British Journal of Pharmacology (1998) 124, 1659–1666
http://www.stockton-press.co.uk/bjp

Structural determinants for binding to CGRP receptors expressed by human SK-N-MC and Col 29 cells: studies with chimeric and other peptides

David R. Poyner[1,4], Ursel Soomets[2,3], Stephen G. Howitt[1] & Ulo Langel[3]

[1]Pharmaceutical Sciences Institute, Aston University, Aston Triangle, Birmingham, B4 7ET, UK; [2]Department of Biochemistry, Tartu University, EE 2400 Tartu, Estonia and [3]The Arrhenius Laboratories for Natural Sciences, Department of Neurochemistry and Neurotoxicology, Stockholm University, S-10691 Stockholm, Sweden

1 Structure-activity relationships for the binding of human α-calcitonin gene-related peptide 8–37 (hαCGRP$_{8-37}$) have been investigated at the CGRP receptors expressed by human SK-N-MC (neuroblastoma) and Col 29 (colonic epithelia) cells by radioligand binding assays and functional assays (hαCGRP stimulation of adenylate cyclase).

2 On SK-N-MC cells the potency order was hαCGRP$_{8-37}$ > hαCGRP$_{19-37}$ = AC187 > rat amylin$_{8-37}$ > hα[Tyr^0]-CGRP$_{28-37}$ (apparent pKBs of 7.49 ± 0.25, 5.89 ± 0.20, 6.18 ± 0.19, 5.85 ± 0.19 and 5.25 ± 0.07). The SK-N-MC receptor appeared CGRP$_1$-like.

3 On Col 29 cells, only hαCGRP$_{8-37}$ of the above compounds was able to antagonize the actions of hαCGRP (apparent pKB = 6.48 ± 0.28). Its receptor appeared CGRP$_2$-like.

4 hα[Ala^{11,18}]-CGRP$_{8-37}$, where the amphipathic nature of the N-terminal α-helix has been reduced, bound to SK-N-MC cells a 100 fold less strongly than hαCGRP$_{8-37}$.

5 On SK-N-MC cells, hαCGRP$_{8-18,\ 28-37}$ (M433) and mastoparan-hαCGRP$_{28-37}$ (M432) had apparent pKBs of 6.64 ± 0.16 and 6.42 ± 0.26, suggesting that residues 19–27 play a minor role in binding. The physico-chemical properties of residues 8–18 may be more important than any specific side-chain interactions.

6 M433 was almost as potent as hαCGRP$_{8-37}$ on Col 29 cells (apparent pKB = 6.17 ± 0.20). Other antagonists were inactive.

Keywords: Calcitonin gene-related peptide; mastoparan; CGRP$_1$ receptor; CGRP$_2$ receptor; CGRP antagonist; chimeric peptide

Introduction

CGRP is a widespread 37 amino acid peptide (Poyner, 1992). Its receptors have been divided into two classes: CGRP$_1$ and CGRP$_2$. CGRP$_1$ receptors have a high affinity for the peptide antagonist CGRP$_{8-37}$, whereas CGRP$_2$ receptors bind this compound at least an order of magnitude less strongly (Poyner, 1995; Quirion *et al*., 1992). This classification is not without controversy; for example there are species variation in the affinities reported for CGRP$_{8-37}$ and in radioligand binding studies it interacts with both classes of receptors with a high affinity (Poyner, 1995; Dennis *et al*., 1990). However, the classification does succeed in accommodating much existing functional data on CGRP$_{8-37}$ and is a useful working model.

The structure-activity relationship for CGRP when interacting with CGRP$_1$ receptors has been addressed in a number of studies (e.g. Dennis *et al*., 1989, 1990; Poyner *et al*., 1992; Mimeault *et al*., 1992). Briefly, these suggest that high affinity binding requires residues 8–37. This consists of an amphipathic α-helix (residues 8–18), an area with little regular structure (19–27) and the C-terminal (28–37) which possibly contains a β-turn. The conformation of the C-terminus may be particularly important in determining the ability of the peptide to interact with its receptor (Wisskirchen *et al*., 1997; Boulanger *et al*., 1996). This work has largely been carried out on rat and guinea-pig CGRP receptors. Although something is known of the importance of both the helical region and C-terminus of CGRP, the role (if any) of the middle section of the molecule is obscure. Very little work has been done on the structure-activity relationship of CGRP$_2$ receptors.

A common model system for human CGRP receptor is provided by the neuroblastoma derived cell line, SK-N-MC (Van Valen *et al*., 1990). These receptors have been shown to bind CGRP$_{8-37}$ with a pKB in excess of 7, consistent with them belonging to the CGRP$_1$ subclass (Semark *et al*., 1992; Muff *et al*., 1992; Longmore *et al*., 1994; Zimmerman *et al*., 1995). It has been suggested that this receptor is likely to be similar in pharmacology to the rat CGRP$_1$-like receptor although this has not been studied in depth. Little is also known of human CGRP$_2$ receptors. The Col 29 cell line is derived from human colonic epithelium and expresses CGRP receptors linked to adenylate cyclase (Kirkland, 1985; Poyner *et al*., 1993). It has been reported that these are insensitive to CGRP$_{8-37}$, but their pharmacology has not been examined in detail (Cox & Tough, 1994).

Relatively recently there has been interest in the properties of chimeric peptides as tools for studying peptide-receptor interactions (Howl *et al*., 1997). Thus galanin$_{1-12}$-neuropeptide Y$_{25-36}$ shows high affinity binding to galanin receptors and neuropeptide Y Y2 receptors (Kahl *et al*., 1994); and galanin$_{1-13}$-bradykinin$_{2-9}$ is a galanin agonist at low concentrations in RIN-M-5F cells, but an antagonist at high concentrations (Kask *et al*., 1995). Mastoparan chimeras have also been synthesized. Galanin$_{1-13}$-mastoparan-amide binds with high affinity to galanin receptors (Langel *et al*., 1996) but

[4] Author for correspondence.

also has actions to release insulin that are independent of either galanin or mastoparan receptors (Ostenson *et al*., 1997). There have been no reports on the activity of chimeric peptides based on CGRP.

The chief aim of this study was to probe the relative importance of the different parts of the CGRP molecule in promoting binding to human $CGRP_1$- and $_2$-like receptors. This has been done by a variety of substituted and chimeric peptides, including mastoparan-CGRP hybrids (Figure 1). In order to do this, it has also been necessary to carry out a detailed pharmacological analysis of the CGRP receptors on SK-N-MC and Col 29 cells. The data demonstrates that only a relatively short stretch of the CGRP sequence is necessary for high affinity binding, provided this is combined with an amphipathic helix.

Methods

Cell culture

SK-N-MC cells were grown in a 1 : 1 mixture of Dulbecco's modified Eagle medium: Ham's F12 medium supplemented with 10% foetal calf serum. Col 29 cells were grown in Dulbecco's modified Eagle medium supplemented with 5% foetal calf serum. Both cell lines were grown in a humid atmosphere containing 5% CO_2 at 37°C and passaged with 0.1% trypsin when confluent. Fresh stocks were used after approximately 10 passages (Col 29) or 20 passages (SK-N-MC).

