 fold decrease in affinity at the $CGRP_1$ receptor (Rovero *et al*., 1992; Howitt & Poyner, 1997; Poyner *et al*., 1998). Introduction of a bend in the helix at position 16 also causes a large decrease in affinity (Wisskirchen *et al*., 1999; 2000). The contribution of individual amino acids to the role of 8-18 in promoting high affinity binding has not been fully elucidated. An alanine scan of the first four amino acids identified Arg^{11} as the most significant residue, but its substitution produced barely a 5 fold potency change (Mimeault *et al*., 1991; 1992). Much of the amphipathic character of the region comes from Arg^{11} and Arg^{18}, the other fully charged residue that is present in the helix. Whilst replacement of either of these alone by alanine produces only

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modest potency decreases, the double alanine substitution reduces affinity by 100 fold (Mimeault *et al*., 1992; Howitt & Poyner, 1997). However, it is not known whether this reduction is due to a decrease in the overall amphipathicity of the peptide or whether it implies that the arginines are involved in specific charge–charge interactions with other residues, either on the receptor or elsewhere on the ligand. Little work has been done to investigate the role of individual amino acids on the hydrophobic face of the peptide.

In the present study, both the hydrophilic and hydrophobic surfaces of the α-helix have been probed. The effects of substituting the positively charged Arg^{11} and Arg^{18} with either serine (uncharged but hydrophilic) and glutamic acid (negatively charged) has been examined, to evaluate whether these make specific contacts with other residues or are simply involved in hydrogen bonding to solvent water molecules. Steric constraints on the hydrophobic side of the α-helix have been investigated by substituting Leu^{12}, Leu^{15} and Leu^{16} with the large benzoyl-phenylalanine (bpa) moiety. These have been incorporated into $CGRP_{8-37}$. The abilities of these derivatives to antagonize CGRP binding and stimulation of cyclic AMP production on human SK-N-MC cells and rat L6 cells, both of which express $CGRP_1$ receptors, has been used to estimate their potency compared with $CGRP_{8-37}$.

A portion of this work has previously appeared in abstract form (Wang *et al*., 2001).

Methods

Cell culture

SK-N-MC human neuroblastoma cells were a gift from Prof. S. Nahorski, University of Leicester. L6 cells were purchased from the European Collection of Animal Cell Cultures (Porton Down, U.K.). Cells were cultured essentially as described previously (Poyner *et al*., 1992; 1998). Briefly L6 cells were grown in Dulbecco's Modified Eagle Medium supplemented with 10% foetal calf serum. SK-N-MC cells were grown in a 1 : 1 mixture of Dulbecco's Modified Eagle Medium/F12 medium supplemented with 10% foetal calf serum. Cells were passaged at confluency with trypsin/EDTA (Sigma) and grown for experiments in 48-well plates at 37°C in 5%CO_2/air in a humidified atmosphere.

Measurement of cyclic AMP

An hour prior to experiments, the medium on the cells was replaced with Kreb's solution supplemented with 0.1% bovine serum albumin and 1 mM isobutyl methyl xanthine. The cells were preincubated with antagonists for 30 min before addition of increasing concentrations of human (h) αCGRP. Incubations were terminated 5 min after addition of agonist by aspiration of the medium and addition of 0.1 ml of absolute ethanol. This was allowed to dry down at room temperature and the cyclic AMP was extracted by addition of 0.25 ml of assay buffer containing (in mM): EDTA 5, HEPES 20, pH 7.5. The samples were agitated for 5 min before 50 μl samples were withdrawn and cyclic AMP measured by a radioreceptor assay as described previously (Poyner *et al*., 1992).

Radioligand binding

This was carried out essentially as described previously (Poyner *et al*., 1998). Briefly, SK-N-MC cell membranes were homogenized in buffer containing (in mM): HEPES 20; EDTA 1; pH 7.5 and stored frozen at −70°C until required. They were then resuspended at approximately 0.25 mg protein per ml containing 50 mM HEPES, 0.1% bovine serum albumin, pH 7.5 and incubated at room temperature for 30 min with 100 pM [^{125}I]-iodohistidyl^8-human αCGRP with various concentrations of displacing ligands in a final volume of 0.5 ml. Non-specific binding was estimated with 1 μM CGRP. Incubations were terminated by centrifugation, pellets washed twice with tap water and counted to determine bound radioactivity.

Analysis of data

For cyclic AMP studies, the data from each concentration-response curve was fitted to a sigmoidal concentration-response curve to obtain the maximum response, Hill coefficient and EC_{50} using the fitting routine PRISM Graphpad. Where data was normalised, the maximum fitted cyclic AMP response to CGRP in the absence of antagonist was taken as 100%. From the individual curves, dose-ratios were calculated. Where several antagonist concentrations were used, a Schild plot was constructed; after confirming that the slope was not significantly different from unity, it was constrained to 1 to obtain the pK_b. Where only a single antagonist concentration was used, an apparent pA_2 was calculated from the formula, log[antagonist]-log(dose ratio-1). For radioligand binding, curves were fitted to obtain Hill coefficient, IC_{50} and non-specific binding. The *pKi* values were calculated from the Cheung-Prussoff equation, log-$[IC_{50}]$-log([radioligand].$Kd_{radioligand}$ + 1).

