STRUCTURE-ACTIVITY RELATIONSHIP OF CALCITONIN GENE-RELATED PEPTIDE AND ITS RECEPTOR

Biochemical and Radioligand binding Studies of CGRP (Calcitonin gene-related peptide) receptors on L6, SK-N-MC and Col29 cells

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Master of Philosophy

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Summary

Calcitonin gene-related peptide (CGRP) is a 37 amino acid peptide, which is widely distributed in the brain and peripheral nervous system. It possesses a wide variety of biological effects in the brain as well as in peripheral tissues including most potent vasodilator actions, pro-inflammatory and nociceptive effects. The various actions are thought to be due to the stimulation of different CGRP receptors. Thus, the discrimination between CGRP receptor subtypes is most important for clinical therapy. Recently, CGRP receptors have briefly been divided into two subtypes, CGRP1 and CGRP2. However, questions still remain about the extent of the receptor diversity.

Structure-activity studies can help to understand how the peptide interacts with receptor. Within the peptide, residues 8-18, especially positively charged arginines at position 11 and 18 are of interest. Most potent CGRP analogues all have those two amino acids. When replaced by the negatively charged glutamic acid, the peptide fragment [11Glu]CGRP8-37 showed weak antagonist activity, while [18Glu]CGRP8-37 failed to block the action of CGRP. Fragment [11, 18Ser]CGRP8-37 where two hydroxyl side-chain containing serines replaced arginine at position 11 and 18 showed weak antagonist ability at the high concentration of 10μM. Those results revealed that 11 arginine may interact with a hydrophilic partner, while 18 arginine may bind with a negatively charged one.

The exact nature of the CGRP receptors remains uncertain. Nevertheless, Calcitonin Receptor-Like Receptor (CRLR) combined with Receptor Activity-Modified Protein (RAMP') seems most likely to be a CGRP receptor. Experiments have been carried out to modify the receptor. Glycosylation seems to be important to allow CGRP producing cAMP. The roles of sulphydryl groups are complicated. Their apparent alkylation by NEM reduces cAMP production but increases CGRP binding.

I would like to dedicate this thesis to my maternal grandparents

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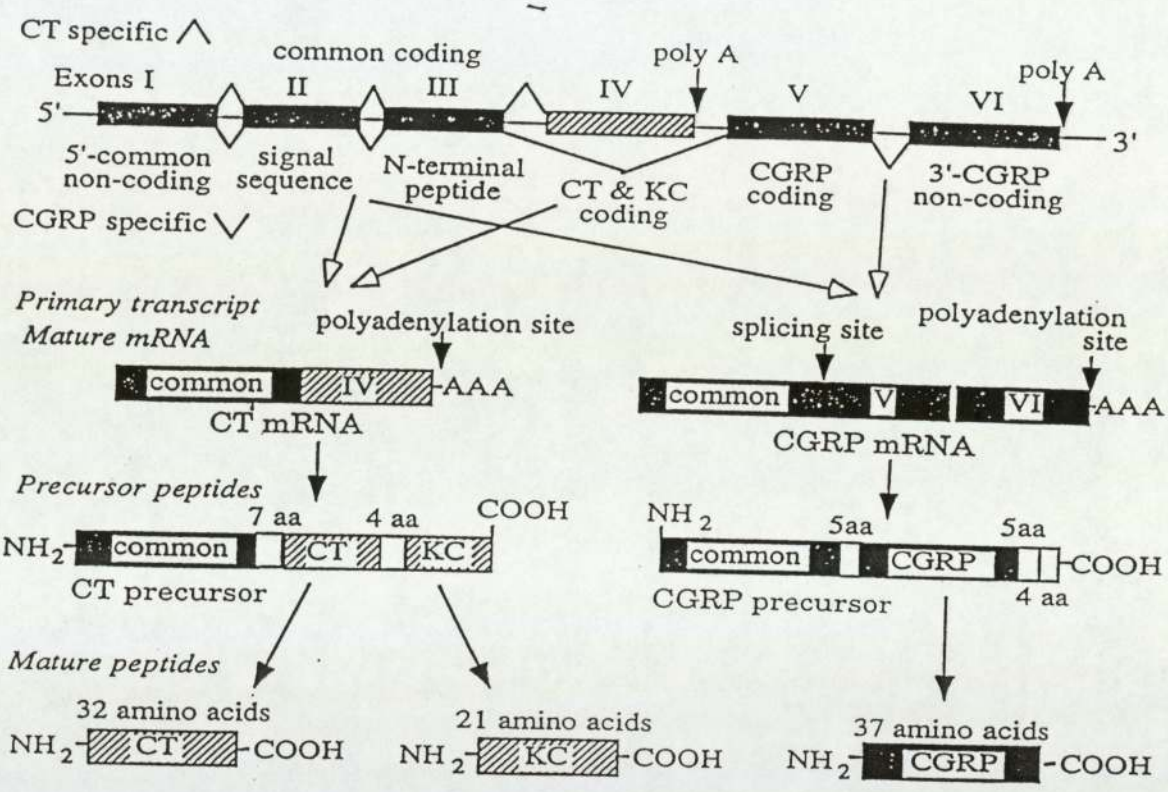
Chapter 1. Introduction

1.1 Discovery of CGRP and related peptides

CGRP (Calcitonin gene-related peptide) is derived from the CGRP/Calcitonin (CT) gene, which is localized to chromosome 11. Alternative splicing of a primary RNA transcript leads to the translation of CGRP and CT peptides in a tissue-specific manner (Rosenfeld et al. 1983). The discovery of CGRP was made from the CT gene. Tumour cells lost the ability to produce CT when they were serially transplanted from rat medullarly thyroid carcinoma. This spontaneous switch from high to low CT production was accompanied by an increase in the size of the mRNA detected by CT cDNA probes that was later determined to be an altered mRNA species. These two mRNAs (original one and altered one) have identical 5' sequences but significantly different 3' sequences. Translation of these mRNA produces either the 17.5kDa Calcitonin precursor protein or the 16kDa CGRP precursor. Proteolytic procession of the precursors yields calcitonin or CGRP plus the flanking N- or C- terminal peptides. The use of calcitonin and CGRP specific DNA probes has shown that the alternate splicing of the calcitonin gene that produces the two mRNAs is regulated tissue-specifically such that calcitonin mRNA is found almost exclusively in the thyroid and CGRP mRNA in the central nervous system (Amara et al. 1982; Rosenfeld et al 1983). Although the existence of a splicing enhancement factor has been postulated, the mechanism of regulation of CT/CGRP production via alternative RNA splicing remains unclear (Diagram 1).