Measurement of cyclic AMP

Cells were grown to confluence in 24 well plates and then the medium exchanged for Krebs solution supplemented with the following (mM): HEPES, 20 (pH 7.4), isobutyl methyl xanthine, 1 and 0.1% bovine serum albumin. They were allowed to pre-incubate in this for 30 min before addition of antagonist (where appropriate). After 5 min, hαCGRP was added to the wells and 5 min later, incubations were terminated by replacing the medium with cold buffer containing (mM): EDTA, 5, HEPES, 20 (pH 7.5) and boiling for 5 min. The cyclic AMP so extracted was assayed by a radioreceptor assay, as described elsewhere (Poyner *et al*., 1992).

Radioligand binding assays

SK-N-MC cells were grown to confluence in 9 cm Petri dishes and membranes were prepared and stored frozen as described previously (Poyner *et al*., 1992). They were resuspended in buffer containing (mM) NaCl, 100; bacitracin, 0.4 mM; HEPES (pH 7.5), 20; 0.1% bovine serum albumin and incubated with approximately 0.1 nM [^{125}I]-iodohistidyl human α-CGRP and antagonists in silanized microcentrifuge tubes for 30 min, followed by centrifugation, as described previously (Poyner *et al*., 1992).

Peptide synthesis

The peptides were synthesized in a stepwise manner in a 0.1 mm mol scale on an Applied Biosystem Model 431A peptide synthesizer on solid support using a dicyclohexylcarbodiimide/hydroxybenzotriazole activation strategy. *tert*-Butyloxycarbonyl amino acids were coupled as hydroxybenzotriazole esters to a *p*-methylbenzylhydrylamine resin (1.1 mmol of amino groups per g, Bachem, Switzerland) to obtain C-terminally amidated peptides. Deprotection of the side chains from formyl and benzyl groups was carried out using the 'low TFMSA' method (Tam *et al*., 1985). The protecting groups on histidine(DNP) were removed by treatment for 1 h at room temperature with 20% (v/v) thiophenol/dimethylformamide. The peptides were finally cleaved from the resin with liquid hydrofluoric acid at 0°C for 30 min. Deprotection of the side chains, cleavage of the peptides and purification on high pressure liquid chromatography (h.p.l.c.) were as described in detail earlier (Langel *et al*., 1992). The purity of the peptides was >99% as demonstrated by h.p.l.c. on an analytical Nucleosil 120–3 C_{18} reverse-phase h.p.l.c. column (0.4 cm × 10 cm). The molecular mass of each synthetic peptide was determined with a Plasma Desorption Mass Spectrometer (Bioion 20, Applied Biosystems, Warrington, UK) and the calculated values were obtained in each case.

Data analysis

Binding curves were fitted by the routine EBDA-LIGAND to obtain estimates of pKd and Hill coefficient. Concentration-response curves were normalized by expressing stimulations as a percentage of that obtained in the presence of 100 nM CGRP (SK-N-MC cells; see Figure 1, Figure 6a) or 300 nM CGRP (Col 29 cells, see Figure 5a). Absolute accumulations of cyclic AMP varied from 160–235 pmol per 10^6 cells (SK-N-MC cells) and 100–145 pmol per 10^6 cells (Col 29 cells). The curves were analysed to obtain pEC_{50} values in the presence and absence of antagonists. Apparent pKB values were calculated from equation $pK_B = \log_{10}((\text{dose ratio} - 1)/[\text{antagonist}])$. Although multiple concentrations were not used, it was

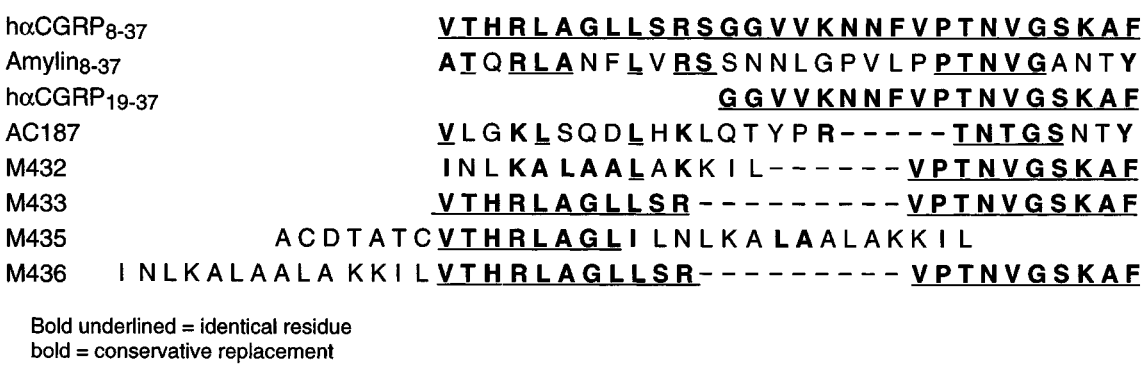

Figure 1 Structures of peptides used in the present study. All peptides are amidated at the C-terminus.

assumed that the Schild plot would have a slope of one; this is the case for the parent antagonist, h$\alpha$$CGRP_{8-37}$ (Poyner *et al.*, 1992).

Statistical analysis was by Student's *t*-test, accepting significance at the $P<0.05$ level. All errors quoted represent s.e.mean.

Drugs

hαCGRP was obtained from Calbiochem and mastoparan from Sigma. Other peptides were synthesized as described above. All CGRP derivatives were based on the structure of hαCGRP. Other reagents were obtained as described previously (Poyner *et al.*, 1992).

Results

Basic characterization of the SK-N-MC cell CGRP receptor

As expected, hαCGRP stimulated cyclic AMP production with a pEC_{50} of about 8. (Figure 2) In the presence of 1 μM $CGRP_{8-37}$, this concentration-response curve was shifted to the right (Figure 2a), consistent with competitive antagonism and from the resulting dose-ratio, an apparent pKB for $CGRP_{8-37}$ of 7.49 ± 0.25 was calculated (Table 1). Preliminary experiments showed that the addition of a cocktail of protease inhibitors (0.4 mM bacitracin, 10 μM thiorphan, 0.1 mg ml leupeptin, 0.1 mg ml aprotinin, 0.1 mg ml pepstatin) made no difference to these results (Figure 3a). $CGRP_{19-37}$, rat $amylin_{8-37}$ and AC187 (an amylin antagonist) also produced rightwards shifts in the concentration-response curve for CGRP, but they were 20–40 fold less potent than $CGRP_{8-37}$ (Figure 2, Table 1). [Tyr^0]-$CGRP_{28-37}$ was nearly 200 fold less potent than $CGRP_{8-37}$.

