

REVIEW

Structure–activity relationships for α -calcitonin gene-related peptide

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Calcitonin gene-related peptide (CGRP) is a member of the calcitonin (CT) family of peptides. It is a widely distributed neuropeptide implicated in conditions such as neurogenic inflammation. With other members of the CT family, it shares an N-terminal disulphide-bonded ring which is essential for biological activity, an area of potential α -helix, and a C-terminal amide. CGRP binds to the calcitonin receptor-like receptor (CLR) in complex with receptor activity-modifying protein 1 (RAMP1), a member of the family B (or secretin-like) GPCRs. It can also activate other CLR or calcitonin-receptor/RAMP complexes. This 37 amino acid peptide comprises the N-terminal ring that is required for receptor activation (residues 1–7); an α -helix (residues 8–18), a region incorporating a β -bend (residues 19–26) and the C-terminal portion (residues 27–37), that is characterized by bends between residues 28–30 and 33–34. A few residues have been identified that seem to make major contributions to receptor binding and activation, with a larger number contributing either to minor interactions (which collectively may be significant), or to maintaining the conformation of the bound peptide. It is not clear if CGRP follows the pattern of other family B GPCRs in binding largely as an α -helix.

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Abbreviations

AM, adrenomedullin; CGRP, calcitonin gene-related peptide; CLR, calcitonin receptor-like receptor; CT, calcitonin; ECD, extracellular domain; ECL, extracellular loop; GLP-1, glucagon-like peptide 1; PACAP, pituitary adenylate cyclase-activating polypeptide; PTH, parathyroid hormone; RAMP, receptor activity-modifying protein; TM, transmembrane domain

Introduction

Calcitonin gene-related peptide (CGRP) is a 37 amino acid neuropeptide belonging to the calcitonin (CT) family of peptides. This family also includes amylin, the calcitonin receptor-stimulating peptides, and the adrenomedullins (AMs), and they are found in vertebrates from fish to mammals (Ogoshi *et al.*, 2006). Figure 1 provides an alignment of CGRP peptides from a representative selection of 19 species. There is a high degree of conservation across these species, with 23 of 37 residues absolutely conserved. The majority of sequence changes in the other positions can be considered to be conservative replacements.

CGRP is found throughout the sensory nervous system; it is a potent vasodilator and a key mediator in neurogenic

inflammation. CGRP antagonists have attracted considerable attention for the treatment of migraine; agonists are of potential interest to treat a range of cardiovascular disorders (Brain and Grant, 2004; Durham and Vause, 2010). There are two forms of CGRP in humans and rodents, α and β ; unless otherwise stated in this review, it is the human α -form that is being considered. This is the most abundant form of CGRP for which most information is available. Generally, there seems little difference between the pharmacology of α - and β -CGRP (Bailey and Hay, 2006) or between rat and human forms (Poyner, 1992).

CGRP has several key structural features, most prominently a disulphide bond between residues 2 and 7 and a C-terminal amide. In common with other peptide ligands for family B GPCRs, it is believed to follow a two-step mechanism

	1	10	20	30																																	
Human	A	C	D	T	A	T	C	V	T	H	R	L	A	G	L	S	R	S	G	G	V	V	K	N	F	V	P	T	N	V	G	S	K	A	F		
Horse	S	C	N	T	A	T	C	V	T	H	R	L	A	G	L	S	R	S	G	G	V	V	K	S	N	F	V	P	T	D	V	G	S	E	A	F	
Rat	S	C	N	T	A	T	C	V	T	H	R	L	A	G	L	S	R	S	G	G	V	V	K	D	N	F	V	P	T	N	V	G	S	E	A	F	
Mouse	S	C	N	T	A	T	C	V	T	H	R	L	A	G	L	S	R	S	G	G	V	V	K	D	N	F	V	P	T	N	V	G	S	E	A	F	
Chicken	A	C	N	T	A	T	C	V	T	H	R	L	A	D	F	L	S	R	S	G	G	V	G	K	N	N	F	V	P	T	N	V	G	S	K	A	F
Pig	S	C	N	T	A	T	C	V	T	H	R	L	A	G	L	S	R	S	G	G	M	V	K	S	N	F	V	P	T	D	V	G	S	E	A	F	
Sheep	S	C	N	T	A	T	C	V	T	H	R	L	A	G	L	S	R	S	G	G	V	V	K	S	N	F	V	P	T	N	V	G	S	Q	A	F	
Cow	S	C	N	T	A	T	C	V	T	H	R	L	A	G	L	S	R	S	G	G	V	V	K	S	N	F	V	P	T	N	V	G	S	E	A	F	
Dog	S	C	N	T	A	T	C	V	T	H	R	L	A	G	L	S	R	S	G	G	V	V	K	N	N	F	V	P	T	N	V	G	S	E	A	F	
Marmoset	A	C	D	T	A	T	C	V	T	H	R	L	A	G	L	S	R	S	G	G	M	V	K	N	N	F	V	P	T	N	V	G	S	E	A	F	
Opossum	G	C	N	T	A	T	C	V	T	H	R	L	A	D	F	L	S	R	S	G	G	V	A	K	S	D	F	V	P	T	N	V	G	A	K	A	F
Gekko	A	C	N	T	A	T	C	V	T	H	R	L	A	D	F	L	S	R	S	G	G	V	G	K	S	N	F	V	P	T	N	V	G	A	K	A	F
Frog	S	C	D	T	S	T	C	A	T	Q	R	L	A	D	F	L	S	R	S	G	G	I	G	S	P	D	F	V	P	T	D	V	S	A	N	S	F
Pufferfish	A	C	N	T	A	T	C	V	T	H	R	L	A	D	F	L	S	R	S	G	G	M	G	N	S	N	F	V	P	T	N	V	G	A	K	A	F
Flounder	G	C	N	T	S	T	C	V	T	H	R	L	A	D	L	S	R	S	G	G	L	G	Y	N	N	F	V	P	T	N	V	G	A	Q	A	F	
Goldfish	A	C	N	T	A	T	C	V	T	H	R	L	A	D	F	L	S	R	S	G	G	I	G	S	S	K	F	V	P	T	N	V	G	S	Q	A	F
Salmon	A	C	N	T	A	T	C	V	T	H	R	L	A	D	F	L	S	R	S	G	G	M	G	N	S	N	F	V	P	T	N	V	G	S	K	A	F
Medalea	A	C	N	T	A	T	C	V	T	H	R	L	A	D	F	L	S	R	S	G	G	L	G	H	S	N	F	V	P	T	N	V	G	A	Q	A	F
Zebrafish	A	C	N	T	A	T	C	V	T	H	R	L	A	D	F	L	S	R	S	G	G	I	G	S	S	K	F	V	P	T	N	V	G	S	Q	A	F

Figure 1

Sequence alignment of CGRP sequences from a selection of species generated using ClustalW and visualized using EsPrint using the %Equivalence algorithm. Conserved regions are shown in red boxes with regions with similar physicochemical parameters in yellow. Species are listed by their common names: human- α (*Homo sapiens*), horse (*Equus caballus*), rat- α (*Rattus norvegicus*), mouse (*Mus musculus*), chicken (*Gallus gallus*), pig (*Sus scrofa*), sheep (*Ovis aries*), cow (*Bos taurus*), dog (*Canis lupus familiaris*), marmoset (*Callithrix jacchus*), opossum (*Monodelphis domestica*), Gekko (*Gekko japonicas*), frog (*Phyllomedusa bicolor*), pufferfish (*Takifugu rubripes*), flounder (*Paralichthys olivaceus*), goldfish (*Carassius auratus*), salmon (*Salmo salar*), medalea (*Oryzias latipes*), zebrafish (*Danio rerio*).

of action to activate its receptor. The C-terminus of the ligand binds to the extracellular domain (ECD) of the receptor and the N-terminus of the ligand (which incorporates the disulphide-bonded loop) binds to the transmembrane domain (TM) and extracellular loop (ECL) region of the receptor to give activation.

Many peptide and non-peptide CGRP antagonists have been described. These were initially based on peptide fragments lacking the N-terminal disulphide-bonded loop, of which the best characterized is CGRP_{8–37} (Chiba *et al.*, 1989; Dennis *et al.*, 1990). More recently, attention has focused on shorter peptides, which are often derivatives of CGRP_{27–37} (Yan *et al.*, 2011). The non-peptide antagonists are typified by olcegepant and telcegepant (Negro *et al.*, 2012). Crystal structures are available for the ECD of the CGRP receptor showing these two antagonists bound but no structure is available of any bound peptide or of the TM region (ter Haar *et al.*, 2010).

The mechanism by which the endogenous ligands for family B GPCRs, like CGRP, bind to and activate their receptors is of considerable interest (Neumann *et al.*, 2008; Parthier *et al.*, 2009; Watkins *et al.*, 2012) because this can lead to the design of novel antagonists and agonists. Understanding the structure–activity relationships of natural peptides has led to the development of several stable and efficacious peptide-based agonists of family B GPCRs that have ultimately become therapeutics (Shimizu *et al.*, 2000; Devigny *et al.*, 2011; Garber, 2012). This review considers recent developments in the structure–activity relationship for CGRP and how these might be interpreted in the light of current knowledge of the binding of ligands to family B GPCRs.

