Structure–activity relationship of human calcitonin-gene-related peptide

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The calcitonin–calcitonin-gene-related peptide (CGRP) gene complex encodes a small family of peptides: calcitonin, CGRP and katacalcin. Calcitonin is a circulating hormone that prevents skeletal breakdown by inhibiting the resorption of bone by osteoclasts. CGRP, a potent vasodilator, is involved in normal regulation of blood flow. The calcitonins structurally resemble the CGRP peptides, and both are known to cross-react at each others' receptors. The present study was undertaken to examine the structural prerequisites for biological activity of the intact CGRP molecule. We therefore prepared eight chymotryptic and tryptic fragments of CGRP and synthesized its acetylated and *S*-carboxy-amidomethylcysteinyl analogues. The analogues were purified by h.p.l.c. and their structures were confirmed by fast-atom bombardment mass spectrometry. We have examined the effects of structurally modified analogues and fragments of human CGRP in a calcitonin-receptor-mediated assay, the osteoclast bone resorption assay, and in one or two CGRP-receptor-mediated assays, the rabbit skin blood flow assay and the oedema formation assay. The results showed that (1) in the osteoclast bone resorption assay, both CGRP peptides, α and β , were equipotent, and were both at least 1000-fold less potent than calcitonin; (2) in the blood flow and oedema assays, both CGRP peptides, α and β , were equipotent and were both approx. 1000-fold more potent than salmon calcitonin; human calcitonin had no effect; (3) the bis- and *N*-acetylated CGRP analogues retained reduced levels of biological activity in all assays, whereas *S*-carboxy-amidomethylcysteinyl-human CGRP was without activity; and (4) all tryptic and chymotryptic fragments of CGRP were without biological activity, with the exception of hCGRP-(Ala¹–Lys³⁵): this fragment had much reduced activity compared with the intact peptide in inhibiting osteoclastic bone resorption and increasing blood flow in the rabbit skin. The results suggest that: (1) calcitonin and CGRP act at distinct receptors to mediate different physiological effects; (2) minor amino acid substitutions, as between the α and β forms of CGRP (these two forms have 94 % structural similarity) do not result in differences in biological activity; (3) the intact peptide is required for full biological activity of the CGRP molecule, and even the loss of two amino acids at the C-terminus of the molecule results in a marked decrease in activity; (4) the disulphide bridge appears to play an important role in the interaction of the intact CGRP molecule with its receptor; and (5) the C-terminal region is probably necessary for the peptide to assume the right conformation in the interaction with the receptor.

INTRODUCTION

The sequence of calcitonin-gene-related peptide (CGRP) was initially predicted for the rat (Amara *et al.*, 1982; Rosenfeld *et al.*, 1983), and its presence was soon established in man (Morris *et al.*, 1984; Edbrooke *et al.*, 1985). More recently, another calcitonin gene has been described (Hoppener *et al.*, 1986), commonly referred to as the β -gene. This gene encodes another CGRP molecule (Jonas *et al.*, 1985) and a second calcitonin-like peptide (Alevizaki *et al.*, 1986), but the latter peptide is not expressed either in the rat or in humans. Human CGRP has 37 amino acids, and has some structural similarity with the 32-amino-acid peptide human calcitonin (hCT) (Fig. 1). Both peptides have an overall positive charge and share a common C-terminal amide and an *N*-terminal disulphide bridge. Whereas the calcitonins exhibit species-related divergence in structure, the known CGRP sequences are highly conserved (for reviews, see Zaidi *et al.*, 1987a; Breimer *et al.*, 1988). Interestingly, salmon (s) CT exhibits a greater structural similarity to human (h) CGRP than to hCT, as shown in Fig. 1.

CT is expressed mainly in the thyroid gland, and CGRP is

expressed chiefly in nerves (Rosenfeld *et al.*, 1983). The primary physiological function of CT is to prevent skeletal loss during periods of potential calcium lack by a direct inhibitory effect on osteoclastic bone resorption. In contrast, CGRP is a neuropeptide which is released from sensory A(δ) and C-fibres and which has a range of biological effects, the most prominent of which is its profound vasodilator activity in man and other species (Brain *et al.*, 1985). As a consequence of its vasodilator activity, CGRP also potentiates oedema produced by endogenous tachykinins in inflammatory models (Brain & Williams, 1985), although the peptide inhibits biosynthesis of the inflammatory mediator leukotriene D₄ in rat lung (Di Marzo *et al.*, 1986) and leukotriene D₄-mediated contractions of guinea pig ileum (Tippins *et al.*, 1986a). In addition, CGRP shares with CT its receptor-mediated inhibitory effects on osteoclastic bone resorption, and thus lowers plasma calcium in young rats (Zaidi *et al.*, 1987b,c, 1988; D'Souza *et al.*, 1987).