Statistical analysis was either by Student's *t*-test or by one-way ANOVA followed by Tukey's test where several values were being compared with each other. Significance was accepted at $P < 0.05$; two-tailed tests were used throughout. All values are quoted as means ± s.e.mean.

Drugs and materials

HαCGRP and h$\alpha CGRP_{8-37}$ were purchased from Calbiochem. [$Ser^{11,18}$]-h$\alpha CGRP_{8-37}$ was a gift of Merck, Sharpe and Dohme U.K. (Harlow). Other peptides were synthesized and purified as described previously (Poyner *et al*., 1998). All radiolabelled compounds were purchased from Amersham Biosciences (Little Chalfont, Buckinghamshire, U.K.). Isobutyl methyl xanthine was purchased from Sigma (Sigma-Aldrich, Gillingham, Dorset, U.K.). Cell culture medium and foetal calf serum were purchased from GIBCO BRL (Life Technologies, Paisley, Renfrewshire, U.K.). Other reagents were purchased from Sigma or Fisher. Peptides were dissolved and stored as frozen aliquots before use as previously described (Poyner *et al*., 1998).

Results

[$Ser^{11,18}$]$CGRP_{8-37}$

[$Ser^{11,18}$]$CGRP_{8-37}$ was investigated for its ability to antagonize CGRP on L6 and SKN-MC cells. In its