CGRP-responsive receptors and their implications for pharmacological studies

The CGRP receptor is a complex between a family B GPCR known as the calcitonin receptor-like receptor (CLR) and a single-pass membrane protein, receptor activity-modifying protein 1 (RAMP1) (McLatchie *et al.*, 1998). A third protein, receptor component protein, is needed for G protein coupling but is of little known relevance for ligand binding (Prado *et al.*, 2002). CGRP also has high affinity at the AMY₁ receptor, a receptor for amylin formed from the calcitonin receptor (CTR) and RAMP1 (Christopoulos *et al.*, 1999; Poyner *et al.*, 2002); indeed it exhibits higher potency than amylin at the receptor formed between RAMP1 and the $\Delta 47$ splice variant of the CTR (Qi *et al.*, 2012). It is also possible that neuronally released CGRP may be able to activate the AMY₁ receptor. At pharmacological concentrations, CGRP can also activate the AM₂ and AMY₂ and AMY₃ receptors and has weak affinity for the AM₁ receptor (Hay *et al.*, 2008). This in turn poses problems for interpreting many studies where the pharmacology of CGRP or its derivatives have been examined on native tissues (where receptor subunit expression often remains undetermined), or cells known to express more than one type of receptor that can be activated by CGRP. There is a possibility that the apparent activity of some derivatives may be due to activation of one of the other CGRP-responsive receptors, not the CLR-RAMP1 complex. In Tables 1–4 where the activity of CGRP derivatives has been reported, the nature of the preparation has been noted; SK-N-MC and L6 cells are commonly used as models

of a CGRP-receptor-expressing system (Hay *et al.*, 2002). Although species differences can influence the potency of non-peptide antagonists, due to their mode of interaction with RAMP1 (Mallee *et al.*, 2002), this seems of much less importance for studies with full-length peptides (Poyner *et al.*, 2002).

The structure of CGRP

A number of NMR and circular dichroism studies have been conducted on CGRP and its derivatives, although no structure of CGRP has been deposited in the protein structure database. The data collectively are consistent with residues 8–18 of CGRP in aqueous solution forming an α -helix; there is some evidence for this for CGRP_{8–37} but the helix is less well defined. For both peptides, the helix is stabilized by increasing the hydrophobicity of the solution (Breeze *et al.*, 1991; Hubbard *et al.*, 1991; Sagoo *et al.*, 1991; Matsuura and Manning, 1993; O'Connell *et al.*, 1993; Boulanger *et al.*, 1995; 1996; Robinson *et al.*, 2009). There is some evidence for a β or γ turnaround residues 18–21 (Sagoo *et al.*, 1991; Boulanger *et al.*, 1995). Thereafter, it becomes difficult to detect any well-defined structure until the last 11 amino acids. A hydrogen bond between the carbonyl of Val²⁸ and the amide of Thr³⁰ has been detected in one study of CGRP (Boulanger *et al.*, 1996). In another study, a β -1 turn was seen between Gly³³ and Val³⁴ (Sagoo *et al.*, 1991). As will be discussed below, these observations of a set of turns at the extreme C-terminus of CGRP fit well with structure activity work on CGRP_{27–37}.

No structure is available for CGRP bound to lipid or detergent micelles; these provide a hydrophobic environment that is often considered to provide a better mimic of the environment that the peptide encounters in association with a receptor. Such structures are available for other peptides that bind to family B GPCRs. They show that the majority has at least some stretch of α -helix (see Watkins *et al.*, 2012, for a recent review). The assumption that micelle-bound peptides resemble receptor-bound peptides has been most rigorously tested for pituitary adenylate cyclase-activating polypeptide (PACAP), where NMR structures are available for the micelle-bound PACAP27 and receptor-bound PACAP21. In this case, the C-terminus of the peptide forms very similar α -helices, but the receptor-bound N-terminus of PACAP21 forms a β -coil not seen in the micelle-bound PACAP27 (Inooka *et al.*, 2001). This suggests that the micelle approach gives a reasonable approximation to the likely structure of parts of the peptide (probably at the C-terminus) but there may be localized changes induced by the receptor. It is not unreasonable to assume that these are most likely to be at either end of the peptide, where secondary structural elements have seldom been observed and the peptide forms an extended conformation. For CGRP, the most relevant structures that can be used for comparison are those of amylin, which has 37% sequence identity with CGRP (55% over the first 20 amino acids). In SDS micelles at pH 7.4, there is a clear helical region in amylin, extending over residues 7–17, immediately beyond the disulphide-bonded loop. At the end of this helix, there is a sharp turn then another helical section involving residues 21–28 or 23–29. There is yet another bend and then a final

section of helix from 33 to 35 (Nanga *et al.*, 2011). This is broadly similar to the pattern of turns and helices found in CGRP. A second set of structures for micelle-bound amylin conducted at pH 4.4 showed amylin to be predominantly helical from residues 5–17 and 18–28, with a bend of around 30° between these segments (Patil *et al.*, 2009). The theme of a helix followed by an area of more flexibility is also seen in structures of micelle-bound AM (Perez-Castells *et al.*, 2012) and CT (Hashimoto *et al.*, 1999).

Overall, if micelle-bound structures provide any guide to the receptor-bound structure, it seems reasonable to assume that CGRP exists as an α -helix from approximately residues 8–18, with the disulphide bond at its N-terminus. It is possible to align the various micelle-bound structures of amylin (2KB8), AM (2L7S) and salmon calcitonin (2GLH), so that the helices and disulphide-bonded loops overlap. Given the sequence overlap in the disulphide-bonded loop (discussed below), it is an attractive notion that all members of this family have this region bound to the receptor in a common orientation. Figure 7A gives a schematic illustration of CGRP showing the main structural features and also a speculative view as to where on the receptor they may interact.

Stabilizing the N-terminus

There has been some discussion about the roles of N-caps in stabilizing helical regions of family B peptide ligands. The N-cap is a structure formed by the amino acids at the N-terminal end of the helix, stabilizing it, and it relies on a combination of hydrogen bonds and hydrophobic interactions. It has been suggested that the disulphide-bonded loop serves this function in the CGRP/CT family (Neumann *et al.*, 2008). On the other hand, a classic N-cap motif is also present (Watkins *et al.*, 2012). Potentially the side chain of Thr⁹ in CGRP could form a hydrogen bond with the carbonyl backbone of the disulphide-bonded ring at Ala⁵; Thr⁹ has been shown to be involved in a hydrogen bond in CGRP in an NMR study, although the authors were not able to identify its partner (Breeze *et al.*, 1991). Thr⁹ is conserved in amylin, although only 10% of the structures of the micelle-bound form show this hydrogen bond (Patil *et al.*, 2009; Nanga *et al.*, 2011). The carbonyl of Ala⁵ could also hydrogen bond to the backbone amides of Thr⁹ and/or Val⁸ and these interactions will also help stabilize the 8–18 helix; in CGRP_{8–37}, N-cap motifs are likely to stabilize this helix (Watkins *et al.*, 2012). Beyond the helical region, the structure of bound CGRP is less certain. By analogy with the known structures of peptides bound to family B GPCRs, it might be expected to adopt a predominantly linear form (Archbold *et al.*, 2011), but as will be argued below, there is good evidence that bound CGRP incorporates a number of bends.

The role of residues 1–7: the disulphide-bonded loop

Removal of residues 1–7 creates CGRP_{8–37}, the prototypical CGRP antagonist. This binds approximately 10-fold less potently than CGRP and has lost all efficacy (Chiba *et al.*,

Table 1

Mutation of residues 1–7

Residue	Mutation	Effect	Comment	Reference
Ala ¹	[Tyr ⁰]-CGRP	Threefold decrease in affinity	SK-N-MC cells	(van Valen <i>et al.</i> , 1989)
Ala ¹	[Tyr ⁰]-CGRP	12-fold increase in affinity	Rat brain membranes	(Dennis <i>et al.</i> , 1989)
Ala ¹	[Tyr ⁰]-CGRP	33-fold decrease in affinity	L6 myocytes	(Poyner <i>et al.</i> , 1992)
Ala ¹	Extension by biotin	150-fold decrease in potency	L6 myocytes	(Howitt and Poyner, 1997)
Asp ³	Coupling to 4 azidoaniline	Twofold decrease in affinity	Solubilized rat cerebellum	(Stangl <i>et al.</i> , 1993)
Asp ³	Long-chain acylation	Sevenfold decrease in potency	CGRP receptor-expressing cell line	Patent WO/2011/051312
Thr ⁴	[Val ⁴]-CGRP	Eightfold decrease in affinity, fivefold decrease in potency	Porcine iris ciliary body	(Heino <i>et al.</i> , 1998)
Thr ⁴	Long-chain acylation	Sevenfold decrease in potency	CGRP receptor-expressing cell line	Patent WO/2011/051312
Thr ⁶	[Val ⁶]-CGRP	Fivefold decrease in affinity, no efficacy	Porcine iris ciliary body	(Heino <i>et al.</i> , 1998)
Cys ² , Cys ⁷	Removal of disulphide	No agonist activity	Rat atria	(Tippins <i>et al.</i> , 1986)
Cys ² , Cys ⁷	Removal of disulphide	Sixfold reduction in affinity	SK-N-MC cells	(Lang <i>et al.</i> , 2006)
Cys ² , Cys ⁷	cyclo [Asp ² , Lys ⁷]-CGRP	Twofold decrease in affinity	Rat spleen	(Dennis <i>et al.</i> , 1989)
Cys ² , Cys ⁷	cyclo [Asp ² , Lys ⁷]-CGRP	No agonist activity	Guinea pig atria	(Dennis <i>et al.</i> , 1989)
Cys ² , Cys ⁷	[Cys(Acm)] ^{2,7} -CGRP	Kd 40 nM ~400-fold decrease in affinity. Partial agonist, ~300-fold decrease in potency where measurable	CGRP receptor Cos 7 cells	(Bailey and Hay, 2006)
Cys ² , Cys ⁷	[Cys(Et) ^{2,7}]-CGRP	10-fold decrease in potency	CGRP receptor Cos 7 cells	(Bailey and Hay, 2006)
Residues 1–5	[Pro ^{7,8} , Cys ^{31,36}]-CGRP _{6–37} (SH992)	Twofold reduction in affinity, threefold reduction in potency, 80% reduction in Emax	Porcine iris ciliary body	(Heino <i>et al.</i> , 1998)