In an attempt to gain some information about the structural prerequisites for biological activity, we have examined the effects of hCT, sCT, hCGRP(α) and hCGRP(β) as well as structurally modified analogues of CGRP in a functional assay for CT (the

Abbreviations used: (h)CGRP, (human) calcitonin-gene-related peptide; hCT, sCT, human and salmon calcitonin respectively; f.a.b.m.s., fast-atom bombardment mass spectrometry; TFA, trifluoroacetic acid; MEM/FCS, minimum essential medium containing 10 % fetal calf serum; ED₅₀, dose causing 50 % of maximal effect.

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Fig. 1. Amino acid sequences of CGRP, rCGRP, hCT and sCT

Positions of tryptic (†) and chymotryptic (‡) cleavage, acetylation (↓) and disulphide bridge (*) are indicated.

osteoclast bone resorption assay), and in assays for CGRP (measurement of microvascular blood flow and oedema formation in rabbit skin). We have also investigated the activity of tryptic and chymotryptic fragments of CGRP; this is relevant since, in a recent study, it has been shown that CGRP is broken down by tryptic and chymotryptic-like enzymes released from mast cells *in vivo* (Brain & Williams, 1988).

MATERIALS AND METHODS

Peptides

hCGRP(α), hCT and sCT were gifts from Dr. J. Pless (Sandoz, Basel, Switzerland), Dr. W. Rittel (Ciba-Geigy, Basel, Switzerland) and Armour Pharmaceuticals respectively. hCGRP(β) and Tyr⁶-hCGRP-(Val²⁸-Phe³⁷) were purchased from Peninsula Laboratories. The peptides were weighed on a Cahn microbalance, dissolved in a solution of aqueous acetic acid (BDH, Poole, Dorset, U.K.; Aristar grade) (0.001%, v/v) containing BSA (0.01%, w/v; recrystallised; Sigma, Poole, Dorset, U.K.), lyophilized and stored at -70 °C. H.p.l.c. analyses were performed on a Waters Associates instrument (Milford, MA, U.S.A.). Fast atom bombardment mass spectrometric (f.a.b.m.s.) analyses were performed on a ZAB-VG analytical instrument equipped with an f.a.b. gun (MScan Ltd.).

Preparation of fragments

hCGRP(α) was incubated with trypsin (0.005%, w/w; Sigma) or with chymotrypsin (0.01%, w/w; Sigma) at 37 °C in ammonium bicarbonate buffer (0.05 M, pH 8.5) for 30–90 s. The products were separated by reverse-phase h.p.l.c. using an Altex Ultrasphere ODS column (0.46 × 25 cm) and a gradient of acetonitrile in aqueous trifluoroacetic acid (TFA; 0.1%, v/v) as solvent. An isocratic gradient of 10% acetonitrile was initially run for 5 min, followed by a convex gradient (10–25% over 60 min) and a linear gradient (25–60% over 60 min). The fragments were quantified by amino acid analysis and their identity was confirmed by f.a.b.m.s. analysis (Morris *et al.*, 1981).

Chemical modifications

hCGRP(α) was acetylated with acetic acid/methanol (1:3, v/v) for 5 min at room temperature. The *N*-acetylated and bis-acetylated (at Lys²⁴ or Lys³⁵) peptides were separated by h.p.l.c., using the column described above with an isocratic gradient of acetonitrile/water/TFA (30:69.9:0.1) for 5 min, followed by a linear gradient to 50% acetonitrile (over 90 min). The disulphide bridge was oxidized with performic acid, or by treating with

iodoacetamide, which produced a mixture containing 20% bis-*S*-carboxyamidomethylcysteinyl-hCGRP(α), 30% of the tri-, 30% of the tetra- and 20% of the penta-substituted products. The reaction products were identified by f.a.b.m.s. before and after h.p.l.c. purification and quantified by amino acid analysis.