Diagram 1. The alternative splicing of CT/CGRP gene (Wimalawansa 1996)



The existence of a second CGRP gene (β-CGRP) was predicted from further analysis of rat and human cDNA clones (Amara et al. 1985; Steenbergh et al. 1985). The former CGRP discovered by Rosenfeld was named α-CGRP. The β-CGRP gene is located on chromosome 12. The second gene is thought to be due to gene duplication and it remains unclear which of the two genes appeared first.

At present, over nine forms of CGRP have been sequenced, α- and β-CGRP from rat and human, and single variants from sheep, pig, chick, salmon and frog (Poyner 1997). The structural differences between α- and β-CGRP in humans and rats are small, three amino acids in humans (positions 3, 22 and 25), and only a single amino acid in rats (position 35) (Diagram 2). Among the mammalian CGRPs, there is a minimum of 84% identity, but even salmon CGRP shows 76% identity with its most distantly related mammalian count part (Poyner 1997). There is a high degree of conservation of CGRP between species. Rat, human and chick CGRPs have broadly similar potencies. CGRP-like immunoreactivity in invertebrates has led to the suggestion that CGRP may share a common evolutionary origin with lobster cystine proteases (Arlot-Bonnemains et al. 1996).

CGRP shows approximately 46% homology to amylin and about 25% homology to adrenomedullin (Diagram 2). These peptides also share key features of their secondary structure, like the 2,7 disulfide bridge, etc. These peptides are all considered as members of the calcitonin super family of peptides (Poyner 1997).

Human-α-CGRP NH_2-ACDTATCVTHRLAGLLSRSGGVVKNNFV PTNVGSKAF-$CONH_2$

Human β-CGGP NH_2-ACNTATCVTHRLAGLLSRSGGMVKSNFV PTNVGSKAF-$CONH_2$

Rat α-CGRP NH_2-SCNTATCVTHRLAGLLSRSGGVVKDNFVP TNVGSEAF-$CONH_2$

Rat β-CGRP NH_2-SCNTATCVTHRLAGLLSRSGGVVKDNFVPT NVGSKAF-$CONH_2$

Chicken CGRP NH_2-ACNTATCVTHRLADFLSRS GGVGKNNFVP TNVGSKAF-$CONH_2$

Human amylin NH_2-KCNTATCATQRLANFLVHSSNNFGAILSST NVGSNTY-$CONH_2$

Salmon calcitonin NH_2- CSNLSTCVLGKLSQDLHKLQTYPR TNTG SGTP-$CONH_2$

[11E] Human α-CGRP NH_2-ACDTATCVTHELAGLLS RSGGVV KNNFVPTNVGS KAE-CONH2

[18E] Human α-CGRP NH_2-ACDTATCVTHRLAGLLSESGGVVK NNFVPTNVGSKAE-$CONH_2$

Diagram 2. Sequences of CGRP and its analogues. (Poyner 1992a for review) A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine Y, tyrosine.

1.2 Structure and activity of CGRP

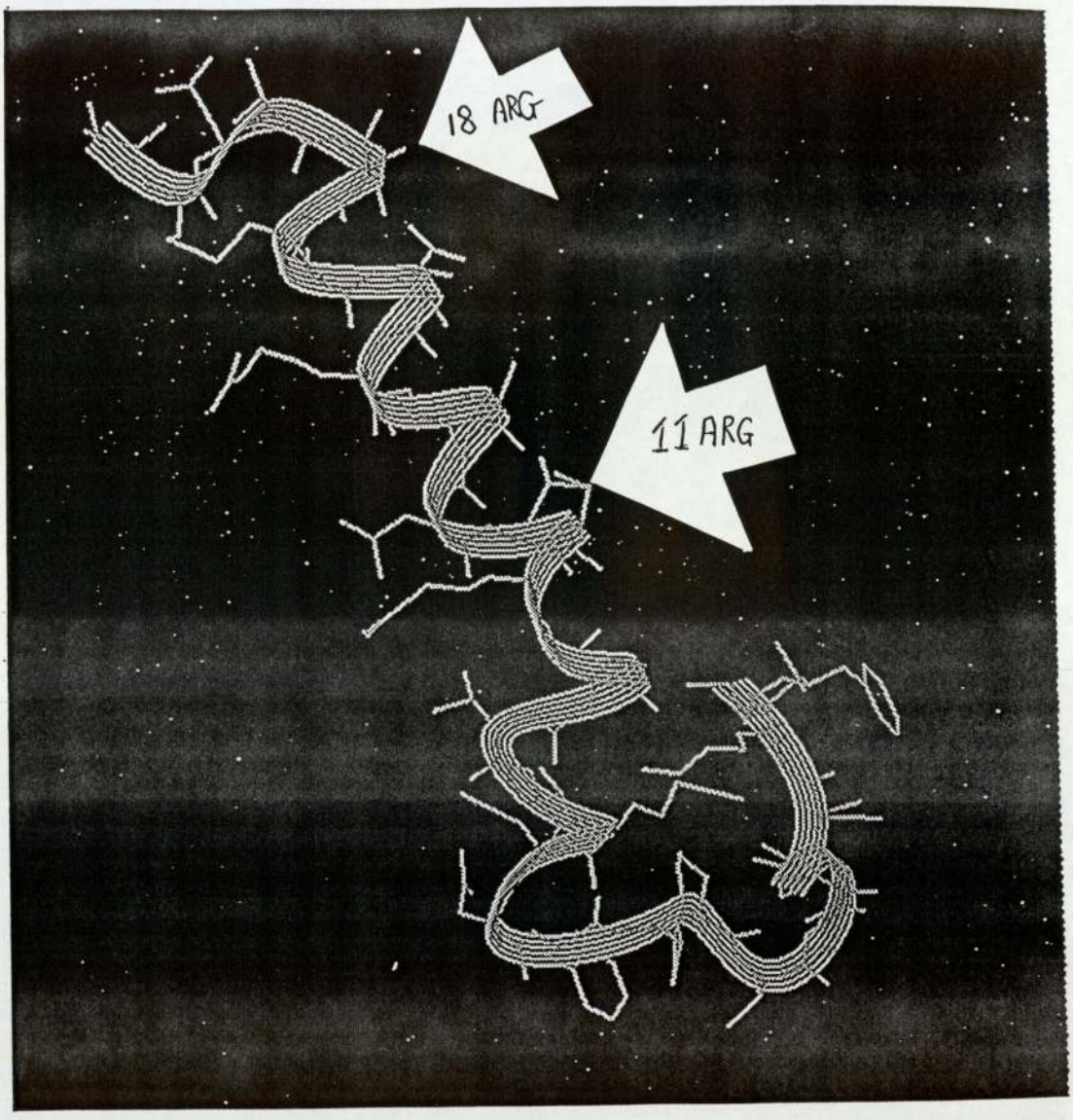
Human α-CGRP is a 37 amino acid peptide. Using circular dichroism (cd) and nuclear magnetic resonance (NMR), a number of obvious structural features have been revealed. Briefly, there is a disulfide bridge between residues 2 and 7 at the N-terminus, an amphipathic α-helix among residues 8-18, and an amidated C-terminus (Diagram 3).