All differences are reported with respect to human α -CGRP.

1989; Dennis *et al.*, 1990). Thus, residues 1–7 have a dual role in activating the receptor, contributing to the overall affinity of bound CGRP, and stabilizing the helix. It seems likely that these residues interact predominantly with the ECLs and associated TM regions of CLR, based on the two domain models for family B GPCRs (Hoare, 2005). A variety of effects have been reported for N-terminal extension of CGRP (Dennis *et al.*, 1989; van Valen *et al.*, 1989; Poyner *et al.*, 1992). Although as Tyr⁰-CGRP can be used successfully as a radioligand, it seems that any reduction in affinity is modest (Table 1). This is consistent with the fact that AM and AM2, both of which bind to CLR, have substantial N-terminal extensions beyond the disulphide-bonded ring, although these can be removed without substantially decreasing potency (Eguchi *et al.*, 1994; Bailey and Hay, 2006). The first residue in CGRP is conserved as a small, uncharged amino acid: serine/glycine/alanine (Figure 1). The conservation suggests that the side chain of this residue is subject to some steric constraints, perhaps adjacent to a hydrophobic surface.

Residue 2 is a cysteine which forms the disulphide bond; the role of this will be considered below. There is extensive conservation of the next four amino acids: Asp/Asn-Thr-Ala-Thr. They are conserved in both CGRP and amylin; in addition, Thr⁶ is conserved in almost every member of the CT

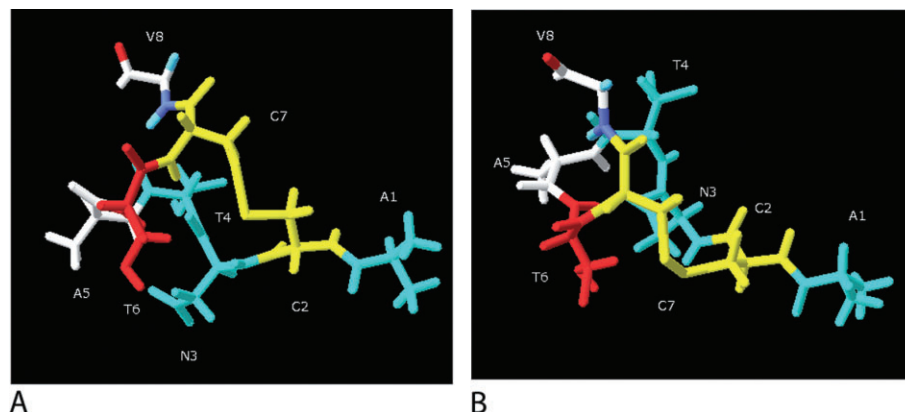
family (Figures 1, 2). It is possible to extend Asp³ by coupling it to 4-azidoaniline to produce a photoaffinity derivative of CGRP that can be used to label the receptor and which shows little change in affinity (Stangl *et al.*, 1993). This derivative could potentially retain any hydrogen bonds formed by the carboxyl or carbonyl amide moieties of aspartic acid and asparagine. However, it does suggest that the residue is not sitting in a particularly constrained pocket. It can also be acylated by a long-chain fatty acid with only a modest change in affinity (Patent WO/2011/051312). The γ -carbonyl of Asp³/Asn³ can potentially hydrogen bond to the backbone amide of Ala⁵, but whether this is of any relevance to bound CGRP is unknown.

The high conservation of the Thr-Ala-Thr motif in positions 4–6 suggests that Thr⁴ and Thr⁶ take part in hydrogen bonds while residue 5 sits in a sterically constrained environment. Substitution of Thr⁴ by valine (Heino *et al.*, 1998) or acylation by a long-chain substituent (Patent WO/2011/051312) causes modest decreases in affinity. [Val⁶]-CGRP is inactive at stimulating cAMP production in porcine iris ciliary body (Heino *et al.*, 1998), suggesting that this position is of especial importance. Interestingly, an AM derivative with substitution of the equivalent of Thr⁶ (Thr²⁰) was reported to have a reduced potency on blood pressure (Kuwasako *et al.*,

	1	10	20	30
CGRP α	AC.DTA	TCVTHRLAG.LL	SRSGGVVKNNF	VPIN.VGSKAF
CGRP β	AC.NTA	TCVTHRLAG.LL	SRSGGMVKSNF	VPIN.VGSKAF
Calcitonin	ACGNLS	TCMLGTYTQDFN	KFHFTF..QTA	IGVG..AP...
AM	YRQSMNMFQGLRSFGC	RFGTCTVQKLAHQIY	QFTDKD.KDNV	APRSKISPQGY
AM2	HSGPRRTQAQLLRVGC	VLGTCTQVQNLSHRLW	QLMGPAQRQDS	APVDPSSPHSY
AM3	RAGC	ALGTCTQVQNLSHRLY	QLIGQSGRDDSS	SPINPKSPHSY
AM4	AAGC	ALFMCAYHDLQRLNHI	YNKQ.KEVT	APKNKILSTGY
CRSP1	AC.DTA	TCVTHRLAG.LL	SRSGGVVKNNF	VPIN.VGSKAF

Figure 2

Sequence alignment from different members of the calcitonin peptide family generated using ClustalW and visualized using EsPript using the %Equivalence algorithm. Conserved regions are shown in red boxes with regions with similar physicochemical parameters in yellow.

**Figure 3**

Residues 1–8 of CGRP. (A) Side view. (B) View looking down on Val⁸. Blue: residues where modification makes a 2–10-fold change in potency; red, modification makes >10-fold change in potency; white, no information; yellow, Cys²–Cys⁷ disulphide bond. The structure is derived from a homology model of SDS-bound amylin (2KB8) and which was then minimized in water for 50 ns using CHARMM, as described previously (Bailey *et al.*, 2010). The orientation of the side chains is intended only to be illustrative.

2011). The residue may play an important role in the activation of CLR and CTR by all members of the peptide family.

The disulphide bond is of course a defining feature of the CGRP/CT family of peptides. Within CGRP, breakage of the disulphide bond usually reduces potency (Lang *et al.*, 2006). However, the effect depends on the nature of the modification. Much of the older literature of necessity relied on *in vivo* or *ex vivo* assays where CGRP and its derivatives could interact with a range of receptors. Using a recombinant CGRP receptor expressed in Cos 7 cells, it has been shown that [Cys(Acm)^{2,7}]-CGRP is a partial agonist with an apparent pK_B of 7.34, suggesting it bound 100–1000 times less well than human α -CGRP. However, [Cys(Et)^{2,7}]-CGRP was a full agonist which only had a 10-fold reduction in potency (Bailey and Hay, 2006). In older studies, analogues such as cyclo^{2,7}[Asp²,Lys⁷] human α -CGRP indicated that modification of the disulphide bridge can give modest effects on affinity, although they are typically more deleterious to efficacy (Dennis *et al.*, 1989). Thus, while the disulphide bridge enhances binding and biological activity in the endogenous peptide, at least some derivatives retain activity without it. It has been reported that a derivative SH992, where the first five amino acids of CGRP were deleted, and Cys⁷–Val⁸ was replaced by diproline, had weak partial agonist activity at stimulating cAMP production on the porcine iris-ciliary body

and rabbit lung (Hakala *et al.*, 1994; Heino *et al.*, 1998). It would be of interest to examine this peptide on a recombinant CGRP receptor to confirm its retention of agonist activity, but it suggests that some form of receptor activation can be achieved with just Thr⁶.

Taken as a whole, some degree of modification is possible for most residues in 1–7, but it is likely that each contributes a modest amount to the binding or efficacy for CGRP. However, Thr⁶ is probably a key residue for activation for all members of the CT family of peptides and may interact with one of the ECLs or TM helices (Figure 3).