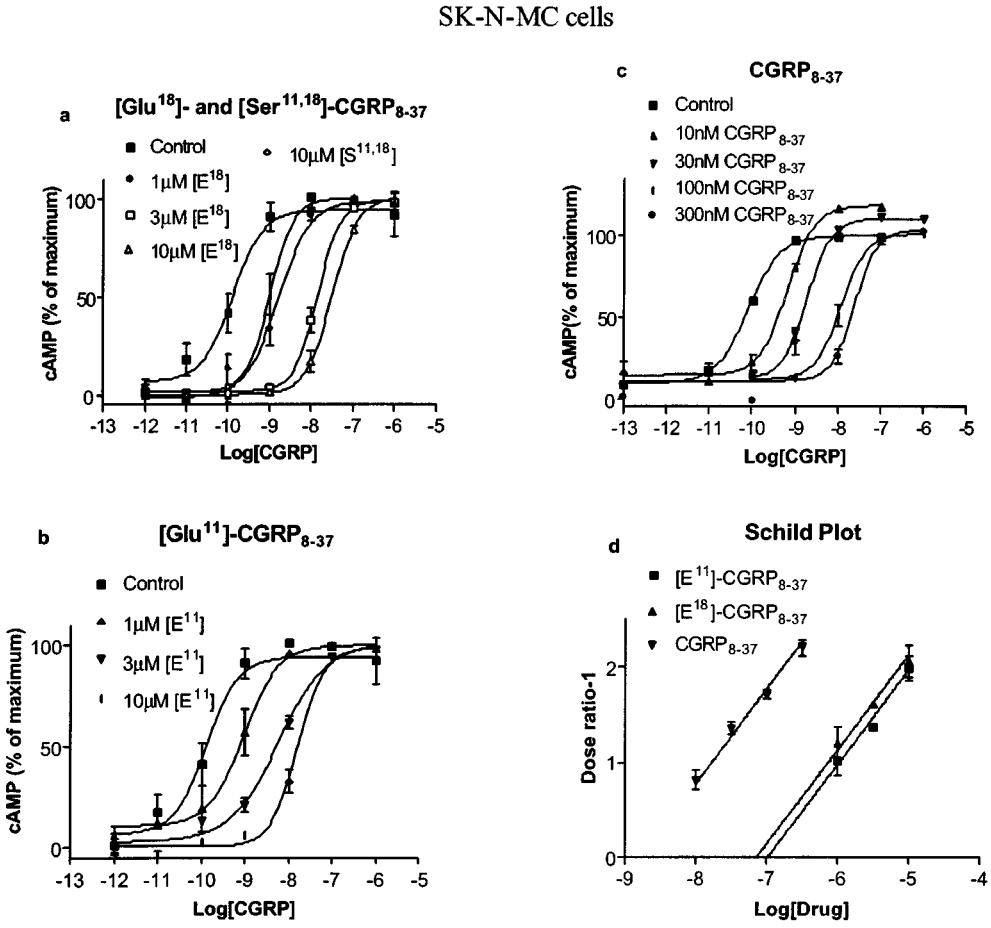

Figure 1 Effects of [Ser^{11,18}] hαCGRP$_{8-37}$, [Glu^{11}] hαCGRP$_{8-37}$, [Glu^{18}] hαCGRP$_{8-37}$ and hαCGRP$_{8-37}$ on the stimulation of cyclic AMP production by hαCGRP in SK-NMC cells. (a) [Ser^{11,18}] hαCGRP$_{8-37}$ and [Glu^{18}] hαCGRP$_{8-37}$; (b) [Glu^{11}] hαCGRP$_{8-37}$; (c) hαCGRP$_{8-37}$ (d) Schild plot for data in parts a, b and c. Parts a, b and c are representative of experiments carried out three or four times. Points were measured in duplicate in each experiment. Data are expressed as percentage of maximum cyclic AMP production caused by CGRP alone, estimated by fitting each line to a logistic Hill equation as described in the Methods. Maximum cyclic AMP values were as follows: (a) 260 ± 20 pmol per 10^6 cells; (b) 250 ± 20 pmol per 10^6 cells; (c) 160 ± 15 pmol per 10^6 cells. Basal values were all below 10 pmol per 10^6 cells. Part (d) includes the data from all experiments.

absence, CGRP increased cyclic AMP in both cell lines (Figure 1, 2: pEC$_{50}$ of 9.41 ± 0.08 in L6 cells; 9.89 ± 0.12 in SK-N-MC cells; $n=3$ in both cases). It had a pK_i of 10.07 measured by radioligand binding on SK-N-MC membranes (Table 2, Figure 3). Schild analysis confirmed that the parent antagonist, CGRP$_{8-37}$ behaved competitively (slopes of 0.90 ± 0.07 for SK-N-MC cells and 0.89 ± 0.07 for L6 cells, neither significantly different from 1). When the slopes were constrained to unity, pK$_b$ values of 8.95 in the L6 cells and 8.76 in SK-N-MC cells were obtained (Table 1, Figures 1, 2) CGRP$_{8-37}$ had a *pKi* of 8.22 on SK-N-MC membranes, in good agreement with the pK_b (Table 2, Figure 3). Only when [Ser^{11,18}]CGRP$_{8-37}$ was present at 10 μM did it produce a significant shift in the concentration-response curve in any of the cell lines (Table 1, Figures 1, 2). The Hill slopes of these curves in the presence of antagonist (2.0 ± 0.2 for SK-N-MC cells and 2.0 ± 0.2 for L6 cells) were not significantly different from the corresponding control curves, suggesting that it was a very weak competitive antagonist. Compared to CGRP$_{8-37}$, in functional assays [Ser^{11,18}]CGRP$_{8-37}$ was over 1000 fold less potent on both cells. Consistent with this data, it had a *pKi* of below 6 on SK-N-MC membranes (Table 2, Figure 3). Thus the replacement of both arginines with serines caused a very substantial reduction in affinity.