Osteoclast bone resorption assay

Slices of human devitalized cortical bone were prepared as described previously (Chambers *et al.*, 1984). The osteoclast suspension was added dropwise on to 30–36 bone slices placed in wells of a Sterilin 100 18 mm multiwell dish. Following incubation (37 °C; 15 min), bone slices were removed, washed gently in Minimal Essential Medium with 10% (v/v) fetal calf serum (MEM/FCS) and placed in separate wells (each well contained five or six slices and 900 μ l of MEM/FCS). After further incubation (37 °C, 10% humidified CO₂, 10 min), 100 μ l of MEM/FCS containing the test substance was added. Finally, after overnight incubation (37 °C, 10% humidified CO₂, 18 h), the bone/osteoclast cultures were fixed in glutaraldehyde and the cells were stained with Toluidine Blue. After assessing the numbers of multinucleate osteoclasts and contaminating mononuclear cells by transmission electron microscope, the slices were bleached by immersion in sodium hypochlorite solution (10%, v/v; 30 min) and dehydrated in aqueous ethanol (80%, v/v; 60 min). The slices were next sputter-coated with gold, randomized and examined in a computer-based scanning electron microscope (Cambridge 360; Cambridge Instruments, Bar Hill, Cambs., U.K.). The number of osteoclastic excavations defined by a continuous border was counted and the area of the resorbed bone surface was calculated by tracing the outline of each resorptive lacuna into an IBM-centred image processor-analyser (Sight Systems, Newbury, Berks., U.K.).

Either one or two homogeneous suspensions of osteoclasts were used for each experiment: when two suspensions were used, the experiment was divided into two blocks, each of which included all treatments with one suspension. Either five or six bone slices were used per dose in all experiments. Finally, the viability of osteoclasts was confirmed by comparing osteoclast numbers on control slices with those on treated slices. The assay has previously been found to be highly specific for CT, and a range of peptides have been found not to inhibit osteoclastic bone resorption (Chambers & Magnus, 1983; Zaidi *et al.*, 1987c). The observed values of precision (s.d./slope) (Daly, 1978) have been found to vary between 0.5 and 1.0 (Zaidi *et al.*, 1990). For the design used in this assay the linear region of the dose-response relationship was in the concentration range of 0.1–10 μ units of hCT (Zaidi *et al.*, 1990).

Rabbit skin blood flow assay

Blood flow was measured in the dorsal skin of male New Zealand White rabbits (3–4 kg, specific-pathogen free; Froxfield Ltd., Petersfield, Hampshire, U.K.) using a multiple site ^{133}Xe clearance technique (Williams, 1979). The peptides were mixed with a solution of ^{133}Xe and rapidly injected intradermally in 100 μl volumes. Phosphate-buffered saline (PBS) was used as a control. In each rabbit, six replicates for each test agent were tested at each dose. After a clearance of 15 min the rabbits were killed by barbiturate overdose. The dorsal skin was removed and the injection sites punched out (17 mm diameter) and counted for radioactivity along with 100 μl aliquots of injection fluid in a γ -radiation counter. Results were calculated as a percentage increase in blood flow in test sites compared with PBS-injected sites, and each result was the mean of the six replicates in each animal.

Oedema formation assay

Male New Zealand White rabbits (specific-pathogen-free; 2.5–3 kg) were used. Local oedema formation in response to bradykinin and its potentiation with hCGRP and CGRP analogues was measured in the skin of these rabbits in response to intradermal injection of the peptides as previously described (Brain & Williams, 1985). The rabbits were anaesthetized by intravenous injection of Saffan (1 mg/kg; Glaxovet, Harefield, Middlx., U.K.) into the marginal ear vein. ^{125}I -labelled human serum albumin (1.5 $\mu\text{Ci/kg}$; Amersham International, Amersham, Bucks., U.K.) and Evans Blue [0.5 ml/kg of 2.5 (w/v); BDH] were injected by the same route. The agents under test were dissolved in saline and injected in 100 μl volumes into the shaved dorsal skin according to a balanced site pattern with six replicates per dose. After a 30 min accumulation period, each rabbit was killed by barbiturate overdose. The dorsal skin was removed and the injection sites punched out (17 mm diameter) and the radioactivity counted. Oedema responses were expressed as equivalent plasma volumes by dividing the radioactivity in each skin sample by that in 1 μl of plasma.

Statistical analysis

For purposes of statistical analysis, responses obtained in the osteoclast bone resorption assays (total area of resorption per bone slice, number of osteoclastic excavations or size of individual excavations) were transformed by taking the square root, to give responses that had approximately equal variances at treatment levels within each experiment. This method of transformation has been shown to produce a normal distribution with values which are otherwise skewed (Zaidi *et al.*, 1990). The responses in the blood flow assay were either expressed as percentage of control or not transformed at all. For all assays, the effect on responses of hormone treatment compared with control and the relationship of responses to $\log(\text{dose of hormone})$ was assessed by analysis of variance. The dose giving 50 % of maximal effect (ED_{50}) and potency estimates were derived using logistic regression analysis of total response at each dose (expressed as % of control) on $\log(\text{dose})$ (Finney, 1978).