The role of residues 8–18: the α -helix

The sequence of the presumed α -helix is highly conserved in CGRP, although in placental mammals residues 14–15 are Gly-Leu, rather than Asp-Phe in almost all other species. Across all members of the family, the general consensus sequence is x-x-polar-polar-Leu-Ala/Ser-x-x-Leu/Ile-polar-polar. This pattern does not create a particularly strongly conserved hydrophobic face but does give a series of hydrophobic and hydrophilic patches, a pattern that is clear in CGRP itself (Figure 4).

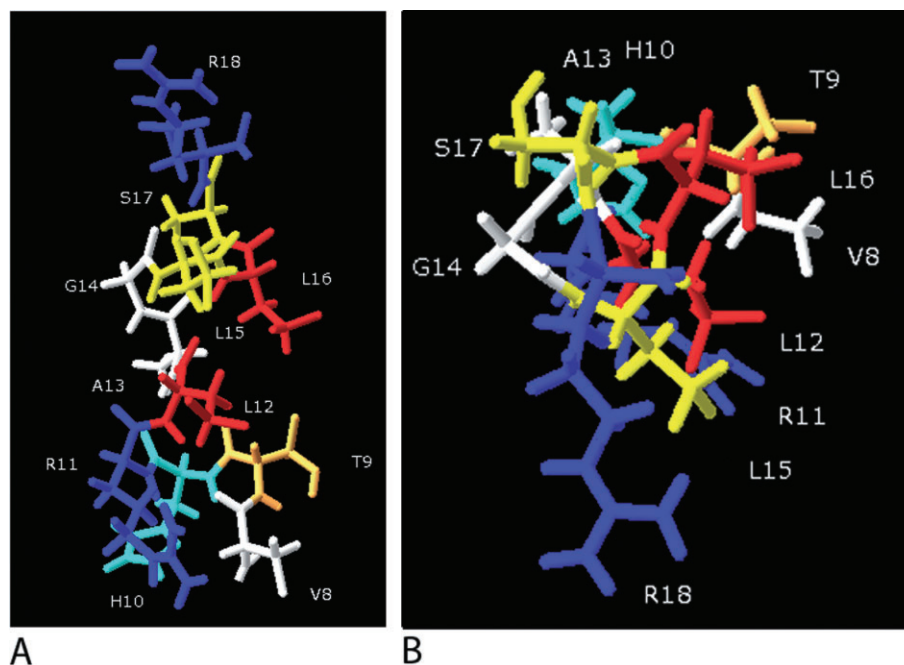


Figure 4

CGRP 8–18. (A) Side view. (B) View looking down on Arg¹⁸. Val⁸, Ala¹³ and Gly¹⁴ (white) have only minor effects on binding of CGRP or CGRP_{8–37} when mutated. His¹⁰ (light blue), Arg¹¹ and Arg¹⁸ (dark blue) potentially form distant coulombic interactions with positively charged residues. Thr⁹ (orange), Leu¹⁵ and Ser¹⁷ make minor contributions to the binding of this region; Ala¹³ and Gly¹⁴ appear of little importance. However, Leu¹² and Leu¹⁶ (red) lie on a sterically constrained face. The structure is derived from a homology model of SDS-bound amylin (2KB8) and which was then minimized in water for 50 ns using CHARMM, as described previously (Bailey *et al.*, 2010). The orientation of the side chains is intended only to be illustrative.

CGRP_{8–37} has a pK_B of around 8; a study of deletion analogues of CGRP_{8–37} indicates that by the time the peptide has been cut back to CGRP_{11–37} the pK_B is close to 6 (Mimeault *et al.*, 1991), although this probably reflects a destabilizing effect on the α -helix (Watkins *et al.*, 2012). CGRP_{19–37} generally binds with an affinity two orders of magnitude lower than CGRP_{8–37} (Rovero *et al.*, 1992; Howitt and Poyner, 1997; Poyner *et al.*, 1998). Thus, the 8–18 helix plays an important part in conferring high affinity to native CGRP. A graphic demonstration of its importance is that when it is substituted into AM_{22–52}, it increases its pA₂ at CGRP receptors from 6 to over 8 (Robinson *et al.*, 2009). Generally, substitutions of individual amino acids cause only modest changes in affinity (Table 2). Sometimes, the results are best interpreted as due to changes in peptide secondary structure of either the helix (by introducing prolines) or elsewhere in the peptide [perturbation of residues 28–37 caused by replacement of Ser¹⁷ by alanine (Boulanger *et al.*, 1996)]. The substitution of the leucines can produce larger effects. Leu¹⁵ can be replaced by a large benzoyl-phenylalanine moiety with only small changes in affinity; however, replacement of Leu¹² causes a 30-fold decrease and replacement of Leu¹⁶ well over a 100-fold decrease in affinity, suggesting both residues are in sterically confined pockets when bound to the receptor (Howitt *et al.*, 2003). The introduction of two lactam bridges between residues 9 and 13, and 15 and 19 resulted in a peptide that bound with around fivefold lower affinity than the parent compound (Miranda *et al.*, 2008). This suggests reasonable toleration of

the modification; the bridges would lie away from the face of the helix defined by Leu¹² and Leu¹⁶, where it is suggested that there are strict steric constraints. Replacement of Arg¹⁸ by alanine has virtually no effect but the double alanine mutant with Arg¹¹ shows 100-fold reduction in affinity. Replacing either of the arginines with glutamic acid caused over a 10-fold reduction in affinity; however, it was possible to substitute with glutamine with retention of high-affinity binding (Poyner *et al.*, 1998; Howitt *et al.*, 2003; Miranda *et al.*, 2008). The arginines may interact with an area of negative charge on the receptor. Potentially, His¹⁰ could also have a role in this interaction, although evidence is lacking.

The affinity of CGRP_{8–37} can be increased by hydrophobic substituents at the N-terminus; for example, by the introduction of a benzoyl group at N1 and a benzene at C4 of the imidazole ring of His¹⁰. Presumably, these are able to interact with a hydrophobic residue on the receptor which is normally too distant to interact with the bound native ligand (Smith *et al.*, 2003). This may be part of the pocket that normally accommodates residues 1–7. His¹⁰ is often exploited in radio-iodinated forms of CGRP; the introduction of an iodine atom here may have some influence on the properties of the resulting derivative, given the effects of benzylation noted above on CGRP_{8–37}.

In summary, the segment from residues 8–18 potentially has two roles: to orientate CGRP to allow efficient binding and to interact directly with the receptor itself [either the upper reaches of the ECLs or the ECD (Figure 7A)]. The