1.2.1 Residues 1-7

The N- terminus includes residues 1-7, and is thought to form a discrete domain for binding and receptor activation, as has been proposed for other peptides (Schwyzer 1987). Removal of the first seven residues, including the disulfide-bonded loop, leaves CGRP8-37, an antagonist (Chiba et al. 1989), while CGRP1-12 acts as agonist (Dennis et al. 1989).

There is a disulfide bond between cysteines at positions 2 and 7, giving the N-terminus a rigid structure (Breeze et al. 1991). The presence of the intact disulfide bond is very important; reduction and derivatisation destroys agonist activity at the CGRP1 receptors. [acetamidomethylcysteine2,7]-CGRP ([Cys(ACM)2,7]-CGRP) with a blocked disulfide-ring structure is less potent than CGRP itself. Replacing the acetamino methyl moiety with an ethylamide group gives another CGRP analogue--[CysEt]-CGRP. It can activate CGRP2 receptors with a potency approaching that of CGRP itself, but it remains largely inactive on CGRP1 receptor (Dumont et al. 1997). It is clear that geometry imposed by the

Diagram 3. 2-D illustration of structure of CGRP 1-18. "Solution structure of human CGRP by H-NMR and distance geometry with restrained molecular dynamics" Breeze A.L., Harvey T.S., Bazzo R. & Campbell I,D. (1991) *Biochemistry* 30 575-582



disulfide is essential for CGRP activity; if an amide linkage replaces it, all agonist activity is abolished at both CGRP receptor subtype (Dennis et al. 1989).

Extension of the N-terminus by tyrosine produces variable affinity values for the peptide, although the changes are usually small. However extension by the biotin group causes a dramatic loss of potency (Howitt & Poyner 1997). Thus limited N-terminal extension is tolerated, but this cannot be taken too far. The N-terminus is likely to reside in some kind of conformationally restricted space on the receptor.

1.2.2 Residues 8-18 (α-helix)

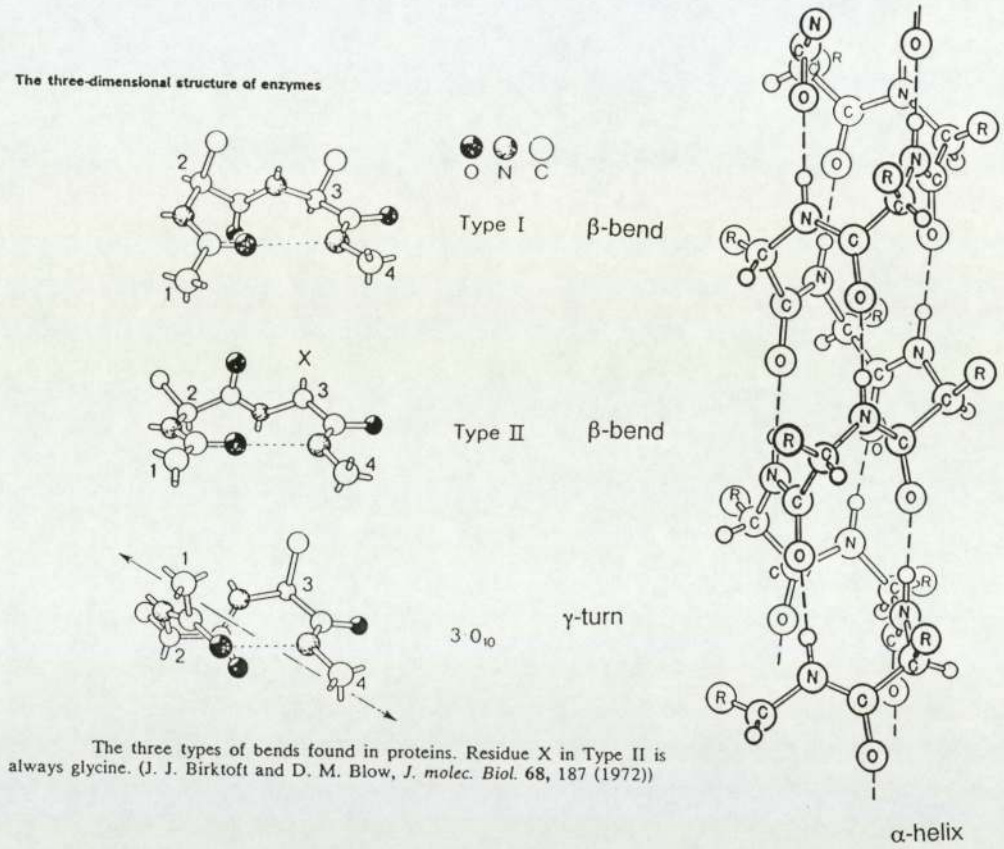
The amphipathic α-helix that is formed by residues 8-18 is thought to be a key feature of CGRP (Lynch & Kaiser 1988). When the residues 8-18 are removed, the resulting fragment is generally 50-100 fold less potent than CGRP8-37 (Howitt & Poyner, 1997; Rovero et al. 1992; Poyner et al. 1998), although in some systems CGRP19-37 shows anomalous behaviour (Tomlinson et al. 1996). However, residues 8-18 by themselves do not bind to the receptor with appreciable affinity (Poyner 1997). They probably work in combination with the other parts of the peptide to bind to CGRP receptors.

For CGRP8-37 acting at the CGRP1 receptors found in the guinea-pig heart, different amino acid residues show different levels of importance. For example, Val 8 is relatively unimportant. Deletion of Thr 9 causes about a five-fold loss of potency (Mimeault et al. 1991; Mimeault et al. 1993). Continuing deletions of Arg

11 and Leu 12 cause further but smaller reductions in affinity. L-alanine substitution of these individual residues reveals small reductions in affinity except at position 11 (arginine). There is also a 40% reduction in helical content with this analogue. This is an interesting result since the alanine substitution would normally be expected to increase helical content. Replacement of Ser17 by alanine increased the potency of CGRP8-37 on the guinea-pig atrium by two fold. This was thought to be due to the changes in the structure of C-terminus between residues 28-37, rather than of the residues 8-18 (Boulanger et al. 1996).