[Glu^{11}]CGRP$_{8-37}$ and [Glu^{18}]CGRP$_{8-37}$

A second series of derivatives replaced the individual arginines with glutamic acid residues to give [Glu^{11}]CGRP$_{8-37}$ and [Glu^{18}]CGRP$_{8-37}$ (Tables 1, 2, Figures 1–3). In both cell lines, these derivatives were less potent than CGRP$_{8-37}$. Due to their modest potencies, only limited Schild analysis could be undertaken, but the slope of the Schild plots was not significantly different from 1, consistent with competitive inhibition (0.97 ± 0.18 Glu^{11}, 0.87 ± 0.21 Glu^{18} for SK-N-MC cells; 0.97 ± 0.24 Glu^{11}, 0.67 ± 0.22 Glu^{18} for L6 cells). From these plots, pK$_b$ values were calculated (Table 1, Figures 1, 2). Based on these values [Glu^{18}]CGRP$_{8-37}$ was approximately 70 and 90 fold less potent on the L6 and SK-N-MC cells respectively; for [Glu^{11}]CGRP$_{8-37}$ the potency reductions were 40 and 70 fold. The *pKi* values measured for the two derivatives on SK-N-MC membranes showed somewhat smaller potency differences (15 fold for [Glu^{11}]CGRP$_{8-37}$

Table 1 pA2 estimates for antagonists

	$CGRP_{8-37}$	$[SER^{11,18}]$-$CGRP_{8-37}$	$[Glu^{11}]$-$CGRP_{8-37}$	$[Glu^{18}]$-$CGRP_{8-37}$	$[Bpa^{12}]$-$CGRP_{8-37}$	$[Bpa^{15}]$-$CGRP_{8-37}$	$[Bpa^{16}]$-$CGRP_{8-37}$
SK-N-MC	8.95±0.04	5.81±0.04***	7.14±0.14***	7.10±0.08***	7.43±0.23***	8.34±0.11*	5.66±0.16***
L6	8.76±0.04	5.31±0.11***	6.99±0.08**	7.12±0.09**	7.96±0.36*	8.28±0.21	6.09±0.4***

Values for $CGRP_{8-37}$, [Glu^{11}]-$CGRP_{8-37}$, [Glu^{18}]-$CGRP_{8-37}$ (on both cell lines) and [Bpa^{12}]-$CGRP_{8-37}$ (on SK-N-MC cells) are pK_b values (mean±s.e.mean) derived from Schild plots. The other values are estimated from single dose ratios and are means of three determinations. Asterisks indicated values significantly different from $CGRP_{8-37}$ (Tukey's test; ***$P<0.001$, **$P<0.01$, *$P<0.05$).