Table 2

Mutation of residues 8–18

Residue	Mutation	Effect	Comment	Reference
Val ⁸	Deletion (CGRP _{9–37})	No change in affinity	Guinea pig atria	(Mimeault <i>et al.</i> , 1991)
Val ⁸	[Pro ⁸]-CGRP _{8–37}	No change in affinity	Rat pulmonary artery	(Wisskirchen <i>et al.</i> , 2000)
Val ⁸	[Gly ⁸]-CGRP _{8–37}	No change in affinity	Rat pulmonary artery	(Wisskirchen <i>et al.</i> , 2000)
Thr ⁹	Deletion (CGRP _{10–37})	Fivefold decrease in affinity	Guinea pig atria	(Mimeault <i>et al.</i> , 1991)
Thr ⁹	[Ala ⁸]-CGRP _{8–37}	Threefold decrease in affinity	Guinea pig atria	(Mimeault <i>et al.</i> , 1992)
Thr ⁹ His ¹⁰	Deletion (CGRP _{11–37})	Further twofold decrease in affinity compared with CGRP _{9–37}	Guinea pig atria	(Mimeault <i>et al.</i> , 1991)
His ¹⁰	[Ala ¹⁰]-CGRP _{8–37}	Threefold decrease in affinity	Guinea pig atria	(Mimeault <i>et al.</i> , 1992)
His ¹⁰	Benzoylation of His	50-fold increase in affinity of CGRP _{8–37}	Porcine coronary artery	(Smith <i>et al.</i> , 2003)
Val ⁸ His ¹⁰ Gly ¹⁴	[Pro ⁸ Glu ¹⁰ Glu ¹⁴]-CGRP _{8–37}	>100-fold decrease in affinity	Rat pulmonary artery	(Wisskirchen <i>et al.</i> , 2000)
Thr ⁹ His ¹⁰ Arg ¹¹	Deletion (CGRP _{12–37})	Further twofold decrease in affinity compared with CGRP _{11–37}	Guinea pig atria	(Mimeault <i>et al.</i> , 1991)
Arg ¹¹	[Ala ¹¹]-CGRP _{8–37}	Fivefold decrease in affinity	Guinea pig atria	(Mimeault <i>et al.</i> , 1992)
Arg ¹¹	[Ala ¹¹]-CGRP _{8–37}	Sixfold decrease in affinity	L6 myocytes	(Howitt and Poyner, 1997)
Arg ¹¹	[Glu ¹¹]-CGRP _{8–37}	60-fold decrease in affinity	SK-N-MC cells	(Howitt <i>et al.</i> , 2003)
Arg ¹¹	Long-chain acylation	Threefold decrease in affinity	CGRP receptor-expressing cell line	Patent WO/2011/051312
Leu ¹²	Replacement by Bpa [Bpa ¹²]-CGRP _{8–37}	30-fold decrease in affinity	SK-N-MC cells	(Howitt <i>et al.</i> , 2003)
Gly ¹⁴	[Ala ¹⁴]-CGRP _{8–37}	No decrease in affinity	Porcine coronary artery	(Li <i>et al.</i> , 1997)
Gly ¹⁴	Replacement by Aib [Aib ¹⁴]-CGRP _{8–37}	Twofold decrease in affinity	Porcine coronary artery	(Li <i>et al.</i> , 1997)
Gly ¹⁴	[Asp ¹⁴]-CGRP _{8–37}	Twofold decrease in affinity	Porcine coronary artery	(Li <i>et al.</i> , 1997)
Gly ¹⁴	[Asn ¹⁴]-CGRP _{8–37}	Threefold decrease in affinity	Porcine coronary artery	(Li <i>et al.</i> , 1997)
Gly ¹⁴	[Pro ¹⁴]-CGRP _{8–37}	100-fold decrease in affinity	Porcine coronary artery	(Li <i>et al.</i> , 1997)
Leu ¹⁵	Replacement by Bpa [Bpa ¹⁵]-CGRP _{8–37}	Fivefold decrease in affinity	SK-N-MC cells	(Howitt <i>et al.</i> , 2003)
Leu ¹⁶	Replacement by Bpa [Bpa ¹⁶]-CGRP _{8–37}	700-fold decrease in affinity	SK-N-MC cells	(Howitt <i>et al.</i> , 2003)
Leu ¹⁶	[Pro ¹⁶]-CGRP _{8–37}	>1000-fold decrease in affinity	Rat pulmonary artery	(Wisskirchen <i>et al.</i> , 2000)
Leu ¹⁶	[Ala ¹⁶]-CGRP _{8–37}	Fivefold decrease in affinity	Rat pulmonary artery	(Wisskirchen <i>et al.</i> , 2000)
Ser ¹⁷	[Ala ¹⁷]-CGRP _{8–37}	Twofold increase in affinity	Guinea pig atrium	(Boulanger <i>et al.</i> , 1996)
Arg ¹⁸	[Ala ¹⁸]-CGRP _{8–37}	No decrease in affinity	L6 myocytes	(Howitt and Poyner, 1997)
Arg ¹⁸	[Glu ¹⁸]-CGRP _{8–37}	60-fold decrease in affinity	SK-N-MC cells	(Howitt <i>et al.</i> , 2003)
Arg ^{11,18}	[Ala ^{11,18}]-CGRP _{8–37}	300-fold decrease in affinity	L6 myocytes	(Howitt and Poyner, 1997)
Arg ^{11,18}	[Ser ^{11,18}]-CGRP _{8–37}	1000-fold decrease in affinity	SK-N-MC cells	(Howitt <i>et al.</i> , 2003)

All differences are with respect to human α -CGRP_{8–37}.
Bpa, 3-(4-benzoylphenyl)alanine.

segment can orientate both the N-terminus of CGRP and may also stabilize the C-terminus in a high-affinity conformation. Individual residues make only small contributions to the overall binding energy but, collectively these may make a substantial contribution to the binding affinity. Leu¹² and Leu¹⁶, which lie on the same face of the helix, (Figure 4), may be in close proximity to the receptor. Arg¹¹, Arg¹⁸ and possibly His¹⁰ may interact with an area of negative charge, probably arising from one or more residues some distance from the bound ligand.

As CGRP_{8–37} is an antagonist, it might be expected that it contributes little to the process of receptor activation, as opposed to ligand binding. However, at the guinea pig atrium, CGRP_{1–7} acts as an antagonist at high concentrations (apparent pK_B of 6.6), not an agonist (Dennis *et al.*, 1989). CGRP_{1–12}, CGRP_{1–15} and CGRP_{1–22} all mimicked the hypotensive effects of CGRP when injected into anaesthetized rats, albeit with considerable loss of potency (Maggi *et al.*, 1990). This suggests that residues 8–12 (and possibly beyond) are needed to promote the efficacy of any activator switches in

Table 3

Mutations of residues 19–26

Residue	Mutation	Effect	Comment	Reference
Ser ¹⁹ Gly ²⁰	Replaced by BTD [BTD ^{19–20}]-CGRP _{8–37}	No decrease in affinity	Rat pulmonary artery	(Wisskirchen <i>et al.</i> , 2000)
Gly ²⁰	[Gly ²⁰]-CGRP _{8–37}	Twofold decrease in affinity	Guinea pig atrium	(Boulanger <i>et al.</i> , 1996)
Lys ²⁴	Long-chain acylation	Threefold decrease in affinity	CGRP receptor-expressing cell line	Patent WO/2011/051312
Asn ²⁵	Long-chain acylation	Threefold decrease in affinity	CGRP receptor-expressing cell line	Patent WO/2011/051312
Asn ²⁶	Long-chain acylation	Threefold decrease in affinity	CGRP receptor-expressing cell line	Patent WO/2011/051312
Residues 1–18	CGRP _{19–37}	40-fold decrease in affinity	SK-N-MC cells	(Poyner <i>et al.</i> , 1998)
Residues 1–7 and 19–27	CGRP _{8–18, 28–37}	18-fold decrease in affinity	SK-N-MC cells	(Poyner <i>et al.</i> , 1998)
Residues 1–27	Tyr ⁰ -CGRP _{28–37}	170-fold decrease in affinity (fourfold decrease with respect to CGRP _{19–37})	SK-N-MC cells	(Poyner <i>et al.</i> , 1998)

All differences are with respect to human α -CGRP_{8–37}.
BTD, β -turn dipeptide.

1–7 of CGRP. There is of course no proof that these effects were mediated by CGRP receptors, but given the data that N-terminal fragments of other family B GPCRs can show agonist activity, it would not be surprising if this was the case. If it is accepted that CGRP_{1–7} is an antagonist but CGRP_{1–12} is an agonist, then this suggests that residues in the regions 8–12 are able to promote receptor activation, perhaps by allowing the correct orientation of 1–7, although the mechanism for this is unclear.

Residues 19–26

The key structural feature of this part of CGRP is the evidence for a bend covering residues 19–21. The sequence of Ser-Gly-Gly is almost completely conserved in all known CGRP sequences. Not only is there evidence for this from the structural studies both on CGRP and other members of the peptide family reviewed above, but introduction of a β -turn dipeptide at residues 19–20 maintained the normal affinity of CGRP_{8–37} at the CGRP receptor of rat pulmonary artery (Table 3) (Wisskirchen *et al.*, 2000). This raises questions about whether residues 8–37 of CGRP bind to its receptor predominantly as a helix as presumed or observed for the equivalent regions of many other peptide ligands for family B GPCRs. The bend at this position is likely to be important for its local effects on the structure of CGRP, but there is also NMR evidence that it helps maintain structure at the extreme C-terminus of the peptide (Boulanger *et al.*, 1996).

Residues 19–26 are important for CGRP binding, as demonstrated by their deletion in a CGRP_{8–37} analogue; this bound with around 10-fold lower affinity than CGRP_{8–37} itself (Poyner *et al.*, 1998). There is however little detailed structure–activity information for this part of CGRP. Replacement of the glycines at positions 20 and 21 reduces affinity, probably by disrupting the α -turn (Boulanger *et al.*, 1996). Introduction of a long-chain acylated lysine at positions 24,

25 or 26 is tolerated with a three- to fourfold loss in potency (Patent WO/2011/051312), suggesting that the residues are subject to a rather loose steric constraint. Substitution of Lys²⁴Asn²⁵ by Arg-Lys gives a small increase in potency, possibly suggesting that the region is influenced by a negative charge on the receptor (Miranda *et al.*, 2008). Substitution of Asn²⁵ by leucine gives only a small change in affinity but the corresponding replacement of Asn²⁶ is reported to give almost a 100-fold reduction in affinity, by far the largest change seen for any substitution in this part of the molecule (Heino *et al.*, 1998). This residue aside, the main function of this part of the molecule may be to orientate CGRP in a hinge-like manner. There may well be contacts between individual amino acids and the receptor, but as with 8–18, singly these may be relatively weak.