Though controversy still exists, there is no doubt that helical content is a factor in determining affinity. Other factors are also significant. The physical size of the N-terminal fragments such as CGRP8-37 or CGRP12-37 may be important. Affinity will suffer if it is too short. Amphipathicity is also an important consideration. A CGRP derivative (mastoparan-CGRP28-37) where the entire region is replaced by the amphipathic peptide mastoparan, binds with identical affinity as its parent compound (CGRP8-18,28-37) (Poyner et al. 1998), and the importance of amphipathicity was also emphasised by Lynch and Kaiser (1988). However, the exact nature and role of the amphipathic region remains unclear, particularly in CGRP8-37. The existence of amphipathic helix has been inferred from CD measurements made in hydrophobic solvent mixtures (Hubbard et al. 1991). However, two-dimensional nuclear magnetic resonance studies have suggested that this region is largely a random coil (Boulanger et al. 1996). Despite all these uncertainties, arginines 11 and 18 in this region are particularly interesting. Details of these two residues will be discussed below (See 1.8 Aims of the work).

Diagram 4. Structure of α-helix, β-bend and γ-turn. Fersht A. (1977) *Enzyme Structure and Mechanism.*



The three types of bends found in proteins. Residue X in Type II is always glycine. (J. J. Birktoft and D. M. Blow, *J. molec. Biol.* **68**, 187 (1972))

. The right-handed α-helix which is found in proteins. (Reprinted from Linus Pauling: *The nature of the chemical bond.* © 1939, 1940, third edition © 1960 by Cornell University. Used by permission of Cornell University Press.)

1.2.3 Residues 19-27

Residues 19-27 have not been investigated in the thesis. However, this part of the mole follows immediately after the α-helix and may interact with it (Boulanger et al. 1996). It is uncertain about the structure of this part of the molecule. Based on NMR and modelling studies of a CGRP19-37 fragment, a possible β-turn (Diagram 4) has been postulated between resides 19-22 (Mimeault et al. 1993). Nevertheless, another NMR study of a CGRP suggested a γ-turn limited to Ser19-Gly21. Deletion of the entire segment causes a seven-fold decrease in affinity (Poyner et al. 1998). CGRP8-21,28-37, and CGRP8-18,21-37 show the same affinity to the receptor, despite the disruption of the bend. (Poyner et al. unpublished). One interpretation of this data is that residues 19-27 predominantly function as a hinge in the binding of CGRP.

1.2.4 Residues 28-37

The C-terminus, chiefly residues 28-37, are essential for high affinity binding. Although much work on the structure-activity relationship has been done, there is no agreement on the structure of this part of CGRP. There is an agreement that removal of the terminal phenylalanine residue results in complete loss of high affinity binding; obviously further truncations also produce inactive species (O'Connell et al. 1993; Poyner 1992a; Smith et al. 1993; Zaidi et al. 1990).

Although most studies have shown that isolated fragments such as CGRP28-37 have very low affinity for the CGRP receptor (Howitt & Poyner 1997; Tomlinson

et al. 1996; Poyner 1992a), Rist et al. (1998) have shown that [Asp^{31}, Pro^{34}, Phe^{35}]CGRP27-37 binds to the CGRP1 receptor on human SK-N-MC cells with a Kd of about 30nM. Thr^{30}, Val^{32}, Gly^{33} and Phe^{37} were crucial to receptor binding. Replacement by alanine, phenylalanine, proline or the corresponding D-amino acids all caused major decreases in affinity (Rist et al. 1998). Furthermore, [Asp^{31}, Pro^{34}, Phe^{35}]CGRP27-37 can significantly decrease the CGRP-induced vascular conductance in rat right femoral artery (Rist et al. 1999). Thus it is of interest to compare the structure of [Asp^{31}, Pro^{34}, Phe^{35}]CGRP27-37 and CGRP8-37 with the corresponding region in the full-length peptide. [Asp^{31},Pro^{34},Phe^{35}]CGRP27-37 may spontaneously have conformation, which the C-terminus of the full-length peptide adopts in the presence of residues 8-18 (as mentioned above). This analogue confirms that residues 27-37 are a key point for peptide binding to the receptor.

1.3 Receptors

1.3.1 Introduction

Much work has been done on the pharmacology of CGRP receptors. Using binding studies, by 1992 over 50 determinations of CGRP affinity had been made (Poyner 1992a). More experiments have been done on the pharmacological analysis using functional assays. However, no simple picture has emerged from these data due to the lack of suitable CGRP analogues. The most available forms of CGRP are human and rat α-and β-CGRP. Amylin also acts as a weak agonist on CGRP receptor although it only shares 46% homology with CGRP. Truncated CGRP fragments, like CGRP8-37, CGRP12-37 and [Tyr°

]-CGRP28-37, play an important role in studying the receptors. Usually, they are antagonists. The experiments using CGRP8-37 and [Cys(ACM)2,7]-CGRP are the basis for CGRP receptor division. Some substituted CGRP fragments like [Ala11]-CGRP8-37, [Ala18]-CGRP8-37, M432 (mastoparan-CGRP28-37), M436 (mastoparan-CGRP8-18,28-37) are useful in structure and receptor research (Howitt & Poyner 1997).

At least three kinds of proteins had been proposed to be CGRP receptors, RDC-1, CGRP-RPC and CRLR, which will be discussed below. By now, CRLR combined with RAMPs is widely accepted as a CGRP receptor. This model does accommodate existing data well. Research has also revealed that accessory protein RAMP may also help to form receptors for related peptides like amylin.

Due to the confused literature, it is not easy to give a receptor division for CGRP that fits all the data. The provisional division was postulated by Quirion et al. (1992). Though it has not met with universal support, it does accommodate a lot of existing work and is a useful working model. However, much work still remains to be done before a clear picture of CGRP receptors will be seen.

1.3.2 Subdivision of CGRP receptors

The existence of CGRP receptor sub-types was proposed on the basis of the differential antagonistic potencies of C-terminal fragments to block the action of CGRP, and the comparative agonistic properties of linear agonists (such as [Cys(ACM)2,7]-CGRP) in a variety of tissues (Dennis et al. 1989). C-terminal fragments such as CGRP8-37, CGRP9-37 and to a lesser extent CGRP12-37 behaved as relatively potent, competitive antagonists of CGRP-induced inotropic

and chronotropic effects in the guinea pig atria while being much less effective in blocking the effects of CGRP in the rat vas deferens (Dennis et al.1989; Dennis et al. 1990; Mimeault et al. 1991). In contrast, the linear agonist [Cys(ACM)2,7]CGRP was a weak agonist (EC50=100nM) in the vas deferens while being mostly inactive in the atrial preparations (Dennis et al. 1989). Similar results were obtained using either rat or guinea pig atrial and vasa deferentia preparations indicating that the observed differential profile of activity are not merely species related (Quirion et al. 1992).