Table 2 Binding parameters for ligands on SK-N-MC cells

	$CGRP$	$CGRP_{8-37}$	$[Ser^{11,18}]$-$CGRP_{8-37}$	$[Glu^{11}]$-$CGRP_{8-37}$	$[Glu^{18}]$-$CGRP_{8-37}$	$[Bpa^{12}]$-$CGRP_{8-37}$	$[Bpa^{15}]$-$CGRP_{8-37}$	$[Bpa^{16}]$-$CGRP_{8-37}$
pKi	10.07±0.26	8.22±0.22	<6***	7.05±0.05***	6.91±0.23***	7.14±0.17*	7.66±0.21	<6***
nH	0.81±0.13	1.16±0.17		0.81±0.14	1.26±0.38	0.95±0.21	0.98±0.19	

Values are mean±s.e.mean. For each value, $n=3-4$. Asterisks indicate values significantly different from $CGRP_{8-37}$ (Tukey's test; ***$P<0.001$, **$P<0.01$, *$P<0.05$).

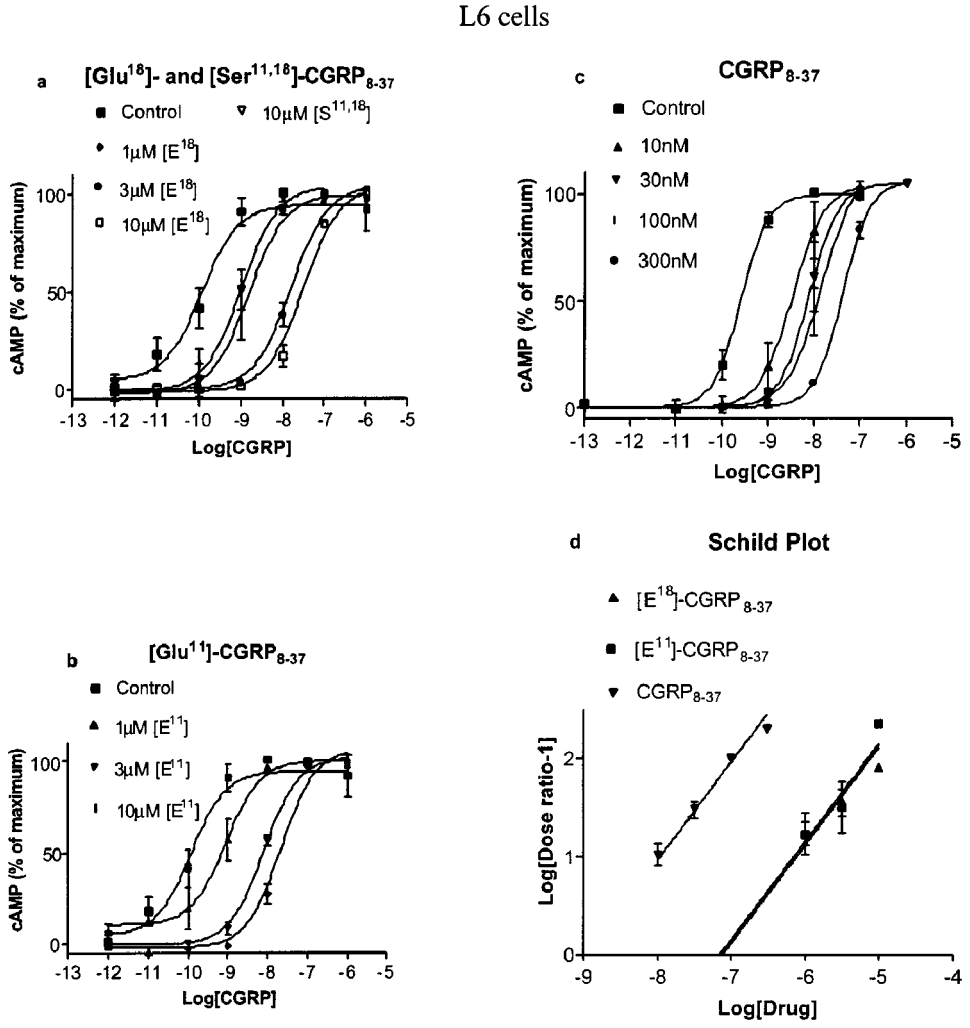

Figure 2 Effects of [$Ser^{11,18}$] hαCGRP$_{8-37}$, [Glu^{11}] hαCGRP$_{8-37}$, [Glu^{18}] hαCGRP$_{8-37}$ and hαCGRP$_{8-37}$ on the stimulation of cyclic AMP production by hαCGRP L6 cells. (a) [$Ser^{11,18}$] hαCGRP$_{8-37}$, [Glu^{18}] hαCGRP$_{8-37}$; (b) [Glu^{11}] hαCGRP$_{8-37}$; (c) hαCGRP$_{8-37}$ (d) Schild plot for data in parts a, b and c. Parts a, b and c are representative of experiments carried out three or four times. Points were measured in duplicate in each experiment. Data are expressed as percentage of maximum cyclic AMP production caused by CGRP alone, estimated by fitting each line to a logistic Hill equation as described in the Methods. Maximum cyclic AMP values were as follows: (a), 205±15 pmol per 10^6 cells; (b) 230±30 pmol per 10^6 cells; (c) 215±20 pmol per 10^6 cells. Basal values were all below 10 pmol per 10^6 cells. Part (d) includes the data from all experiments.

and 20 fold for [Glu[18]]CGRP$_{8-37}$; Table 2, Figure 3). However, all the measurements demonstrated a significant decrease in affinity for both derivatives compared to CGRP$_{8-37}$.

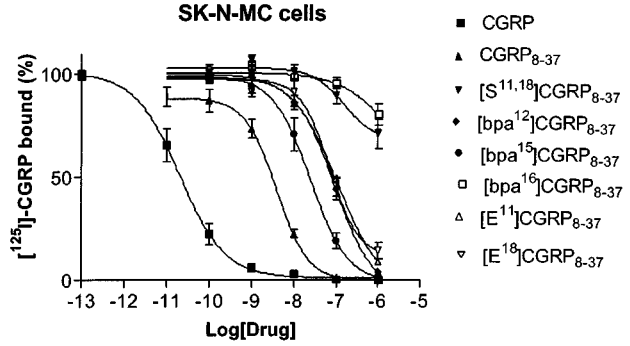

Figure 3 Displacement of [^{125}I]-hαCGRP by CGRP and derivatives. Each point represents mean$\pm$s.e.mean of three or four experiments. Maximum specific binding represents 0.5 fmol ligand bound mg^{-1} membrane protein.

Bpa-substitutents

The ability of [bpa[12]]-, [bpa[15]]- and [bpa[16]]CGRP$_{8-37}$ to inhibit CGRP action on SK-NMC (Figure 3) and L6 cells (Tables 1, 2, Figures 3–5) were examined. [Bpa[12]]CGRP$_{8-37}$ was selected for detailed analysis in SK-N-MC cells (Table 1, Figure 4). The slope of the Schild plot was not significantly different from 1 (0.85 ± 0.10); when constrained to unity it allowed a pK_b estimate of 7.43. This was in good agreement with the *pKi* estimated from binding studies of 7.14. On L6 cells, the apparent pA_2 estimated from a single antagonist concentration was 7.96 (Figure 5), in line with the values found on SK-N-MC cells. Based on the results with this compound, it was concluded that the bpa derivatives were likely to be competitive antagonists and that it should be possible to estimate their affinities from apparent pA_2's calculated from single dose-ratios and *pKi*'s measured in radioligand binding assays. Neither the maximum responses (92% to 103%) nor the Hill slopes of the concentration-response curves in the presence of the antagonists (0.67 ± 0.23 to 1.68 ± 0.32) were significantly different from the corresponding controls (0.58 ± 0.04 to 1.20 ± 0.20), consistent with this assumption. The pA_2/pK_b and *pKi* estimates for [bpa[12]]CGRP$_{8-37}$ were all