Residues 27–37; the C-terminus

Human α -CGRP_{27–37} and Tyr⁰-CGRP_{28–37} have only low affinity for the CGRP receptor (pK_B 6–7) (Howitt and Poyner, 1997; Heino *et al.*, 1998). However, analogues comprising this region have been produced which have nanomolar-binding affinity; CGRP_{27–37} derived from the frog *Phyllomedusa bicolor* also has a high affinity (Ladram *et al.*, 2008), but generally substitution is necessary to achieve this. *P. bicolor* CGRP naturally contains many of the substitutions that are used to increase the affinity of human α -CGRP_{27–37} (Figure 1). In some studies, constraints have been introduced such as with a disulphide bond, or others have used amino acid substitutions to promote bends, either in fragments or full-length CGRP (Hakala *et al.*, 1994; Heino *et al.*, 1998; Rist *et al.*, 1998; 1999; Wisskirchen *et al.*, 2000; Carpenter *et al.*, 2001; Lang *et al.*, 2006; Boeglin *et al.*, 2007; Yan *et al.*, 2011) (Table 4, Figure 5). The most significant residues seem to be in the 30–37 segment of peptide, but with a turn, around residue 29 being important to orientate Thr³⁰ (Carpenter *et al.*, 2001;

Table 4

Mutations of residues 27–37

Residue	Mutation	Effect	Comment	Reference
Phe ²⁷	[Phe ²⁷]-CGRP _{27–37}	Eightfold decrease in affinity	SK-N-MC cells	(Rist <i>et al.</i> , 1998)
Val ²⁸	[Ala ²⁸]-Tyr ⁰ CGRP _{27–37}	Threefold decrease in affinity	SK-N-MC cells	(Rist <i>et al.</i> , 1998)
Pro ²⁹	[Ala ²⁹]-Tyr ⁰ CGRP _{27–37}	No binding	SK-N-MC cells	(Rist <i>et al.</i> , 1998)
Pro ²⁹	[Ala ²⁹ Ala ³⁴ Phe ³⁵]-CGRP _{27–37}	Sevenfold decrease in affinity	SK-N-MC cells	(Carpenter <i>et al.</i> , 2001)
Pro ²⁹	[^Δ ²⁹ D ³¹ P ³⁴ F ³⁵]-CGRP _{27–37}	No change in affinity compared with CGRP _{8–37}	SK-N-MC cells	(Lang <i>et al.</i> , 2006)
Thr ³⁰	[Ala ³⁰]-Tyr ⁰ CGRP _{27–37}	No binding	SK-N-MC cells	(Rist <i>et al.</i> , 1998)
Thr ³⁰	[Ser ³⁰]-Tyr ⁰ CGRP _{27–37}	>30-fold decrease in affinity	SK-N-MC cells	(Rist <i>et al.</i> , 1998)
Thr ³⁰	[Ala ³⁰ Ala ³⁴ Phe ³⁵]-CGRP _{27–37}	100-fold less potent than [Ala ³⁴ Phe ³⁵]-CGRP _{27–37}	SK-N-MC cells	(Carpenter <i>et al.</i> , 2001)
Asn ³¹	[Ala ³¹]-Tyr ⁰ CGRP _{27–37}	>30-fold decrease in affinity	SK-N-MC cells	(Rist <i>et al.</i> , 1998)
Asn ³¹	[Leu ³¹]-Tyr ⁰ CGRP _{27–37}	Twofold decrease in affinity	SK-N-MC cells	(Rist <i>et al.</i> , 1998)
Asn ³¹	[Gln ³¹]-Tyr ⁰ CGRP _{27–37}	30-fold decrease in affinity	SK-N-MC cells	(Rist <i>et al.</i> , 1998)
Asn ³¹	[Asp ³¹]-Tyr ⁰ CGRP _{27–37}	Twofold decrease in affinity	SK-N-MC cells	(Rist <i>et al.</i> , 1998)
Asn ³¹	[Ala ³¹ Ala ³⁴ Phe ³⁵]-CGRP _{27–37}	Fivefold less potent than [Ala ³⁴ Phe ³⁵]-CGRP _{27–37}	SK-N-MC cells	(Carpenter <i>et al.</i> , 2001)
Val ³²	[Ala ³²]-Tyr ⁰ CGRP _{27–37}	No binding	SK-N-MC cells	(Rist <i>et al.</i> , 1998)
Val ³²	[Ala ³² Ala ³⁴ Phe ³⁵]-CGRP _{27–37}	240-fold decrease in affinity compared with [Ala ³⁴ Phe ³⁵]-CGRP _{27–37}	SK-N-MC cells	(Carpenter <i>et al.</i> , 2001)
Gly ³³	[Ala ³³]-Tyr ⁰ CGRP _{27–37}	No binding	SK-N-MC cells	(Rist <i>et al.</i> , 1998)
Gly ³³	[Ala ³³ Ala ³⁴ Phe ³⁵]-CGRP _{27–37}	23-fold decrease in affinity	SK-N-MC cells	(Carpenter <i>et al.</i> , 2001)
Gly ³³	[Asp ³¹ ,azaGly ³³ ,Pro ³⁴ ,Phe ³⁵]-CGRP _{29–37}	No binding	CGRP receptor, HEK293 cells	(Boeglin <i>et al.</i> , 2007)
Gly ³³	[Asp ³¹ ,azaGly ³³ ,Pro ³⁴ ,Phe ³⁵]-CGRP _{27–37}	10-fold increase in affinity compared with [Asp ³¹ ,Pro ³⁴ ,Phe ³⁵]-CGRP _{27–37}	CGRP receptor, HEK293 cells	(Boeglin <i>et al.</i> , 2007)
Ser ³⁴	[Ala ³⁴]-Tyr ⁰ CGRP _{27–37}	No change in affinity	SK-N-MC cells	(Rist <i>et al.</i> , 1998)
Ser ³⁴	[D ³¹ ▼ ³⁴ F ³⁵]-CGRP _{8–37}	Threefold decrease in affinity compared with CGRP _{8–37}	SK-N-MC cells	(Lang <i>et al.</i> , 2006)
Glu ³⁵	[Ala ³⁵]-Tyr ⁰ CGRP _{27–37}	Threefold decrease in affinity	SK-N-MC cells	(Rist <i>et al.</i> , 1998)
Glu ³⁵	[His ³⁵]-Tyr ⁰ CGRP _{27–37}	No change in affinity	SK-N-MC cells	(Rist <i>et al.</i> , 1998)
Glu ³⁵	[Gln ³⁵]-Tyr ⁰ CGRP _{27–37}	Twofold decrease in affinity	SK-N-MC cells	(Rist <i>et al.</i> , 1998)
Glu ³⁵	[Leu ³⁵]-Tyr ⁰ CGRP _{27–37}	Threefold decrease in affinity	SK-N-MC cells	(Rist <i>et al.</i> , 1998)
Glu ³⁵	[Ala ³⁵ Ala ³⁴ Phe ³⁵]-CGRP _{27–37}	14-fold less potent than [Ala ³⁴ Phe ³⁵]-CGRP _{27–37}	SK-N-MC cells	(Carpenter <i>et al.</i> , 2001)
Ala ³⁶	[Phe ³⁶]-Tyr ⁰ CGRP _{27–37}	No binding	SK-N-MC cells	(Rist <i>et al.</i> , 1998)
Ala ³⁶	[Gly ³⁶]-Tyr ⁰ CGRP _{27–37}	16-fold decrease in affinity	SK-N-MC cells	(Rist <i>et al.</i> , 1998)
Phe ³⁷	[Ala ³⁷]-Tyr ⁰ CGRP _{27–37}	No binding	SK-N-MC cells	(Rist <i>et al.</i> , 1998)
Phe ³⁷	[Tyr ³⁷]-Tyr ⁰ CGRP _{27–37}	11-fold decrease in affinity	SK-N-MC cells	(Rist <i>et al.</i> , 1998)
Phe ³⁷	[Ala ³⁷ Ala ³⁴ Phe ³⁵]-CGRP _{27–37}	>340-fold decrease in affinity compared with [Ala ³⁴ Phe ³⁵]-CGRP _{27–37}	SK-N-MC cells	(Carpenter <i>et al.</i> , 2001)
Phe ³⁷	[Gly ³⁷]-CGRP _{8–37}	2050-fold decrease in affinity of CGRP _{8–37}	Porcine coronary artery	(Smith <i>et al.</i> , 2003)
Phe ³⁷	[Tyr ³⁷]-CGRP _{8–37}	No decrease in affinity of CGRP _{8–37}	Porcine coronary artery	(Smith <i>et al.</i> , 2003)
Phe ³⁷	Replacement by rotational restricted Phe analogues	40–60-fold decrease in affinity of CGRP _{8–37}	Porcine coronary artery	(Smith <i>et al.</i> , 2003)
Phe ³⁷	[Ala ³⁷]-CGRP _{8–37}	>100-fold decrease in affinity of CGRP _{8–37}	Rat pulmonary artery	(Wisskirchen <i>et al.</i> , 2000)
Asn ³¹ Ser ³⁴ Glu ³⁵	[Asp ³¹ Ala ³⁴ Phe ³⁵]-CGRP _{27–37}	10-fold decrease in affinity compared with CGRP	SK-N-MC cells	(Carpenter <i>et al.</i> , 2001)
Asn ³¹ Ser ³⁴ Glu ³⁵	[Asp ³¹ Phe ³⁴ Phe ³⁵]-CGRP _{27–37}	Twofold decrease in affinity compared with CGRP	SK-N-MC cells	(Carpenter <i>et al.</i> , 2001)
Ser ¹⁹ Gly ²⁰ and Gly ³³ Ser ³⁴	Both replaced by BTD	No decrease in affinity	Rat pulmonary artery	(Wisskirchen <i>et al.</i> , 2000)
Residues 1–27	CGRP _{27–37}	16000-fold decrease in affinity compared with CGRP _{8–37}	SK-N-MC cells	(Ladram <i>et al.</i> , 2008)
Residues 1–27	Tyr ⁰ -CGRP _{19–37}	900-fold decrease in affinity to CGRP _{8–37}	SK-N-MC cells	(Heino <i>et al.</i> , 1998)
Phyllomedusa CGRP _{27–37}	Residues 1–28	Ki 95 nM (fivefold less potent than human CGRP)	SK-N-MC cells	(Ladram <i>et al.</i> , 2008)

Unless otherwise stated, all differences are with respect to the parent compound used to create the analogue: human α -CGRP_{27–37}, Tyr⁰CGRP_{27–37} or CGRP_{8–37}. ▼, ▲: the ▼ and ▲ conformers of β -aminocyclopropane carboxylic acid. BTD, β -turn dipeptide.