There is little doubt that there are multiple receptors for CGRP. The problem is the extent of this diversity. Various groups have reported on the antagonistic properties of C-terminal CGRP fragments, especially CGRP8-37, in a variety of *in vitro* and *in vivo* bioassays. On that basis, a provisional division was postulated by Quirion et al. (1992). On the basis of the effects of CGRP antagonists, it was proposed that CGRP receptors be divided into CGRP1 and CGRP2 classes. CGRP1 receptors are more sensitive to CGRP8-37 than CGRP2 receptors and are 10 times less sensitive to [Cys(ACM)2,7]-CGRP than CGRP2 receptors. Those in the guinea pig heart typify CGRP1 receptors, CGRP2 receptors are typified by those in the rat vas deferens (Dennis et al. 1990). Although it has been suggested that there is a much smaller difference in CGRP8-37 affinity between the two subtypes when account is taken of protease activity (Longmore et al. 1992), consistent differences have been found in the sensitivity of subtypes to the CGRP fragments 9-37, 10-37, 11-37 and 12-37 (Dennis et al. 1989; Mimeault et al 1991), when allowing for this. In most cases, it seems that CGRP8-37 has a pA2 of 7.0-8.0 at CGRP1 receptors, while [Cys(ACM)2,7]-CGRP is a selective agonist at CGRP2 receptors (Dennis et al. 1989).

1.3.3 Antagonists and agonists

CGRP8-37 is the most important antagonist that has been found on CGRP receptors. It has a very high affinity at some CGRP receptors, e.g. pA2 value of 8.35 in rat liver plasma membranes (For more data on CGRP8-37, see Poyner 1992a review). It was first described by Chiba et al. (1989), where it produced a parallel rightwards shift in the does-response curve of human α-CGRP (acting to stimulate cAMP production in plasma membranes of rat liver). CGRP8-37 had no agonist activity at concentrations of up to 10μM. At the same time, a second antagonist CGRP12-37 was reported by Dennis et al. (1989). CGRP12-37 was a much better antagonist in the guinea-pig atrium than the rat vas deferens. This was an important study because for the first time it indicated that an antagonist could discriminate between different CGRP receptors. CGRP12-37 has a lower affinity than CGRP8-37 and is not generally used.

One experiment suggests that [Tyr°] CGRP28-37 may have an affinity as great as that of CGRP8-37 in the opossum internal anal sphincter (Chakder and Rattan, 1990). However, in other systems it was shown to have a low pA2 (Howitt & Poyner, 1997). [Asp^{31}, Pro^{34}, Phe^{35}]CGRP27-37 has been synthesised by Rist et al. (1998), and has been shown to be a potent antagonist. It is interesting, since the whole peptide only contains 11 amino acids. It demonstrates the importance of the C-terminus of CGRP in binding.

Beside human and rat α-and β-CGRP, [Cys(ACM)2,7]-CGRP is an important CGRP agonist. [Cys(ACM)2,7]-CGRP as a selective agonist was first identified by Dennis et al. (1989). It was inactive on cardiac tissue (EC50>710nM), but had

an EC50 of 76nM on the vas deferens. Thus, they proposed that it was selective for CGRP2 receptors. This has been supported by studies on L6 cells (rat myocytes), which are sensitive to CGRP8-37, but are not activated by [Cys(ACM)2,7]-CGRP (Poyner et al. 1992b). [Cys(ACM)2,7]-CGRP can also discriminate between receptor subtypes in radioligand binding assays. It binds with similar affinities to rat brain and spleen membranes (IC50s of 3nM and 6.8nM, respectively) (Dennis et al. 1989), but in the nucleus accumbens the binding of [Cys(ACM)2,7]-CGRP is weaker than to medial frontal cortex. However, it is not clear how far this reflects the CGRP1/CGRP2 division of receptors, particularly as the nucleus accumbens binding site might be an amylin receptor (For details, see below).

In some experiments, β-CGRP appears noticeably more potent than hα-CGRP and rα-CGRP (e.g. Van Valen et al. 1989). A number of studies have examined [Tyr°]-CGRP. This is generally less potent than CGRP (Poyner et al. 1992b), although Dennis et al. (1989) found it was 10-fold more potent in binding to rat brain and cerebellum.

CGRP8-37 is a widely used antagonist. However, its pA2 value varies from group to group. It is difficult to say which one is near the truth. As discussed above, the classification of CGRP receptor is based on the differences between its affinities on the different preparations. Given the variation of the pA2 values, it is difficult to define a precise pKd for a particular CGRP receptor subtype. Although CGRP receptors are currently divided into two types, more potent antagonists might show this to be an oversimplification. Non-peptide antagonists are required to exclude the possible involvement of various peptidases.

1.4 Amylin and adrenomedullin

Members of the CGRP family also include amylin (AMY) and adrenomedullin (ADM), which share about 46% and 25% sequence homologies with CGRP, respectively.

1.4.1 Amylin

Amylin is a 37-amino acid peptide synthesised and co-secreted with insulin from pancreatic β cells (Wimalawansa 1997). The peptide shares with CGRP an N-terminal disulphide bridge, an amphipathic α-helix and an amidated C terminus. Deposits of amylin are found in the pancreases of patients with type II diabetes and have been implicated in the pathogenesis of this disease. Biological actions of amylin include inhibition of insulin-stimulated glucose uptake and glycogen synthesis in the skeletal musculature.

The similarity between the structures and metabolic effects of CGRP and amylin initially suggested that they could act via common receptors. Amylin can act as a weak agonist at CGRP1 receptors, stimulating adenylate cyclase in L6 skeletal myocytes and producing vasodilation in rat kidney (Poyner et al. 1992b; Chin et al. 1994). However, there is good evidence that on soleus muscle, amylin acts through a receptor that is distinct from the CGRP1 receptor. Here amylin actions are preferentially inhibited by antagonists such as AC187; by contrast, CGRP is preferentially inhibited by CGRP8-37 (Beaumont et al. 1995).

Specific high affinity binding sties for amylin have been identified in the brain and peripheral tissues (e.g. Bhogal et al. 1993). In brain, amylin binds with high

affinity to a site, which seems identical to the "C3" site previously identified by Sexton et al. (1986) as having a high affinity for both CGRP and calcitonin. This seems to be a good model of the amylin receptor (Beaumont et al. 1993). Recent evidences suggest that the amylin receptor involves a complex between the calcitonin receptor and an accessory protein (RAMP1 or RAMP3). This will be discussed below.

1.4.2 Adrenomedullin

Adrenomedullin was first isolated from human pheochromocytoma and is a potent, vasorelaxant and hypotensive peptide (Kitamura et al. 1993; Ishiyama et al. 1993; Nuki et al 1993). Adrenomedullin, moreover, showed diuretic and natriuretic activity (Ebara et al. 1994). Increased plasma levels in hypertensive patients, compared with normal subjects, suggest a role for circulating adrenomedullin in blood pressure control (Kitamuta et al. 1994). The tissues of human and rat, which express adrenomedullin encoding messenger RNA (mRNA), include the adrenal medulla, lung, kidney, and heart; and the peptide has been identified in endothelial and vascular smooth muscle cells (Kitamura et al. 1993).