Binding experiments were carried out on membranes prepared from SK-N-MC cells and these essentially gave similar results to those obtained from the functional studies. CGRP binding was of high affinity, but the Hill coefficient was significantly less than 1, suggesting the presence of high and low affinity sites (Figure 4a, Table 1). The displacement curve could be resolved into two components; 27% with an affinity of 0.23 nM and the remainder with an affinity of 9.35 nM. In the presence of 0.1 mM guanylyl-imidodiphosphate (GppNHp) specific binding was reduced by $60\pm3\%$ ($n=3$). The other antagonists all bound with Hill coefficients not significantly different from unity, indicating that they interacted with a single population of sites. $CGRP_{8-37}$ had the highest affinity of the antagonists, followed by AC187 and then $CGRP_{19-37}$ and $amylin_{8-37}$ (Figure 4a, Table 1).

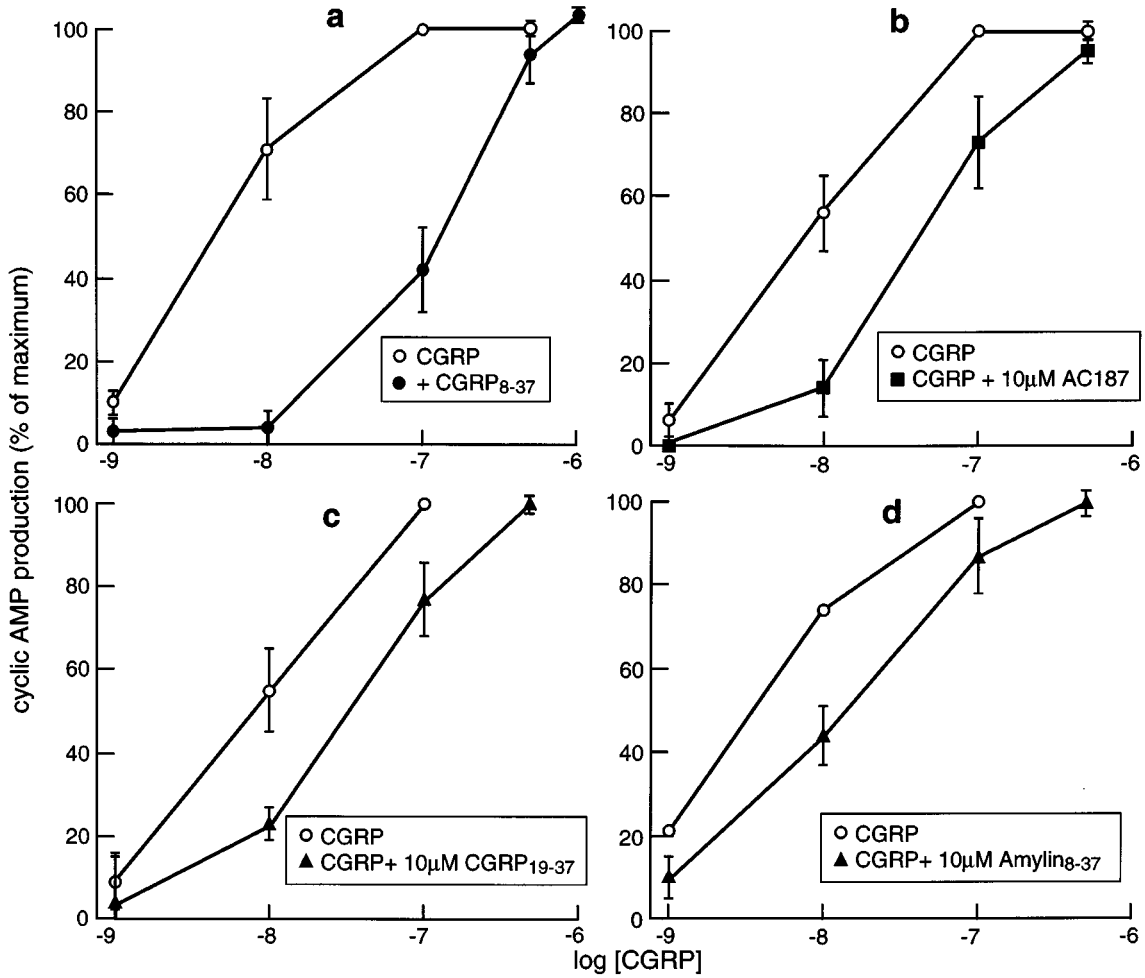

Figure 2 Stimulation of cyclic AMP production in SK-N-MC cells by CGRP in the absence and presence of (a) $CGRP_{8-37}$, (b) AC187, (c) $CGRP_{19-37}$ and (d) $amylin_{8-37}$. Points represent mean$\pm$s.e.mean of 3–5 determinations. Cyclic AMP levels (pmol per 10^6 cells) in these experiments ranged as follows: 65–75 (resting), 260–300 (stimulated).

Table 1 Potencies of CGRP and amylin antagonists at the CGRP receptors of SK-N-MC and Col 29 cells

Compound	*SK-N-MC* pKd/n_H, *binding*	*Apparent pKB*	*Col 29* *Apparent pKB*
CGRP	8.03±0.12/0.63±0.13 (3)		
$CGRP_{8-37}$	7.29±0.18/1.42±0.20 (4)	7.49±0.25 (5)	6.48±0.28 (6)
$CGRP_{19-37}$	6.23±0.13/1.07±0.06 (3)	5.89±0.20 (3)	<5.25
$[Tyr^0]$-$CGRP_{28-37}$	ND	5.25±0.07 (3)	ND
AC187	6.78±0.08/1.10±0.10 (4)	6.18±0.19 (3)	<5.25
$Amylin_{8-37}$	5.60±0.07/1.59±0.72 (3)	5.85±0.19 (3)	<5.25

ND, Not determined: n_H, Hill coefficient. Values are means±s.e.mean, number of determinations in brackets.