RESULTS

Purity of chemically modified analogues and fragments of CGRP

The purity and identity of the analogues and fragments was determined from the fact that each eluted as a single peak from h.p.l.c., gave a molecular ion peak ($M + H$)⁺ in f.a.b.m.s. and had a predicted amino acid composition from amino acid analysis which, by comparison with an internal norleucine standard, also gave the molar concentrations of the peptides (Tippins *et al.*,

Fig. 2. Effects of hCGRP(α) ($\square$), hCGRP(β) ($\blacksquare$), hCT ($\bullet$) and sCT ($\circ$) on the area of bone resorbed by isolated rat osteoclasts

Results are expressed as percentages of control resorption $\pm$ S.E.M.; $n = 8$ slices per variable).

1986b). The bis-acetylated form of hCGRP(α) was a mixture of various forms, as detailed in the Materials and methods section.

Effects of hCT, sCT, hCGRP(α) and hCGRP(β)

There was a concentration-dependent inhibition of osteoclastic bone resorption with each peptide; the total area of bone resorption per slice and the number of osteoclastic excavations were found to significantly ($P < 0.01$) regress on $\log(\text{dose})$ (assessed by logistic regression analysis) (Fig. 2). Using the estimated common slope, hCT was found to be 682 times (fiducial limits, 95 % confidence: 448–916) as potent, pmol/pmol, as hCGRP(α). Using logistic regression analysis, the ED_{50} values were calculated as: hCT, 0.25 pmol/l, hCGRP(α), 0.225 nmol, hCGRP(β), 0.15 nmol/. sCT was 34 times (range 12–56) as potent as hCT. The four peptides also increased blood flow in the rabbit skin in a dose-dependent manner, assessed using a ^{133}Xe -clearance technique. In contrast, in rabbit skin hCGRP(α) and (β) were the most potent agents tested in the blood flow assay, and are equipotent as vasodilators (Brain *et al.*, 1986). sCT was at least 10000-times less potent and hCT had no detectable vasodilator activity at the doses tested. The potentiation of oedema formation by vasodilators in rabbit skin is proportional to their vasodilatory potency (Williams, 1979). Therefore hCGRP(α) and (β) potentiated oedema formation to a similar extent, as shown in Fig. 3. sCT had no oedema-potentiating activity at doses below 1 nmol per site.

Effect of chemically modified analogues

Acetylation with acetic anhydride/methanol produced an *N*-acetylated product and a bis-acetylated peptide (at the *N*-terminal and at Lys²⁴ or Lys³⁵). In comparison with the intact peptide, the *N*-acetylated product was approx. 0.8 times (fiducial limits, 95 % confidence: 0.1–1.5) as active as intact CGRP in inhibiting osteoclastic bone resorption. Although this difference represented a parallel shift of the $\log(\text{dose})$ –response curve of the *N*-acetylated peptide compared with the intact peptide (Fig. 4a), the difference [derived using the common slope of the $\log(\text{dose})$ –response lines] was not statistically significant when assessed by logistic regression analysis ($P > 0.1$). Again, the *N*-acetylated peptide (ED_{50} 0.31 nmol/l) was approx. 3 times (fiducial limits, 95 % confidence: 1–5) more potent than the bis-acetylated peptide (ED_{50} 0.95 nmol/l); this difference was not statistically significant ($P > 0.1$) (Fig. 4a). The data derived from these experiments,

Fig. 3. Effects of CGRP and CT in skin blood flow (a) and oedema formation (b) assays

(a) Effects of hCGRP(α) (□), hCT (●) and sCT (○) on the change in blood flow in the rabbit skin. Results are means $\pm$ S.E.M.; $n = 5$. (b) Effects of hCGRP(α) (□) and hCGRP(β) (■) on the potentiation of skin oedema induced by bradykinin. The results are expressed as an increase in skin plasma volume. The peptides when injected without bradykinin did not cause oedema formation at a concentration of 10^{-11} M. The effects of bradykinin on skin plasma volume (○) are shown. Results are means $\pm$ S.E.M.; $n = 4$.

with the appropriate 95% confidence limits, are shown in Table 1. At the concentrations tested, both analogues caused a significant dose-dependent decrease in the total area of bone resorbed per slice ($P < 0.01$), the total number of osteoclastic excavations ($P < 0.01$) and the size of individual excavations ($P < 0.05$).