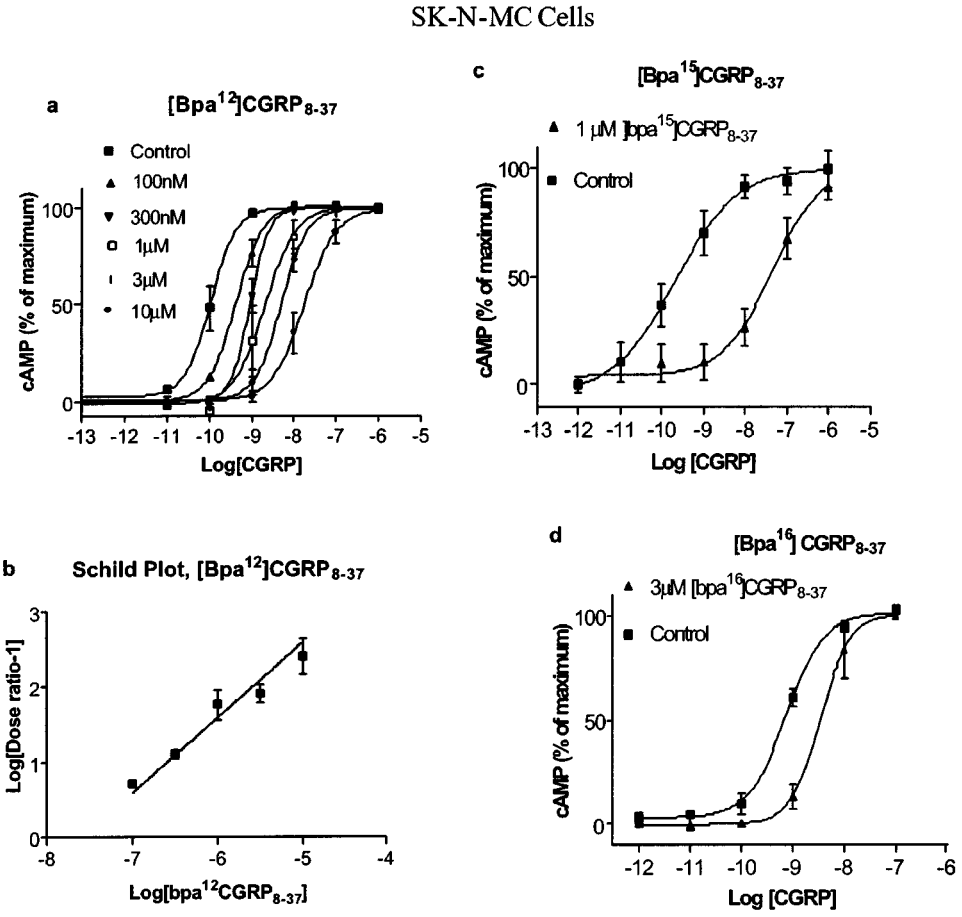

Figure 4 Effects of bpa derivatives on the stimulation of cyclic AMP production by hαCGRP in SK-N-MC cells. (a) [bpa[12]]-hαCGRP$_{8-37}$, (b) Schild plot for [bpa[12]]-hαCGRP$_{8-37}$, (c) [bpa[15]]-hαCGRP$_{8-37}$ and (d) [bpa[16]]-hαCGRP$_{8-37}$. Data represent mean$\pm$s.e.mean of 3–8 experiments. Points were measured in duplicate in each experiment. Data are expressed as percentage of maximum cyclic AMP production caused by CGRP alone, estimated by fitting each line to a logistic Hill equation as described in the Methods. Maximum cyclic AMP values were as follows: (a) 220 ± 25 pmol per 10^6 cells; (c) 255 ± 30 pmol per 10^6 cells; (d) 240 ± 35 pmol per 10^6 cells; basal values were all below 10 pmol per 10^6 cells.

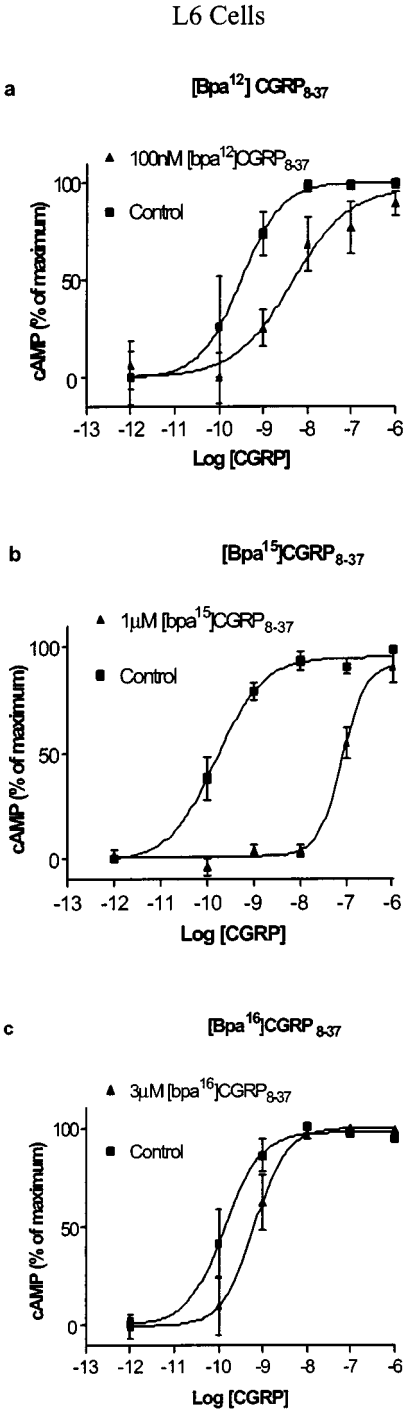

Figure 5 Effects of: (a) [bpa^{12}]-, (b) [bpa^{15}]- and (c) [bpa^{16}]-hαCGRP$_{8-37}$ on the stimulation of cyclic AMP production by human αCGRP in L6 cells. Data represent mean$\pm$s.e.mean of 3–6 experiments. Points were measured in duplicate in each experiment. Data are expressed as percentage of maximum cyclic AMP production caused by CGRP alone, estimated by fitting each line to a logistic Hill equation as described in the Methods. Maximum cyclic AMP values were as follows: (a) 245$\pm$40 pmol per 10^6 cells; (b) 265$\pm$30 pmol per 10^6 cells; (c) 255$\pm$45 pmol per 10^6 cells; basal values were all below 10 pmol per 10^6 cells.

significantly different from those for CGRP$_{8-37}$. [Bpa^{15}]CGRP$_{8-37}$ appeared slightly more potent than [bpa^{12}]CGRP$_{8-37}$; neither the apparent pA$_2$ on L6 cells nor the *pKi* on SK-N-MC membranes were significantly different from the corresponding values for CGRP$_{8-37}$ (Tables 1,2 Figures 3–5). By contrast, all the affinity estimates for [bpa^{16}]CGRP$_{8-37}$ on both cell lines showed that it was much reduced in potency compared to CGRP$_{8-37}$, being $\sim$500 fold less potent than CGRP$_{8-37}$ in the L6 cells and $\sim$2000 fold less potent in the SK-N-MC cells.