The existence of unique adrenomedullin receptors was demonstrated further with the cloning of an adrenomedullin preferring receptor (McLatchie et al. 1998). The clone belongs to a seven-transmembrane domain receptor super family. This receptor can also bind CGRP in the presence of an appropriate accessory protein, which will be discussed below.

1.5 Biochemistry of receptors

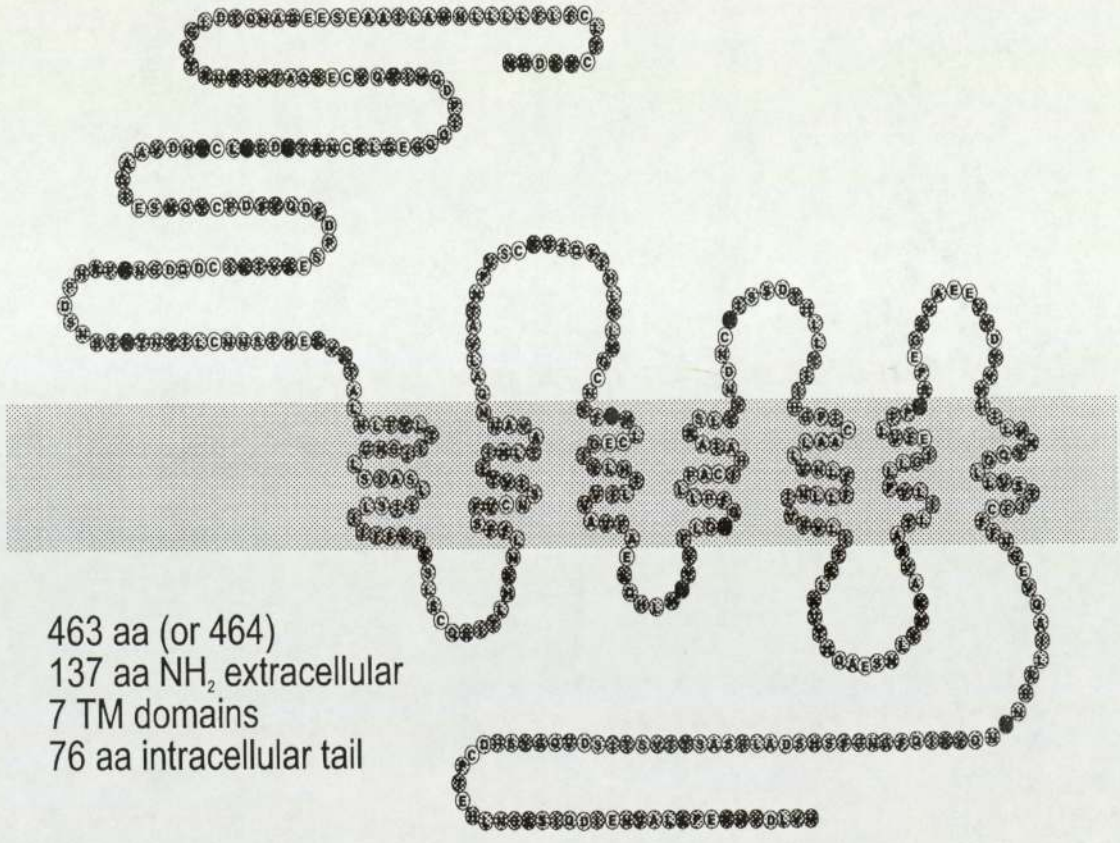
1.5.1 Molecular aspects of receptors

1.5.1.1 History of CGRP receptor cloning

The cloning of a CGRP receptor has taken a long time. The efforts made to discover the CGRP receptor has also led to the discovering of receptors for other members of calcitonin family of peptides. Following the discovery of the calcitonin receptor, various other receptors or complexes were suggested to be able to bind CGRP or related peptides. These include RDC-1, G10d, RCP and CRLR (combined with RAMP1, 2 and 3). CRLR combined with RAMP1 is widely thought of as a CGRP receptor and has most support from different research groups (Han et al 1997; Buhlmann et al. 1999; Sams et al. 1999).

The first receptor of Calcitonin family--CTR was first found by Lin et al. in 1991. They screened a cDNA library from LLC-PK1 cells for binding to radio-iodinated salmon calcitonin (SCT). One clone of 2.2-kilo bases was found to be positive for the ligand. The peptide expressed by this gene was a seven-transmembrane receptor. Analysis of the deduced amino acid sequence of the CTR revealed a molecule with an unusual structure and searches of nucleic acid and protein

Diagram 5. Snake-like plot of Calcitonin Receptor–Like Receptor (Rat α). For details of the amino acids, see diagram 6 page 32. (Copyright from Dr. David Smith, Imperial College). For details of amino acids, see diagram 2, page 16.



HRAMP$_1$

MARALCRLPRRGLWLLLAHHLFMTTACQEANYGALLRELCLTQFQV

|←----------Signal peptide ----------→|

DMEAVGETLWCDWGRTIRSYRELADCTWHMAEKLGCFWPNAEV

DRFFLAVHGRYFRSCPISGRAVRDPPGSILYPFIVVPITVTLLVTALVV

|←------ TM domain --

WQSKRTEGIV

→|

Diagram 6 Sequence of Receptor Activity Modifying Protein. Each character represents one amino acid using single letter code (for details of the single letter amino acid code, see diagram 2). McLatchie L.M. (1998) *Nature*, See references.

sequence databases did not identify sequences similar to CTR. Nevertheless, there was a striking degree of amino acid sequence similarity observed between the CTR and the PTH-PTHrP (parathyroid hormone) receptor and rat secretin receptor (Ishihara et al. 1991). CTR was thought to be a member of a new receptor family containing secretin and PTH/PTHrP. This has subsequently been called the Type II family of G-protein coupled peptide receptors (GPCRs).

Later two receptors for CGRP and ADM (adrenomedullin), RDC-1 and G10d, were identified by Kapas et al. 1995 (a, b), although these have not been confirmed by the further research. G10d was first discovered as an orphan receptor (Harrison et al. 1993; Eva & Spengel 1993) It was screened against many different peptides, but none activated it. Later, Kapas et al. (1995b) independently identified a gene from rat lung encoding a peptide of 395 residues. When expressed in COS-7 cells, it was sensitive to ADM, with an EC50 of 7nM for cAMP accumulation and Kd of 8.3nM for radioligand binding. It was 1.8-kilo bases in length. Its sequence revealed that it was actually G10d. RDC-1 was first discovered by Libert et al. 1989. An approach based on the polymerase chain reaction had been used to clone new members of the family from thyroid cDNA, which was thought to encode the G-protein coupled receptors. RDC-1 shows 30% homology to G10d and showed sensitivity to both CGRP and ADM, but was antagonised by CGRP8-37 when expressed in COS-7 cells. It also showed no response to [Cys(ACM)2,7]hαCGRP, and was identified as a CGRP-1 receptor.