In the blood flow experiments, the *N*-acetylated peptide was active, and produced a similar increase in blood flow when compared with CGRP and the bis-acetylated peptide. It was not possible to estimate a potency difference, as the dose-response curves were obviously non-parallel (Fig. 4b). The destruction of the disulphide bridge between Cys² and Cys⁷, either by performic acid oxidation or by reduction and alkylation, abolished biological activity in the blood flow assay at doses of up to 0.1 nmol per site and also in the bone resorption assays (Fig. 5). There was insufficient material to allow assessment of these products in the oedema formation assay.

Effect of tryptic and chymotryptic fragments

A 30 s tryptic digest resulted in the production of fragments Ala¹-Arg¹¹, Ala¹-Arg¹⁸, Ser¹⁹-Phe³⁷-NH₂, Asn²⁵-Lys³⁵ and Ser¹⁹-Lys³⁵. The 90 s chymotryptic digest produced fragments Ala¹-Leu¹⁶ and Ser¹⁷-Phe³⁷. Fragment Ala¹-Lys³⁵ was produced by a 10 s tryptic digestion (Fig. 1). Fragments Ala¹-Arg¹¹, Ala¹-Arg¹⁸, Ser¹⁹-Phe³⁷-NH₂, Ala¹-Leu¹⁶ and Ser¹⁷-Phe³⁷ and synthetic peptides were tested on the osteoclast bone resorption assay and found to be inactive at 250 nm concentrations (Fig. 5). Fragments Ala¹-Arg¹¹, Ala¹-Arg¹⁸, Ser¹⁹-Phe³⁵, Ser¹⁹-Phe³⁷.

Fig. 4. Effects of hCGRP and analogues in bone resorption (a) and skin blood flow (b) assays

The effects of hCGRP(α) (■) and its structurally modified analogues, bis-acetyl-hCGRP(α) (□) and *N*-acetyl-hCGRP(α) (○) on the area of bone resorbed by isolated osteoclasts (a) and on blood flow in the rabbit skin (b) were measured. Results are means $\pm$ S.E.M.; $n = 4$.

Table 1. Effects of chemically modified CGRP analogues

Dose (nm)...	0	0.25	2.5	25	250
<i>N</i>-acetyl-hCGRP(α):					
Area (%)	100	68	47	15	11
	(82, 118)	(51, 85)	(37, 54)	(7, 23)	(2, 20)
Number	24	16	12	8	2
Size (%)	100	89	96	73	43
	(92, 108)	(77, 101)	(81, 111)	(52, 94)	(33, 53)
Bis-acetyl-hCGRP(α):					
Area (%)	100	72	41	20	8
	(91, 109)	(54, 90)	(32, 50)	(11, 29)	(0, 18)
Number	31	18	12	5	1
Size (%)	100	94	87	50	43
	(87, 113)	(82, 106)	(74, 100)	(29, 71)	(30, 56)

Fig. 5. Effects of CGRP fragments in the bone resorption assay

The effects of tryptic and chymotryptic fragments, synthetic Tyr⁰-Val²⁸-Phe³⁷ and *S*-carboxyamidomethylcysteinyl-hCGRP(α) (SCAMT) on the area of bone resorbed by isolated osteoclasts ($\square$), the number of osteoclastic excavations ($\blacksquare$) and the size of individual excavations ($\blacksquare$) are shown. The results are expressed as means $\pm$ S.E.M.; $n = 6$ slices per group.

NH₂ and Asn²⁵-Lys³⁵ were tested on rabbit skin blood flow and oedema formation assays and were also found to be inactive when tested at doses of up to 0.1–0.24 nmol per site. In addition, fragments Tyr⁰, Val²⁸-Phe³⁷ (synthesized by Peninsula Laboratories) and Ala¹-Leu¹⁶ were found to be without activity on the rabbit blood flow assay. The chymotryptic fragment Ala¹-Lys³⁵ retained biological activity in both bone resorption assays (Fig. 5) and blood flow assays.

DISCUSSION

Several previous studies have focused on the CGRP receptor signal transduction mechanisms (Seifert *et al.*, 1985; Laufer & Changeux, 1989) and have led to important insights into the post-receptor events in various tissues. However, very little is known about the structural prerequisites necessary for the interaction of CGRP with its receptor.