Discussion

The amphipathic helix formed by residues 8-18 in CGRP is an important factor in high affinity binding of the peptide to its receptor (Lynch & Kaiser, 1988). This paper provides new detail on how the helix fulfils this function.

The characteristics of the CGRP receptors expressed on L6 and SK-N-MC cells in this study are in agreement with previous work by ourselves and others (Van Valen *et al*., 1990; Muff *et al*., 1992; Semark *et al*., 1992; Longmore *et al*., 1994; Poyner *et al*., 1992; 1998; Howitt & Poyner, 1997). In radioligand binding studies, CGRP shows high affinity binding; the Hill slopes for it and its derivatives suggest they interact predominantly with a single class of sites. The pK_b values for CGRP$_{8-37}$ on SK-N-MC cells is higher than we have previously reported (7.49, Poyner *et al*., 1998) but it is in line with values found by other groups (8.7 to 8.9, Semark *et al*., 1992; Longmore *et al*., 1994). In the current study we have used a longer pre-incubation with antagonist (30 min as opposed to 5 min); this may be significant although other studies have suggested that CGRP$_{8-37}$ reaches equilibrium at its receptor within 5 min (Wisskirchen *et al*., 1998). We confirmed that CGRP$_{8-37}$, the glutamate-substituted analogues and [bpa^{12}]CGRP$_{8-37}$ all behaved as competitive antagonists.

Previous studies have shown that the Arg^{11} and Arg^{18} collectively play a key role in the high affinity binding of CGRP (Howitt & Poyner, 1997; Poyner *et al*., 1998). However, those studies did not address how the arginines worked in promoting high affinity binding. At least two models are possible. On the one hand, the arginines could act to reinforce the amphipathic nature of residues 8-18. This might be expected if the hydrophilic face of the helix faced the aqueous solvent, with the hydrophobic face making peptide–peptide or protein contact. In this case, replacement with other hydrophilic residues such as serine or glutamic acid would not be expected to cause a reduction in affinity. Indeed, the fact that 8-18 can be replaced by other amphipathic peptides with relatively little loss in affinity (Lynch & Kaiser, 1988; Poyner *et al*., 1998) might be considered as supporting this proposal. The alternative is that the arginines interact electrostatically with groups carrying either a full or partial negative charge, either elsewhere on the CGRP molecule or on the receptor. Replacement with the negatively charged glutamic acid will result in electrostatic repulsion and hence a decrease in affinity; serines, lacking full charges will also not fully promote these interactions. It is this latter pattern that is observed, suggesting that Arg^{11} and Arg^{18} are involved in well-defined electrostatic interactions.

Previously, it had been shown that the single replacement of Arg^{11} with alanine produced, at most, only a 6 fold decrease in affinity; replacement of Arg^{18} alone had a negligible effect on affinity (Mimeault *et al*., 1991; Howitt &

Poyner, 1997; Poyner *et al*., 1998). The most likely explanation for this pattern of activity is that, whilst both arginines are involved in charge–charge interactions, there is redundancy so that one or other of these can be disrupted with minimal loss of affinity. When the amphipathic peptide mastoparan is substituted for residues 8-18, the two arginines are replaced by lysines, suggesting the reason why this derivative remains active (Poyner *et al*., 1998). The introduction of electrostatic repulsion at either site is sufficient to produce larger changes in affinity, so that marked affinity decreases are seen with either of the singly substituted derivatives.

In principle, Arg^{11} and Arg^{18} may be involved in ionic interactions or hydrogen bonds. [$Ser^{11,18}$]$CGRP_{8-37}$ has the potential to form hydrogen bonds and this binds much more weakly than CGRP8-37. However, the hydrogen bonds formed by serine are likely to be weaker than those formed by arginine. Not only will there be a decrease in coulombic interactions when replacing the positively charged arginine by the neutral serine, but as the side chain of serine is much smaller than that of arginine, it is very unlikely that the serines will be as favourably placed sterically as the arginines to maintain a hydrogen bond interaction. The decrease in free energy of binding seen when [$Ser^{11,18}$]$CGRP_{8-37}$ is used instead of $CGRP_{8-37}$ is about 12KJ per mole. This value is consistent with either loss of hydrogen bonds or weak ionic interactions (where the charges are shielded by water molecules and other ions in solution) (Ferscht, 1977; Howell *et al*., 1986). Thus the arginines may be involved in either of these interactions. If they are involved in hydrogen bonds, these could be between acceptors either on the receptor or elsewhere on $CGRP_{8-37}$. There is evidence for interactions between the 8-18 helix and the C-terminus of $CGRP_{8-37}$; replacement of Ser^{17} by alanine increases potency 2 fold but this can be correlated with changes in the structure of residues 28–37 (Boulanger *et al*., 1995; 1996). If however ionic interactions are involved, these must be between 8-18 and the receptor, as there is no negatively charged residue in $CGRP_{8-37}$.