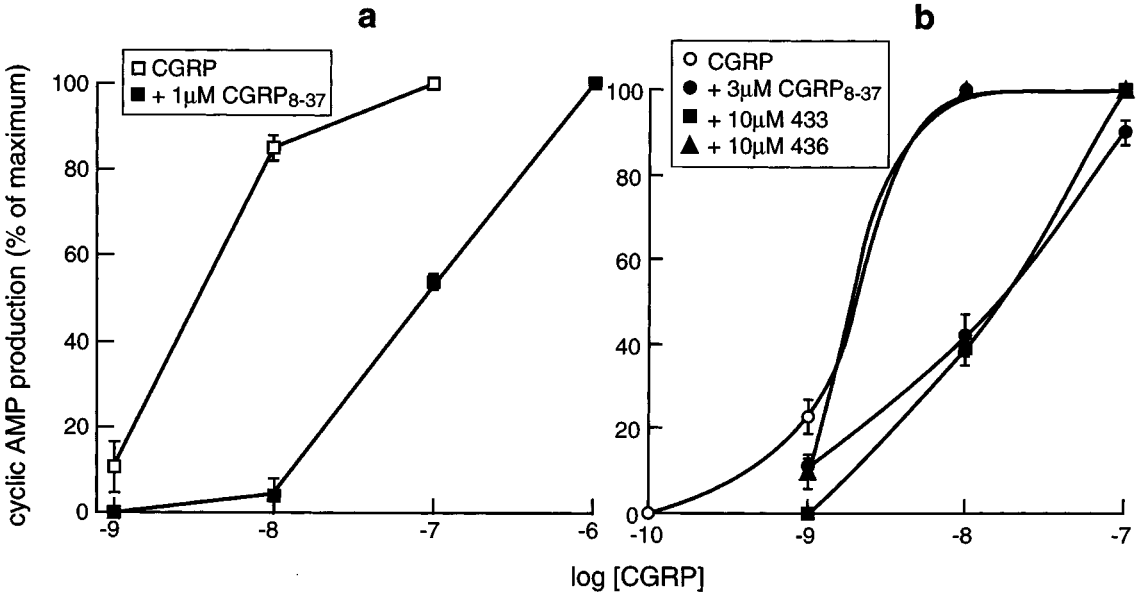

Figure 3 Stimulation of cyclic AMP production by CGRP in the presence of protease inhibitors in (a) SK-N-MC cells, (b) Col 29 cells. Points represent means±s.e.mean of three determinations. Cyclic AMP levels (pmol per 10^6 cells) were as follows: SK-N-MC cells, <10 (resting) and 190±20 (stimulated); Col 29 cells, <10 (resting) and 205±15 (stimulated).

Characterization of the Col 29 CGRP receptor

As previously reported, CGRP increased cyclic AMP levels within Col 29 cells with a pEC_{50} of about 8. $CGRP_{8-37}$ at 1 and 3 μM produced progressive rightward shifts in the CGRP concentration-response curve, consistent with a pKB of 6.48±0.28 (Figure 5a, Table 1). All other agents were inactive. Addition of the same cocktail of peptidase inhibitors as used with the SK-N-MC cells caused a fourfold enhancement of CGRP potency, but failed to increase the potency of any of the putative antagonists (Figure 3b).

It did not prove possible to obtain satisfactory [^{125}I]-CGRP binding to Col 29 cell membranes, due to the low level of specific binding (<20% of total).

Activity of substituted and chimeric CGRP derivatives

The role of the different sections of the CGRP molecule were examined in a series of derivatives (Table 2; see Figure 1 for structures). The amphipathicity of the section 8–18 was varied by substituting arginines at positions 11 and 18 with alanines. On SK-N-MC cells, single substituents made little difference to binding, but the affinity of the double substituent was reduced about 100 fold relative to $CGRP_{8-37}$. (Table 2, Figure 4b). The role of residues 19–27 were investigated with M433, $CGRP_{8-18,\ 28-37}$. This had an affinity intermediate between $CGRP_{8-37}$ and $CGRP_{19-37}$ (Table 2, Figures 4c and 6b). The specific role of the C-terminus was investigated by substituting residues 8–18 with the amphipathic peptide mastoparan to give M432 (mastoparan-$CGRP_{28-37}$). This was of similar affinity to M433. M436 (mastoparan-$CGRP_{8-18,\ 28-37}$) had an extended N-terminus but it behaved similarly to the parent compound M433 (Figure 6). Mastoparan at 3 μM did not antagonize the responses to CGRP (pEC_{50}s; control=8.02±0.14, with mastoparan=7.75±0.03, $n=3$). The key determinants for CGRP receptor activation are likely to be found in the N-terminus of the molecule. Accordingly M435 ($CGRPD_{1-15}$-mastoparan-amide) was tested. It bound to SK-N-MC cells with low affinity (Table 2), but at concentrations of up to 10 μM, it failed to cause any reproducible activation of adenylate cyclase.

The compounds were also tested on Col 29 cells. Both $[Ala^{11}]$-$CGRP^{8-37}$ and $[Ala^{11,18}]$-$CGRP_{8-37}$, were inactive at 10 μM, the highest concentration tested. Of the other antagonists, only M433 showed antagonist activity at 10 μM, the highest concentration it was feasible to use (Figure 5). M435 failed to activate adenylate cyclase.

Discussion

This paper provides new information on the selectivity of the human $CGRP_1$-like receptor, the first detailed account of the pharmacology of a human $CGRP_2$-like receptor and also