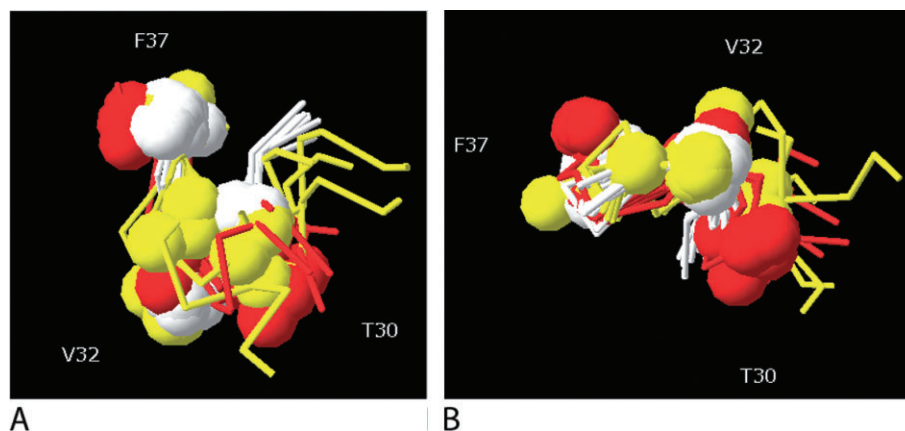


Figure 5

Overlay of residues 27–37 for CGRP derivatives. (A) Side view. (B) View looking up on Val³². White, cyclo-[Cys²⁹, Cys³⁶]CGRP_{27–37}; yellow, cyclo-[Cys³¹, Cys³⁶]CGRP_{27–37}; red, [D³¹,A³⁴,F³⁵]CGRP_{27–37}. Homology models were created from SDS-bound amylin (2KB8) and this was then minimized in water using CHARMM, as described previously (Bailey *et al.*, 2010). For cyclo-[Cys²⁹, Cys³⁶]CGRP_{27–37}, structures at 50 s intervals to 350 ns are superimposed. For cyclo-[Cys³¹, Cys³⁶]CGRP_{27–37}, structures after 10, 15, 20, 22, 27, 32, 37, 41 and 89 ns are superimposed. For [D³¹,A³⁴,F³⁵]CGRP_{27–37}, structures at 50 s intervals to 300 ns are superimposed. Spheres indicate the position of the α -carbons of Thr³⁰, Val³² and Phe³⁷.

Lang *et al.*, 2006). Within residues 30–37, a β -turn between amino acids 33 and 34 is extremely important for high-affinity interactions (Wisskirchen *et al.*, 2000; Carpenter *et al.*, 2001; Lang *et al.*, 2006; Boeglin *et al.*, 2007). As noted above, these two elements of secondary structure can be detected among the assemblages of NMR structures obtained for CGRP or CGRP_{8–37}.

Two key short peptide antagonists have been identified: [D³¹,P³⁴,F³⁵]CGRP_{27–37} and [D³¹,A³⁴,F³⁵]CGRP_{27–37}. Structure–activity studies have shown that Thr³⁰, Val³², Gly³³ and Phe³⁷ are key residues (Rist *et al.*, 1998; 1999). While the Phe²⁷-Val²⁸-Pro²⁹ sequence is highly conserved in all forms of CGRP, its role is probably chiefly to orientate the subsequent C-terminal residues. Gly³³ is likely to be involved in the β -turn; the side chains of Thr³⁰, Val³² and Phe³⁷ are plausibly involved in receptor contacts. Thr³⁰ cannot be replaced by serine without some loss of affinity, and Val³² cannot be replaced by alanine or phenylalanine, indicating extremely strict steric constraints at these positions. Asn³¹ can tolerate replacement with Asp or Leu but not Glu, suggesting steric constraints; substitution with Ala reduces affinity, but there is no agreement on the magnitude of the effect (Rist *et al.*, 1998; Carpenter *et al.*, 2001) depending on whether A³⁴P³⁵CGRP_{28–37} or Tyr⁰CGRP_{28–37} is used. The effect of substitutions at Gly³³ also differs depending on the peptide analogue being studied (Carpenter *et al.*, 2001; Boeglin *et al.*, 2007). Position 35 tolerates not only phenylalanine but numerous bulky synthetic analogues such as β -(2-naphthyl)alanine (Miranda *et al.*, 2008); in native CGRP this position can either be positively or negatively charged or uncharged (Figure 1), suggesting that the side chain is probably not normally in contact with the receptor. Phe³⁷ is another residue where the structure–activity relationship depends on the nature of the peptide used. It is possible to substitute this with Tyr or Leu in CGRP_{8–37} with little loss of affinity, but attempts to constrain the rotational flexibility of the aromatic ring are not tolerated (Heino *et al.*,

1998; Smith *et al.*, 2003). By contrast, Tyr substitution is not tolerated in Tyr⁰-CGRP_{28–37} (Rist *et al.*, 1999). In a disulphide-constrained analogue, Phe³⁷ can be replaced by pyridylalanine (Yan *et al.*, 2011). There is agreement that the C-terminal amide is essential for all analogues (Rist *et al.*, 1999; Smith *et al.*, 2003).

Constraining the conformation of the C-terminus by a disulphide bond or other bridge unsurprisingly can change affinity. However, as with amino acid substitutions, the details of the effects depend on the analogue. A bridge between residues 31 and 36 is well tolerated for CGRP_{8–37} but not in a CGRP_{27–37} derivative, where only a 29–36 bridge was effective (Heino *et al.*, 1998; Yan *et al.*, 2011).

A number of conclusions can be drawn about the role of the C-terminus. It is of major significance for the binding affinity of CGRP; there is good evidence for a number of key contacts between residue side chains and the receptor, particularly involving Thr³⁰, Val³², Phe³⁷ and the C-terminal amide. There are strong steric constraints to ensure these residues are orientated correctly, and other parts of the C-terminus such as Gly³³Ser³⁴ help orientate the contact residues. It is possible to overlay the C-termini of the two highest affinity disulphide-constrained CGRP analogues (cyclo-[Cys²⁹, Cys³⁶]CGRP_{27–37} and [Pro^{7,8},Cys^{31,36}]-CGRP_{6–37}) with CGRP_{27–37} incorporating a backbone hydrogen bond between Val²⁸ and Thr³⁰, and β -turn between Gly³³ and Ser³⁴ (Figure 5); in all of these structures, there is good correlation between the positions of individual amino acids and these are probably a fair approximation of the structure of the bound peptide. There are differences in detail as to the structure activity relationships between different peptides, indicating they bind in different ways. This can be seen in Figure 6, where comparison is made between the effects of equivalent substitutions at either Tyr⁰-CGRP_{27–37} and [Ala³⁴Phe³⁵]-CGRP_{27–37} or [Asp³¹Pro³⁴Phe³⁵]-CGRP_{27–37} and [Asp³¹Pro³⁴Phe³⁵]-CGRP_{29–37}. The most striking example is with the latter pair of peptides,