The bpa derivatives give information on the hydrophobic face of the 8-18 helix. Although care must be taken not to over-interpret this data, as apparent pA_2 values were estimated from single dose-ratio measurements, there is agreement between these values measured on both L6 and SK-N-MC cells and pKi values estimated from radioligand binding. Three leucine residues were replaced by the larger bpa moiety.

The most dramatic changes were seen with [bpa^{16}]$CGRP_{8-37}$, with over a 100 fold decrease in potency. This suggests that Leu^{16} sits in a conformationally restricted space. No such constraints were apparent for the adjacent residue Leu^{15}. A small decrease was seen when Leu^{12} was substituted, but this was much less marked than that for Leu^{16}. If Leu^{16} is substituted by alanine there is little change in affinity (Wisskirchen *et al*., 1999; 2000), indicating that any contribution from the packing energy of the side chain is not by itself crucial to the binding of $CGRP_{8-37}$. As with the arginines, it is difficult to say whether bpa^{16} interacts with either the receptor or another portion of $CGRP_{8-37}$. However, it is difficult to envisage how both faces of the 8-18 helix could simultaneously interact with the rest of $CGRP_{8-37}$, and so it is likely that either the arginines or Leu^{16} are in proximity to the receptor.

All the derivatives show similar potency reductions on the $CGRP_1$ receptor of the SKN-MC and L6 cells. Thus the 8-18 helix is not particularly sensitive to species differences between human and rat. This is consistent with the observation that $CGRP_{8-37}$ itself has a similar affinity at both rat and human CGRP receptors. By contrast the low molecular weight CGRP antagonist BIBN4096BS shows well over 100 fold higher affinity for the human $CGRP_1$ receptors compared to that of the rat (Doods *et al*., 2000). Human and rat CRLR have over a 91% identity (Njuki *et al*., 1993; Flühmann *et al*., 1995) whereas the two RAMP1s show only 71% identity (Foord *et al*., 2000). It has been shown that the species selectivity of BIBN4096BS is due to a single residue in RAMP1 (Mallee *et al*., 2002). The 8-18 helix must be promoting high affinity CGRP binding by (directly or indirectly) interacting with a more conserved part of the CRLR/RAMP1 heterodimer.

It is possible to integrate the results of the present study with other work to build a fuller picture of the key features of the 8-18 helix. Alanine scans have revealed that replacement of any of the first three residues (Val^8, Thr^9, His^{10}) causes no more than a 3 fold potency reduction; however, their complete removal causes a 10 fold reduction in affinity (Mimeault *et al*., 1991; 1992). Thus within the first turn of the helix, no single residue is of particular importance, but collectively they are significant, perhaps in stabilising a helical conformation (Mimeault *et al*., 1992). Wisskirchen *et al*., (1999; 2000) demonstrated that substitution of His^{10} and Gly^{14} with aspartic acid residues caused a large drop in the affinity of $CGRP_{8-37}$. As it has also been shown that Gly^{14} can be replaced by aspartic acid with less than a 2 fold affinity shift (Li *et al*., 1997), it seems that the replacement of the electropositive His^{10} was mainly responsible for the affinity decrease. The pattern of activity seen with His^{10} is similar to that seen with Arg^{18}, suggesting that the two may have similar roles in interacting with electronegative residues. Arg^{11} is involved in a coulombic interaction but Leu^{12} is not subject to stringent steric constraints. A study on Gly^{14} suggests that it can be substituted freely by charged and non-polar amino acids (Li *et al*., 1997). Leu^{15} is not subject to spatial constraints but Leu^{16} is in a conformationally restricted environment. Ser^{17} plays a minor role, perhaps in stabilising the C-terminus of CGRP (Boulanger *et al*., 1995; 1996) but Arg^{18} is involved in a charge–charge interaction. Thus, it appears that the residues highlighted in this study, Arg^{11}, Leu^{16} and Arg^{18} are of particular significance. It will be important to discover how they interact with either the receptor or with other parts of CGRP.

This work was supported by the British Heart Foundation and the Wellcome Trust. We thank Dr J. Longmore of Merck, Sharpe and Dohme (U.K.) for the gift of [$Ser^{11,18}$]$CGRP_{8-37}$ and Dr C.H. Schwalbe and Prof. I.L. Martin for discussions about the results.

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(Received September 13, 2002
Accepted October 9, 2002)