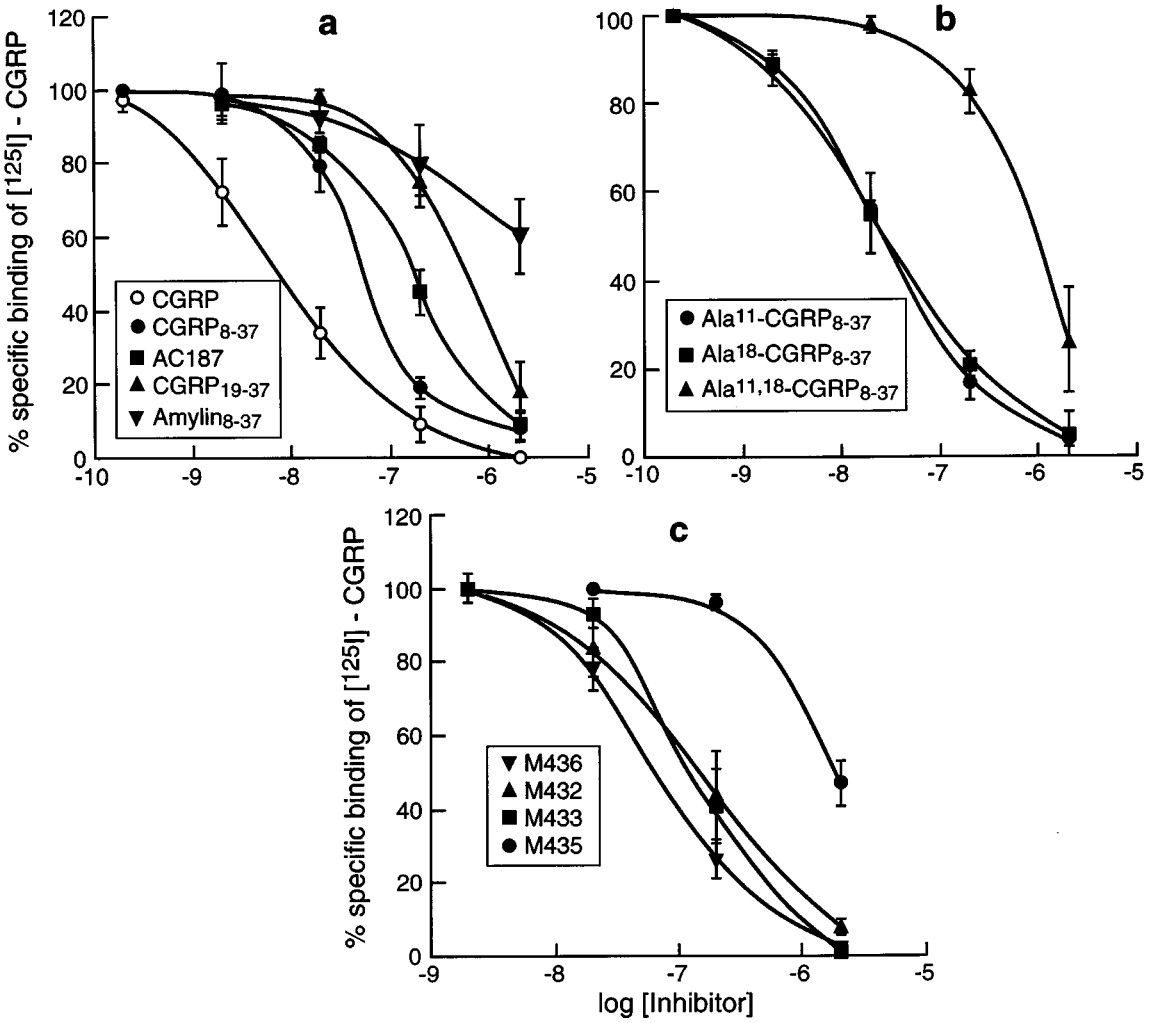

Figure 4 Displacement of 0.1 nM [^{125}I]-iodohistidyl CGRP by (a) human α-CGRP, $CGRP_{8-37}$, AC187, $CGRP_{19-37}$ and $amylin_{8-37}$; (b) [Ala^{11}]-$CGRP_{8-37}$, [Ala^{18}]-$CGRP_{8-37}$ and [$Ala^{11,18}$]-$CGRP_{8-37}$; (c) M432, M433, M435 and M436. Points represent means ± s.e.mean of three to four determinations.

provides new insights into the role of the N-terminal amphipathic α-helix of CGRP in promoting high affinity binding to its receptors.

Previous workers have examined the actions of CGRP and $CGRP_{8-37}$ at SK-N-MC cells and their results are broadly in line with the data of the present study (Van Valen *et al.*, 1990; Semark *et al.*, 1992; Muff *et al.*, 1992; Longmore *et al.*, 1994; Zimmerman *et al.*, 1995; Barrett *et al.*, 1997). CGRP binds with high affinity to its receptor on these cells and $CGRP_{8-37}$ is a potent antagonist. In this study, CGRP binding appears to be heterogeneous. This may reflect partial coupling of receptor to G-protein and G-protein coupling is consistent with the actions of GppNHp in reducing CGRP binding. However in the cerebellum an additional conformational state of the receptor has also been postulated (Chatterjee & Fisher, 1991). As a consequence of the heterogeneity, no attempt has been made to convert IC_{50} values for the other inhibitors into Kd values. Despite this, there is generally good agreement between the IC_{50}s estimated from the binding assays and the affinity constants estimated from the functional assays. The detailed pharmacology of the receptor is similar to the $CGRP_1$-like receptor found on rat, L6 myocytes (Howitt & Poyner, 1997), consistent with them having a close relationship.

There are a number of differences of detail between this and previous studies. Thus, Semark *et al.* (1992) reported that CGRP bound with sub-nanomolar affinity to a single class of binding sites, whereas in this study two subclasses of sites are present. This may be a consequence of the different conditions used in each study (e.g. Semark *et al.* used a buffer containing 5 mM $MgCl_2$, which in other systems can promote receptor G-protein coupling [Hulme *et al.*, 1983]). The apparent pKB for $CGRP_{8-37}$ found in this study is low compared with the values of 8.7 and 8.9 reported previously (Semark *et al.*, 1992; Longmore *et al.*, 1994). However, these values were obtained on cell membrane fragments using buffers containing 5 mM $MgCl_2$. The affinity of the CGRP receptor for $CGRP_{8-37}$ is known to be sensitive to experimental conditions (Poyner *et al.*, 1992).

M433, $CGRP_{8-18,28-37}$, was tested in an attempt to assess the importance of the middle of the molecule. Deletion of this section did lead to a reduction in affinity of between two and seven fold, as measured either by radioligand binding or functional assays. However, this should be compared with the 11–20 fold reduction seen on deletion of the residues 8–18. Whilst residues 19–27 clearly have a role in promoting high affinity binding, they would appear of less relative importance than other parts of the CGRP molecule. There is evidence that residues 19–21 form part of a bend in CGRP (Sagoo *et al.*, 1991) and it may be that the absence of this structure largely accounts for the decreased affinity shown by M433 compared to $CGRP_{8-37}$.