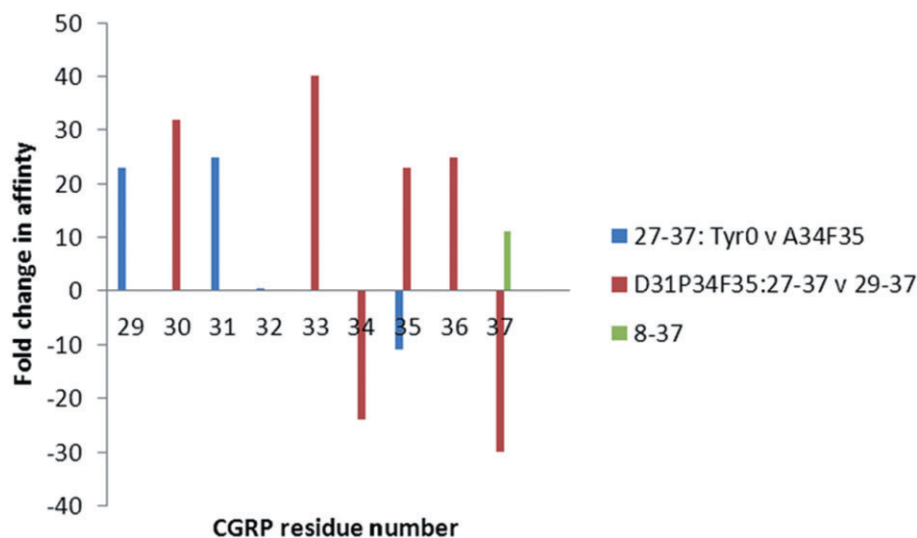


Figure 6

Effect of analogue on the C-terminal CGRP structure activity relationship. For each set of comparisons, the change in affinity between pairs of analogues at individual residues has been plotted. Blue, comparison between Tyr⁰-CGRP₂₇₋₃₇ and [Ala³⁴Phe³⁵]-CGRP₂₇₋₃₇ replacing residues with alanine (Rist *et al.*, 1998; Carpenter *et al.*, 2001). Blue dots indicate no difference, no comparison was made at positions 34 and 35. Red, comparison between [Asp³¹Pro³⁴Phe³⁵]-CGRP₂₇₋₃₇ and [Asp³¹Pro³⁴Phe³⁵]-CGRP₂₉₋₃₇ replacing residues with aza Gly (Boeglin *et al.*, 2007). No comparisons were done at positions 29 and 32. Green, comparison between [Tyr^{0,37}]-CGRP₂₇₋₃₇ and [Tyr³⁷]-CGRP₈₋₃₇ (Smith *et al.*, 2003). There are no data for CGRP₈₋₃₇ at other positions.

where substitution of Gly³³ with its aza equivalent either abolishes high-affinity binding or increases it 10-fold (Boeglin *et al.*, 2007). For these short analogues, it is possible that the principle-binding interactions seen with CGRP are conserved, but that the substitutions act to strengthen weak interactions by providing extra or better contacts which are of little significance for the longer peptides.

CGRP-receptor contacts

It is very difficult to predict the contact points on the receptor for CGRP with any level of useful detail. This is especially the case as a number of binding modes have been either identified or proposed for other family B GPCRs for both the N- and C-termini of the peptides. (Archbold *et al.*, 2011; Barwell *et al.*, 2011; Couvineau and Laburthe, 2011). We have previously suggested that the N-terminus of CGRP may contact the CGRP receptor in a pocket formed chiefly by ECLs 1 and 2 (Barwell *et al.*, 2012). Residues 8–18 and 19–27 are most likely to contact the ECD of CLR; by analogy with bound glucagon-like peptide 1 (GLP-1), exendin, glucose-dependent insulinotropic polypeptide, parathyroid hormone (PTH) and PTHrP (Parthier *et al.*, 2007; Pioszak and Xu, 2008; Runge *et al.*, 2008; Pioszak *et al.*, 2009; Underwood *et al.*, 2010). The main interactions are likely to be with the N-terminal helix and the second and fourth loops. For most of the above structures, the C-termini of the ligands make contacts in the vicinity of loop 4 of their receptors. However, in the CGRP receptor, we suggest that the β -bend around residues 20–21 and the turns at the extreme C-terminus of the peptide act to direct the latter part of the molecule towards a cavity formed

from helices 2 and 3 of RAMP1 and the interconnecting loop 2 and the adjacent portions of the ECD of CLR, particularly the N-terminal helix and the second loop (Barwell *et al.*, 2010; Moore *et al.*, 2010). It is interesting that the C-terminus of exendin, which forms a pronounced loop stabilizing a Trp-cage, also points away from loops 2 and 4 of the GLP-1 receptor into the space where it is suggested that the C-terminal loop of CGRP may sit (Figure 7). These predictions are of course speculative, although the model is consistent with the structures of the bound non-peptide CGRP antagonists, which also occupy the space between loop 2 of RAMP1 and the N-terminus and loops 2 and 4 of CLR (ter Haar *et al.*, 2010).

Conclusions

There is enough evidence in the literature to identify both areas of CGRP that make key contacts with the receptor and those that are more distant. A case can be made that there are relatively few individual high-affinity or efficacy contacts. These might include Thr⁶, important for receptor activation, a hydrophobic patch involving Leu¹² and Leu¹⁶, Asn²⁶, Thr³⁰, Val³², Phe³⁷ and the C-terminal amide. There are likely to be a more extensive range of minor contacts, for example, His¹⁰ and Arg¹¹, possibly responding to negatively charged amino acids on the ECD of CLR some distance (>5 Å) from the bound ligand. Collectively, these probably make a substantial contribution to the binding affinity. A third set of residues are those that are required for stabilizing an element of secondary structure: Cys², Cys⁷, residues 9–12 for the 8–18 α -helix, 19–21, 28–30 and 33–34 for bends.

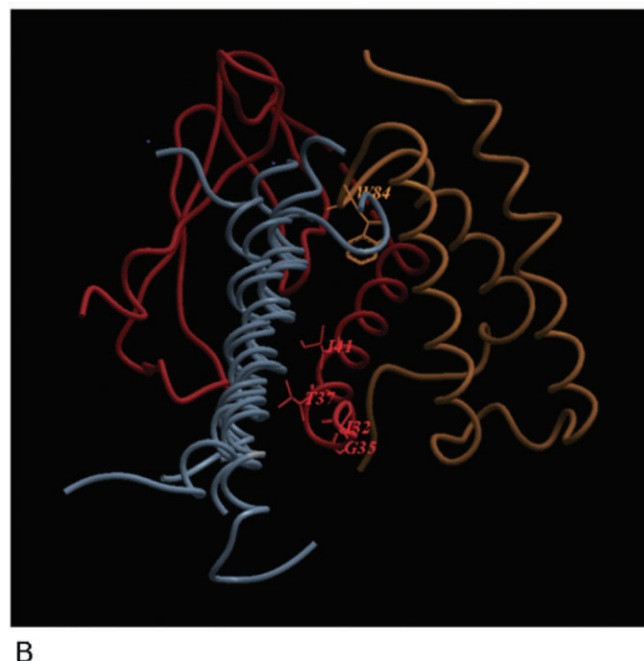
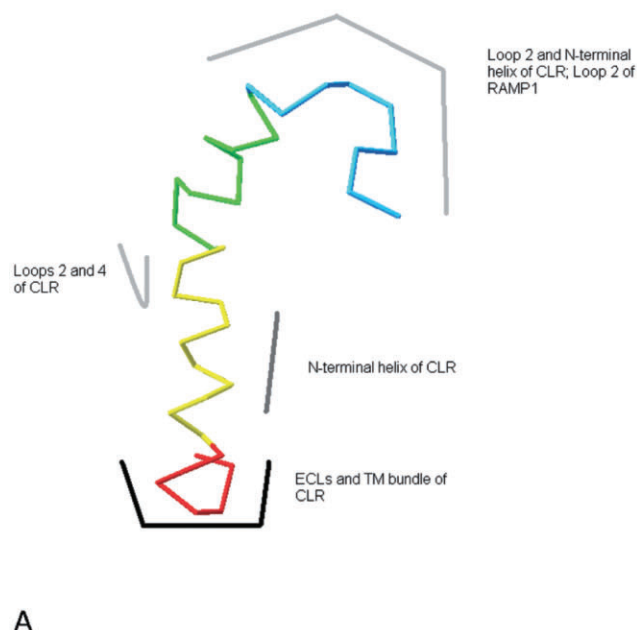


Figure 7

(A) Schematic model of the possible interaction of CGRP with its receptor. Red, residues 1–7 of CGRP; yellow, residues 8–18; green, residues 19–27; blue, residues 28–37. The structure shown of CGRP is simply for illustrative purposes. (B) Superimposition of the bound structures of PTH, GIP, GLP-1 and exendin (all grey) on the extracellular domains of CLR (red) and RAMP1 (orange). Residues on CLR and RAMP1 known to be important for CGRP binding are shown.

The importance of bends for high-affinity binding of CGRP raises questions as to the nature of the bound peptide. The conventional model for ligand binding of family B GPCRs to their receptors envisages either helical or slightly bent helices binding to the N-terminus. It is not clear how far this can be applied to CGRP; the β -bend that seems likely to exist over residues 19–21 and the potential sharp turns at the C-terminus may imply a more complicated structure for the bound peptide. Of course, the alternative is that in the bound receptor, the bends can be accommodated within a broadly linear binding mode.

Clearly, a crystal structure of CGRP bound to its receptor will clarify many of the points raised in this review. Given progress with GPCR crystallization, this is not unrealistic, although it is disappointing that it has not so far been possible to co-crystallize any part of the peptide with the ECD of the receptor; an alternative would be an NMR structure of bound CGRP, as has been reported for other family B GPCR peptide ligands (Inooka *et al.*, 2001; Watkins *et al.*, 2011). Even if crystals are obtained, these are unlikely to solve all problems. A crystal structure is simply a single picture of what is likely to be a dynamic process; the predictions need to be verified by mutagenesis and extended by NMR, molecular modelling and other allied techniques. In terms of therapeutics, there remains a need for additional CGRP antagonists and especially, novel agonists. Work to understand how the N-terminus of CGRP interacts and activates its receptor is particularly needed, especially if molecules can be produced, which retain selectivity for CGRP

receptors (or other receptors for which CGRP is a physiological agonist).

Conflict of interest

None.

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