In previous studies on the effect of CGRP in various biological systems, we have shown that CGRP and CT, though derived from the same gene, appear to act preferentially at distinct receptors. For example, in our initial report on the vasodilator activity of CGRP (Brain *et al.*, 1985), we showed that CGRP produced a significant increase in blood flow in rabbit skin with doses as low as 100 fmol, whereas CT was without effect at a dose of 10 pmol. On the other hand, when the plasma-calcium-lowering effects of CGRP and CT were compared in the rat, CGRP was about 1000-fold less potent than CT (Tippins *et al.*, 1984; Zaidi *et al.*, 1988). Furthermore, the resorption of bone by rat osteoclasts *in vitro* was inhibited by CGRP at high doses and by CT at low doses (Zaidi *et al.*, 1987b,c, 1988). Similar evidence for discrete CGRP and CT receptors has been reported by many other authors (Goltzman & Mitchell, 1985; Wohlend *et al.*, 1985; Marshall *et al.*, 1986; Michelangeli *et al.*, 1986; Hirata *et al.*, 1988; Yamaguchi *et al.*, 1988).

In a preliminary investigation of the structure-activity relationship of CGRP, a study of the effects of enzymic digest fragments and chemically modified products was limited to one bioassay system, the rat paired atrial preparation (Tippins *et al.*, 1986). In the present study we have looked at the activity of CGRP fragments and chemically modified analogues in three biological assays. In one of these, the osteoclast bone resorption assay, the activity of CGRP is mediated via CT receptors (Roos

et al., 1986; Zaidi *et al.*, 1987b,c), while in the other two, the blood flow and oedema assays, the effect is mediated via CGRP receptors. The results reported here clearly indicate that the intact CGRP molecule is required for its full biological activity. However, the structurally similar sCT also mediated CGRP-like vasodilator responses in rabbit skin when injected at high concentrations. None of the fragments produced by either trypsin- or chymotrypsin-catalysed hydrolysis of specific peptide bonds showed a significant effect in any of the three assay systems used, with the only exception of fragment hCGRP-(Ala¹-Lys³⁵), which exhibited a low activity in the blood flow and bone resorption assays. The reduced potency shown by *N*-acetyl-hCGRP and the complete destruction of biological activity upon either oxidation or reduction followed by methylation seem to indicate that the *N*-terminal region of the peptide, and in particular the Cys²-Cys⁷ disulphide bridge, play an important part in the interaction of the molecule with the CGRP receptor. However, the lack of biological activity of the *N*-terminal fragments Ala¹-Arg¹¹, Ala¹-Ser¹⁶ and Ala¹-Arg¹⁸ suggests that the remaining part of the molecule is probably necessary for the peptide to assume the right conformation and therefore to ensure a correct interaction with the receptor. The fact that partial modifications such as acetylation of Lys²⁴ or Lys³⁵ or substitution, in CGRP(β), of Val²² and Asn²⁵ with Met and Ser respectively, do not cause relevant changes in biological activity, corroborates the hypothesis of this secondary, but nevertheless important, role played by the *C*-terminal region of the peptide.

These findings are supported by a recent report of the CGRP receptor antagonist activity of hCGRP-(Val⁸-Phe³⁷) (Chiba *et al.*, 1989). These authors reported that this fragment was essential for the binding of intact CGRP to its receptor; nevertheless, without the *N*-terminal disulphide bridge and ring structure, this *C*-terminal fragment was unable to induce the subsequent intracellular signal transduction event. The fragment was, however, able to promote adenylate cyclase activity in LLC-PK₁ cells, which indicates an action at CT receptors (Wohlend *et al.*, 1985). Chiba *et al.* (1986) concluded that the *C*-terminus of CT is more important than the *N*-terminus in binding to the CT receptor, which may have a broad specificity in recognizing the *C*-terminal portions of binding agonists. From their results we might have expected that *C*-terminal fragments that we tested would possess some agonist activity in the osteoclast assay and antagonist activity in the blood flow and oedema assays. This was not the case. However, the *C*-terminal fragments that we tested were much shorter than that reported by Chiba and co-workers.

In conclusion, our results show that similar structural prerequisites are needed for the interaction of CGRP with receptors in vascular and bone tissues. The results confirm preliminary studies carried out in cardiac tissue (Tippins *et al.*, 1986b). The present suggestion that the *N*-terminal region of the peptide is essential for the binding of CGRP to its receptor, and that the *C*-terminal region also has an important role, is relevant to further studies aimed at developing chemically different agents which stimulate vasodilation via the CGRP receptor.

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