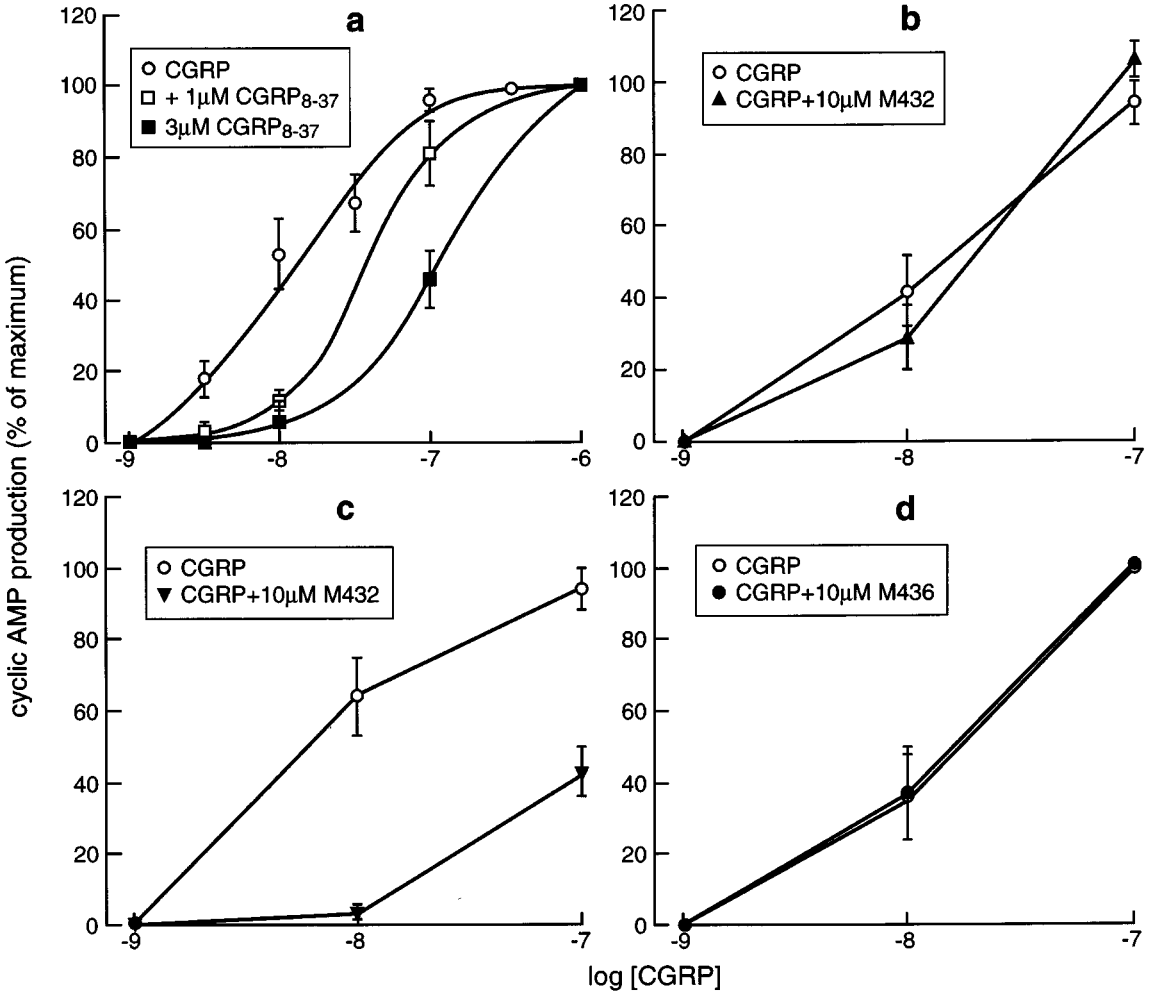

Figure 5 Stimulation of cyclic AMP production in Col 29 cells by CGRP in the absence and presence of (a) $CGRP^{8-37}$, (b) M432, (c) M433 and (d) M436. Points represent means ± s.e.mean of three to six determinations. Cyclic AMP levels (pmol per 10^6 cells) in these experiments ranged as follows: 11–31 (resting), 140–155 (stimulated).

Table 2 Affinities of substituted and chimeric CGRP analogues at the CGRP receptors of SK-N-MC and col 29 cells

Compound	*SK-N-MC* pKd/n_H, *binding*	*Apparent pKB*	*Col 29* *Apparent pKB*
$[Ala^{11}]$-$CGRP_{8-37}$	7.51 ± 0.08/1.28 ± 0.30 (3)	ND	< 5.25
$[Ala^{18}]$-$CGRP_{8-37}$	7.55 ± 0.18/0.75 ± 0.05 (4)	ND	< 5.25
$[Ala^{11,18}]$-$CGRP_{8-37}$	6.60 ± 0.33/0.87 ± 0.25 (3)	ND	ND
M432	6.87 ± 0.13/1.18 ± 0.38 (4)	6.64 ± 0.16 (3)	< 5.25
M433	6.92 ± 0.27/1.06 ± 0.18 (4)	6.42 ± 0.26 (3)	6.17 ± 0.20 (3)
M435	5.77 ± 0.13/1.17 ± 0.12 (4)	NA	NA
M436	7.51 ± 0.18/0.93 ± 0.13 (4)	6.19 ± 0.37 (3)	< 5.25

ND, Not determined: NA, not applicable. Values are means ± s.e.mean, number of determinations in brackets.

M432, mastoparan-$CGRP_{28-37}$, is particularly interesting. It emphasizes the potential importance of the C-terminus of CGRP for high affinity binding. It behaves essentially identically to M433, despite the substitution of CGRP residues 8–18 by mastoparan. Mastoparan, $[Tyr^0]$-$CGRP_{28-37}$ and $CGRP_{28-37}$ (Muff *et al.*, 1992) do not show such high affinity interactions with the CGRP receptor. On the best alignment between mastoparan and the N-terminus of CGRP there is 1/13 identical residues, with a further seven conservative replacements. The chief similarity between the peptides is their amphipathic nature. The actual sequence of residues 8–18 may be of minor importance in CGRP binding compared to its overall physico-chemical properties. This is reinforced by the results from replacing the arginines at positions 11 and 18 by alanines. This greatly reduces both amphipathicity and binding. (We have previously reported similar findings for the $CGRP_1$-like receptor on rat L6 cells [Howitt & Poyner, 1997].) The optimum length for the amphipathic helix is probably about 10 amino acids, as M436, mastoparan-$CGRP_{8-18,28-37}$ had a similar affinity to mastoparan-$CGRP_{28-37}$.

M432 represents a modified decapeptide CGRP fragment but with an affinity for the CGRP receptor previously only seen in longer CGRP fragments. Of course, the modification consists of the addition of a substantial peptide chain, but it