At about the same time, there were other reports of CGRP receptors. One was CGRP-RCP, (CGRP receptor component protein) (Luebke et al. 1996). Using an expression-cloning strategy to screen the guinea pig organ of Corti (the hearing organ), a cDNA was isolated, which caused CGRP responsiveness when expressed in *Xenopus laevis* oocytes. It caused an inward Cl- current (a phenomenon caused by CFTR when activated via cAMP). The protein encoded by that gene was short and not hydrophobic, it did not belong to the class of G-protein coupled receptors that contain seven membrane-spanning helices. It was hypothesised that CGRP-RCP could (1) be CGRP receptor itself or (2) be a part of a complex of proteins that constitute the CGRP receptor. No further relevant results are yet available about this interesting protein.

1.5.1.2 CRLR combined with RAMP may act as CGRP receptor

Another calcitonin-receptor-like structure was identified (Chang et al. 1993; Fluhmann et al. 1995). This was a 461 amino acid protein with seven putative transmembrane domains. The protein showed 56% identity to the human calcitonin receptor and was predominantly expressed in the lung, heart and kidney. This protein was termed CRLR (Calcitonin Receptor-Like Receptor). Rat and porcine versions of CRLR have been cloned (Njuki et al. 1993) (Diagram 5).

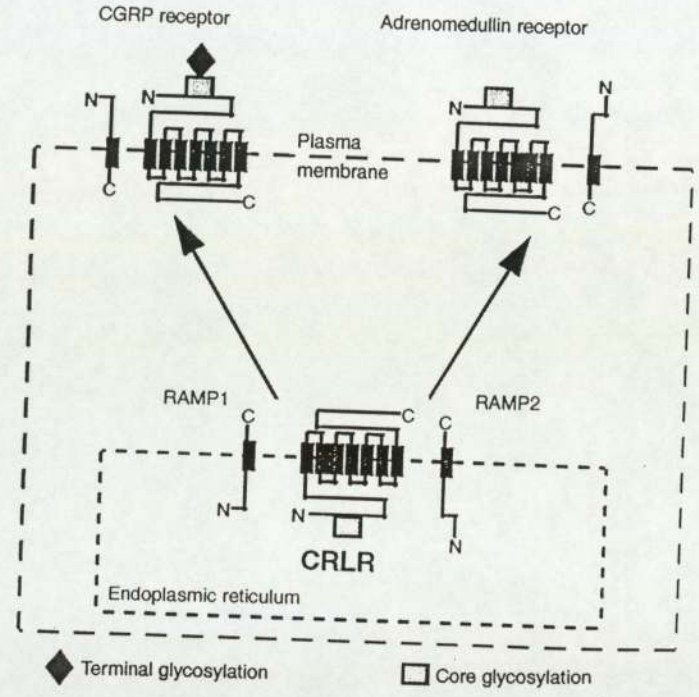
CRLR showed no response when transfected into COS-7 cells and treated with calcitonin, hCGRP (α or β), human amylin, human adrenomedullin, lizard helodermin, salmon stanniocalcin and chicken parathyroid hormone-related protein. Nevertheless, some controversial results were presented by Aiyar et al. (1996). They found the receptor, when stably expressed in HEK293 cells (human embryonic kidney 293), gave specific, high affinity binding sites for CGRP that

displayed pharmacological and functional properties very similar to native human CGRP1 receptor. Exposure of these cells to CGRP resulted in a 60-fold increase in cAMP production, which was inhibited in a competitive manner by the CGRP1 receptor antagonist, CGRP8-37. Northern blot analysis revealed that the mRNA for this receptor was predominantly expressed in the lung and heart. This was confirmed by Han et al. (1997), who used transiently transfected cells. At that stage, it was unclear why other researchers failed to see a response to CGRP when the receptor was expressed.

The problem why CRLR only produced a CGRP receptor in HEK293 cells remained unsolved. When Han (1997) confirmed the results of Aiyar (1996), they supposed that HEK293 cells contained an extra factor necessary for functional CGRP receptor expression. This factor was identified as the result of co-expression of receptor activity modifying proteins (RAMPs) by McLatchie et al. (1998). Three RAMPs, namely RAMP1, 2 and 3, have been found. They concluded that RAMP1 and CRLR form a CGRP receptor, while RAMP 2 or RAMP 3 and CRLR form an ADM receptor (for details, see review by Foord et al. 1999).

The actions of RAMPs on CRLR to produce either CGRP or adrenomedullin receptor have been confirmed by others (Sams et al. 1998; Kamitani et al. 1999; Buhlmann et al. 1999; Muff et al. 1999). Nevertheless, the idea has not acceptable to everyone. Drake et al. (1999) challenged the hypothesis. They presumed that if the receptor showed specify of CGRP and ADM is determined by RAMPs, then the peptide should desensitise each other. They failed to observed cross-desensitisation, a result which needs further

. Diagram 7. The role of RAMPs 1 and 2. RAMPs 1 and 2 and CRLR in generating CGRP or ADM receptors



The role of RAMPs 1 and 2 and CRLR in generating CGRP or ADM receptors.

investigation. However the observation that CRLR and RAMP produces a CGRP receptor remains widely accepted. The reason why CRLR can act as a CGRP receptor on HEK293 cells is thought to be that HEK 293 cells happened to express endogenous RAMP1 (McLatchie et al. 1998). The hydrophobicity plot of the RAMP protein indicates that it has an amino-terminal signal sequence and single putative transmembrane domain close to the carboxyl terminus. However, RAMP is not, by itself, a CGRP receptor.

RAMP may transport CRLR to cell surface and produces terminally glycosylated CRLR (McLachtie et al. 1998). The mechanism of RAMP1 activity is consistent with a transport event. In summary, CRLR functions as a CGRP receptor after it appears at the plasma membrane as a mature glycoprotein. Nevertheless, RAMP1 may contribute more directly to the ligand-binding site of the receptor (Diagram 7).

Buhlmann et al. (1999) have confirmed that RAMP2 expression with CRLR gives an adrenomedullin receptor. They also demonstrated that CRLR preferentially associates with RAMP1 rather than RAMP2. The nature of the amylin receptor has also been investigated (Muff et al. 1999). hCTR2, a human calcitonin receptor, when expressed by itself has a low affinity for amylin, but when co-expressed with RAMP1 or 3 in rabbit aortic endothelial cells or COS-7 cells produced a specific amylin receptor. Co-expression of hCTR2 with RAMP2 gives a receptor with high affinities for both calcitonin and amylin.