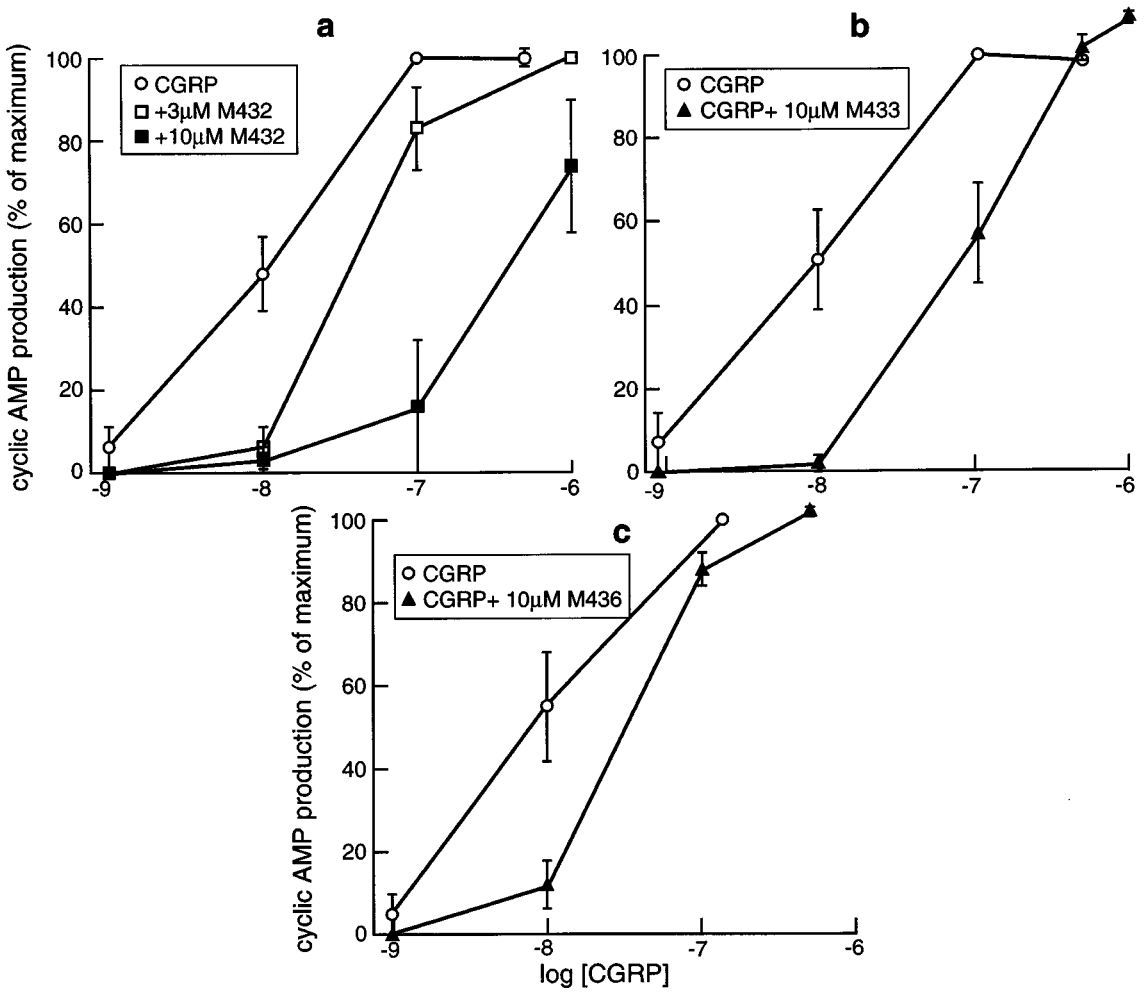

Figure 6 Stimulation of cyclic AMP production in SK-N-MC cells by CGRP in the absence and presence of (a) M432, (b) M433 and (c) M436. Points represent means ± s.e.mean of three to four determinations. Cyclic AMP levels (pmol per 10^6 cells) in these experiments ranged as follows: < 10 (resting), 163 – 195 (stimulated).

will be of considerable interest if residues 8 – 18 can also be replaced by a bulky amphipathic non-peptide substituent.

Other chimeric peptides involving mastoparan have a variety of actions either as a result of the mastoparan moiety, or entirely novel in origin (e.g. Ostenson *et al.*, 1997). Indeed, mastoparan can inhibit forskolin-induced cyclic AMP accumulation in PC12 cells (Murayama *et al.*, 1996). As mastoparan itself failed to inhibit the response to CGRP and the mastoparan-CGRP chimeras were able to displace specifically bound CGRP from SK-N-MC membranes, it is unlikely that they antagonize CGRP by any indirect mechanism. However no attempt has been made to investigate non-CGRP related activities of M432 or M436 and they may have additional properties. Accordingly some caution is required in their use.

The role of the C-terminus of CGRP as a crucial determinant in high affinity binding to its receptor has received support from a number of sources. Thus Boulanger *et al.* (1996) were able to correlate the n.m.r. structure of this part of the molecule and several derivatives with their binding affinities. A recent study has shown that a derivative of $h\alpha CGRP_{27-37}$ has a Ki of 29 nM in radioligand binding studies (Rist *et al.*, 1998). An alanine scan of residues 28 – 37 of $CGRP_{8-37}$ has shown that several specific residues are important for binding to the $CGRP_1$-like receptor of rat L6 cells (Howitt *et al.*, 1998). We have preliminary data that shows a similar pattern for SK-N-MC cells (apparent pKBs for $[Ala_{37}]$-$CGRP_{8-37} = 6.96 \pm 0.3$, $[Ser_{36}]$-$CGRP_{8-37} = 7.42 \pm 0.15$, $[Ala_{35}]$-$CGRP_{8-37} = 6.15 \pm 0.15$, $[Ala_{34}]$-$CGRP_{8-37} = 6.95 \pm 0.25$, all $n = 3$).

The Col 29 CGRP receptor is clearly distinct from that found in the SK-N-MC cells. It is much less sensitive to most antagonists and fits well into the $CGRP_2$ classification. It is interesting to note that M433, $CGRP_{8-18,28-37}$, is only slightly less potent than $CGRP_{8-37}$, suggesting that residues 19 – 27 are of even less significance for binding to $CGRP_2$-like receptors than $CGRP_1$-like receptors. Replacement of residues 8 – 18 by mastoparan does cause a decrease in affinity, perhaps indicating that these do more than simply provide amphipathic character when interacting with $CGRP_2$-like receptors. It may be significant that replacement of alanine 11 by arginine, a substitution tolerated at the SK-N-MC CGRP receptor, causes loss of affinity at the Col 29 CGRP receptor. However, these results are somewhat limited because it was not practical to measure apparent pKBs of much less than 5.25 because of the high concentrations of antagonists that would be required. Consequently it was not possible to determine the magnitude of most decreases in affinity. Whilst there are differences of detail between the structure-activity relationships for the Col 29 and SK-N-MC CGRP receptors, the overall picture may well be broadly similar. It is clear that, despite its low affinity, $CGRP_{8-37}$ remains the only widely available

antagonist for this receptor, although some truncation of its structure may be tolerated. Amylin antagonists are not useful tools.

In summary, these results support the suggestion that the C-terminus of CGRP is a key determinant of high affinity binding to $CGRP_1$-like receptors. Residues 19–27 probably play a more minor role in binding. Residues 8–18 are also needed for high affinity binding, but the key property of this part of the molecule may simply be its amphipathicity. A somewhat similar situation may exist at $CGRP_2$-like receptors, where residues 19–27 seem to be of secondary importance. Further work here requires antagonists of higher affinity.

We would like to thank Miss Mina Varsani, who carried out some preliminary experiments, Prof. S. Nahorski, Leicester University for supply of SK-N-MC cells, Dr S. Kirkland, London University for supply of Col 29 cells and Dr H. Cox, London University for advice and encouragement with the Col 29 cells.

References

BARRETT, V.J., RAGHIB, A., BUTLER, A. & CONNOR, H.E. (1997). Effect of analogues of $CGRP_{8-37}$ on piglet isolated basilar arteries and ^{125}I-CGRP binding to SK-N-MC cell membranes. *Br. J. Pharmacol.,* **120,** 234P.

BOULANGER, Y., KHIAT, A., CHEN, Y., SENECAL, L., TU, Y., ST. PIERRE, S. & FOURNIER, A. (1996). Structure of the human calcitonin gene-related peptide and of its antagonist human calcitonin gene-related peptide 8–37 by nmr and molecular modelling. *Peptide Res.,* **8,** 206–213.

CHATERJEE, T.K. & FISHER, R.A. (1991). Multiple affinity forms of the calcitonin gene-related peptide receptor in rat cerebellum. *Mol. Pharmacol.,* **39,** 798–804.

COX, H.M. & TOUGH, I.R. (1994). Calcitonin gene related peptide receptors in human gastrointestinal epithelia. *Br. J. Pharmacol.,* **113,** 1243–1248.

DENNIS, T.B., FOURNIER, A., CADIUEX, A., POMERLEAU, F., JOLICOEUR, F.B. & QUIRION, R. (1990). hCGRP8–37, a calcitonin gene-related peptide antagonist revealing CGRP receptor heterogeneity in brain and periphery. *J. Pharmacol. Exp. Ther.,* **254,** 123–128.

DENNIS, T.B., FOURNIER, A., ST. PIERRE, S. & QUIRION, R. (1989). Structure-activity profile of calcitonin gene-related peptide in peripheral and brain tissues. Evidence for receptor multiplicity. *J. Pharmacol. Exp. Ther.,* **251,** 718–725.

HOWITT, S.G. & POYNER, D.R. (1997). The selectivity and structural determinants of peptide antagonists at the CGRP receptor of rat, L6 myocytes. *Br. J. Pharmacol.,* **121,** 1000–1004.

HOWITT, S.G., BILLINGTON, D.C. & POYNER, D.R. (1998). Effects of C-terminal alanine or serine substitutions on the binding of calcitonin gene-related peptide 8–37 to its receptors on rat L6 skeletal myocytes. *Br. J. Pharmacol.,* **123,** 129P.

HOWL, J., LANGEL, U., HAWTIN, S.R., VALKNA, A., YARWOOD, N.J., SAAR, K. & WHEATLEY, M. (1997). Chimeric peptides for the rational design of bioactive analogues of small peptide hormones. *FASEB J.,* **11,** 582–590.

HULME, E.C., BERRIE, C.P., BIRDSALL, N.J.M., JAMESON, M. & STOCKTON, J.M. (1983). Regulation of muscarinic agonist binding by cations and guanine nucleotides. *Eur. J. Pharmacol.,* **94,** 59–72.

KAHL, U., LANGEL, U., BARTFAI, T. & GRUDEMAR, L. (1994). Functional effects and ligand binding of chimeric galanin-Neuropeptide Y (NPY) peptides on NPY and galanin receptor types. *Br. J. Pharmacol.,* **111,** 1129–1134.

KASK, K., BERTHOLD, M., BOURNE, J., ANDELL, S., LANGEL, U. & BARTFAI, T. (1995). Binding and agonist antagonist actions of M35, Gal1-13-BK2–9 amide chimeric peptide in RIN-M-SF insulinoma cells. *Reg. Pep.,* **59,** 341–348.

KIRKLAND, S.C. (1985). Dome formation by a human colonic adenocarcinoma cell line (HCA-7). *Cancer Res.,* **45,** 3790–3795.

LANGEL, U., LAND, T. & BARTFAI, T. (1992). Design of chimeric peptide ligands to galanin receptors and substance P receptors. *Int. J. Pept. Protein Res.,* **39,** 516–522.

LANGEL, U., POOGA, M., KAIRANE, C., ZILMER, M. & BARTFAI, T. (1996). A galanin-mastoparan chimeric peptide activates the Na^+-K^+-ATPase and reverse its inhibition by oubain. *Reg. Pep.,* **62,** 47–52.

LONGMORE, J., HOGG, J.E., HUTSON, P.H. & HILL, R.G. (1994). Effects of two truncated forms of the human calcitonin gene-related peptide: implications for receptor classification. *Eur. J. Pharmacol.,* **265,** 53–59.

MIMEAULT, M., QUIRION, R., DUMONT, Y., ST. PIERRE, S. & FOURNIER, A. (1992). Structure-activity study of hCGRP8–37, a CGRP receptor antagonist. *J. Med. Chem.,* **35,** 2163–2168.

MUFF, R., STANGL, D., BORN, W. & FISCHER, J.A. (1992). Comparison of a calcitonin gene-related peptide receptor in a human neuroblastoma cell line (SK-N-MC) with a calcitonin receptor in a human breast carcinoma cell line (T47D). *Ann. N.Y. Acad. Sci.,* **657,** 106–116.

MURAYAMA, T., ODA, H. & NOMURA, Y. (1996). Pertussis toxin-insensitive effects of mastoparan, a wasp venom in PC12 cells. *J. Cell Physiol.,* **169,** 448–454.

OSTENSON, C.G., ZAITSEU, S., BERGGREN, P.O., EFENDIC, S., LANGEL, U. & BARTFAI, T. (1997). Galparan, a powerful insulin-releasing chimeric peptide acting at a novel site. *Endocrinology,* **138,** 3308–3313.

POYNER, D.R. (1995). The pharmacology of receptors for calcitonin gene-related peptide and amylin. *Trends Pharmacol. Sci.,* **16,** 424–428.

POYNER, D.R. (1992). Calcitonin gene-related peptide; multiple functions, multiple receptors. *Pharm. Ther.,* **56,** 23–51.

POYNER, D.R., ANDREW, D., BROWN, D., BOSE, C. & HANLEY, M.R. (1992). Pharmacological characterization of a receptor for calcitonin gene-related peptide on rat L6 skeletal myocytes. *Br. J. Pharmacol.,* **105,** 441–447.

POYNER, D.R., TOMLINSON, A.E., GOSLING, M., TOUGH, I.R. & COX, H.M. (1993). Stimulation of chloride secretion and adenylate cyclase in human colonic derived cell lines by calcitonin gene-related peptide. *Biochem. Soc. Trans.,* **21,** 434S.

QUIRION, R., VAN ROSSUM, D., DUMONT, Y., ST. PIERRE, S. & FOURNIER, A. (1992). Characterization of CGRP1 and CGRP2 receptors. *Ann. N.Y. Acad. Sci.,* **657,** 88–105.

RIST, B., ENTZEROTH, M. & BECK-SICKINGER, A.G. (1998). From micromolar to nanomolar affinity; a systematic approach to identify the binding site of CGRP at the human calcitonin gene-related peptide 1 receptor. *J. Med. Chem.,* **41,** 117–123.

SAGOO, J.K., BOSE, C., BEELEY, N.R.A. & TENDLER, S.J.B. (1991). Structural studies on the [tBu-cys^{18}]-19–37 fragment of human β-CGRP. *Biochem. J.,* **280,** 147–150.

SEMARK, J.E., MIDDLEMISS, D.N. & HUTSON, P.H. (1992). Comparison of calcitonin gene-related peptide receptors in rat brain and a human neuroblastoma cell line, SK-N-MC. *Mol. Neuropharmacol.,* **2,** 311–317.

TAM, J.P., HEATH, W.F. & MERRIFIELD, R.B. (1985). Mechanisms for removal of benzyl protecting groups in synthetic peptides by trifluoromethanesulfonic acid-dimethyl sulfide. *J. Am. Chem. Soc.,* **108,** 5242–5251.

VAN VALEN, F., PEICHOT, G. & JURGHENS, H. (1990). Calcitonin gene-related peptide receptors are linked to cyclic adenosine monophosphate production in SK-N-MC neuroblastoma cells. *Neurosci. Lett.,* **119,** 195–198.

WISSKIRRCHEN, F.M., DOYLE, P.M., GOUGH, S.L., HARRIS, C.J. & MARSHALL, I. (1997). Conformational restraints to find biologically relevant structures of CGRP8–37 in rat prostatic vas deferens, pulmonary artery and internal anal sphincter. *Br. J. Pharmacol.,* **120,** 209P.

ZIMMERMAN, U., FISCHER, J.A. & MUFF, R.A. (1995). Adrenomedullin and calcitonin gene-related peptide interact with the same receptor in cultured hyman endothelial neuroblastoma SK-N-MC cells. *Peptides,* **16,** 421–424.

(Received January 15, 1998
Revised May 26th 1998
Accepted June 8, 1998)