Although a putative CGRP1 receptor has been cloned, the CRLR combined with RAMP does not tell the whole story. Neither CRLR, nor RAMP1 mRNAs (McLatchie et al. 1998) are found in the cerebellum, an area with many specific

CGRP binding sites (VanRossum et al. 1997). On the other hand, the problem still remains about the nature of the CGRP2 receptor as well as CGRP receptors in other tissues. A cDNA library made from Col 29 cells may lead to the discovery of a CGRP2 receptor.

1.5.2 Studies on isolated CGRP receptors

Partial biochemical purification has provided insights into the molecular structure of CGRP receptors. Single CGRP binding proteins have been isolated from a variety of tissues including the porcine spinal cord (Sano et al. 1989), cultured rat vascular smooth muscle cells and bovine endothelial cells (Hirata et al. 1988), guinea pig gastric smooth muscle and pancreatic acinar cells, rat cerebellum and lung. An extensive study using a variety of rat tissues suggested an apparent molecular weight of 44kD following enzymatic N-deglycosylation. However, various other studies reported more than one cross-linked molecular weight bands in tissues such as porcine coronary arteries, rat atrium, porcine kidney rat liver and skeletal muscle. Moreover, the human cerebellum apparently contains two CGRP binding proteins (50 and 13.7kD: Dumont et al. 1997) while Stangl et al. (1993) reported three different masses of 60, 54 and 17kD respectively with evidence that the higher molecular weight components were glycosylated. The presence of a low molecular weight component is most interesting in the context of the recent identification of the RAMPs (Foord et al. 1999).

1.5.3 Transduction pathway of CGRP receptors

CGRP receptor transduction appears to involve coupling to a G-protein (Gsα) to stimulate adenylate cyclase and increase cAMP production (Van Rossum et al. 1997; Wimalawansa et al. 1996; Poyner et al. 1992b; Chatterjee et al. 1993). Evidence for this coupling comes from binding studies where agonist affinity has been altered by adding GTPα or its analogues, which promote receptor G-protein uncoupling. However in the studies of Chatterjee (1993), it is suggested that the CGRP receptor-G-protein complex can itself show multiple agonist affinities. Despite this complexity, it is clear that in some conditions at least, the receptors show the characteristic of a G-protein coupled receptor.

The most widely reported action of CGRP is to increase cAMP levels. It can probably also activate other pathways. The stimulation of CGRP receptors has also been shown to activate muscarinic K^+ channels in rat atrial cells (Kim 1991) and enhance Ca^{2+} currents in nodose ganglion neurons (Wiley et al. 1992) via a pertussis toxin-sensitive G-protein. Little is known about the desensitisation of CGRP receptors but recent data suggest the involvement of a G-protein receptor kinase (GRK-6; Aiyar et al. 1999).

1.6 Physiology of CGRP

1.6.1 Distribution and Actions

Compared with calcitonin, CGRP is the predominant product expressed in the central nervous system and is widely distributed in the nervous system and the cardiovascular system (Amara et al. 1982; Rosenfeld et al 1983). Here the distribution of CGRP will be discussed briefly. For details, see review (Poyner 1992a).

CGRP immunoreactivity and binding site have revealed that the peptide is widely distributed in the central and peripheral nervous system (Yamamoto et al 1989). In the central nervous system, CGRP is found in the dorsal horn receiving sensory inputs and the ventral horn of some motor nerves. In brain, the peptide is present in the nuclei of sensory nerves and in cell bodies in the hypothalamus, preoptic area, ventromedial thalamus and medial amygdala. In the peripheral nervous system, CGRP is distributed in both sensory and motor nerves. Thus the peptide is found in the skin, blood vessels, the heart, the gastrointestinal tract, the tongue, the oesophagus and pancreas, salivary glands, the lungs, the kidney and other organs (Yamamoto et al. 1989; Skolitsch et al. 1985; Mulderry et al 1985, 1988; Kummer et al. 1991). CGRP immunoreactive nerve fibres are found in the heart, especially in the coronary arteries, sino-atrial node and right atrium (Saito et al. 1986).

Just like its widespread distribution, CGRP has multiple activities throughout body. In CNS, it can activate neuronal pathways. For example, the peptide increases noradrenergic sympathetic outflow (Fisher et al. 1983). CGRP is one of the most potent (lowest EC50) vasodilators known (Brain et al. 1985). In skeletal muscle, it can rapidly increase phosphorylation of the nicotinic receptor. In rat primary myotube cultures, CGRP caused a 30-60% increase in [P^{32}]-labelling of the nicotinic receptor δ-subunit (EC50=10nM) that was maximal by 5 min. There was a 10-fold greater increase in phosphorylation of the α-subunit, which was maximal by 40 min (Miles et al. 1989). It might function as a neurotrophic agent (Denis-Donini 1989). CGRP also has a number of metabolic effects, which are largely confined to skeletal muscle (Leighton & Cooper 1988). CGRP can inhibit insulin-stimulated glucose uptake in the rat diaphragm

(Hothersall et al. 1990). In blood vessels, CGRP is one of the most potent vasodilators known (Brain et al. 1985), while in heart, CGRP can increase cAMP levels in atrial homogenates and myocytes grown in culture (Sigrist et al. 1986; Ishikawa et al. 1988; Wang & Fiscus 1989; Fisher et al. 1988). Since cAMP is an important second messanger, it is not surprising that CGRP can increase both the force and rate of contraction of heart (Marshall et al 1986).

CGRP is frequently localised with other substances, like acetylcholine, 5-hydroxytryptamine and substance P (Mora et al 1989; Arvidsson et al. 1991; Ekblad et al. 1988). CGRP can activate neuronal pathways and has a number of interactions with substance P at the level of the spinal cord (Poyner 1992a). In the cardiovascular system a variety of additional interactions are seen. It seems that substance P can stimulate mast cells to secret proteases that destroy the CGRP, thereby attenuating its effects (Brain et al. 1988). In contrast to that CGRP desensitises skeletal muscle arteries to substance P *in vitro* (Ohlen et al. 1988).

When CGRP is released peripherally, it causes long-lasting vasodilation (Brain et al. 1985), which is responsible for many of the pro-inflammatory actions of CGRP. The vasodilation greatly potentiates the action of mediators that can increase permeability of blood vessels. Thus it increases the oedema and neutrophil accumulation in rabbit skin seen after interleukin treatment (Buckley et al 1991a b; Hughes et al 1991). The vasodilator actions of CGRP can also contribute to its inflammatory actions. There is a body of evidence to implicate CGRP in pain transmission in the CNS. Although the majority of CGRP's action as an inflammatory mediator is secondary to its actions as a vasodilator, it can

have more direct effects, as there are CGRP receptors on lymphocytes (Umeda et al. 1988).

1.6.2 Therapeutic potential

CGRP is of potential clinical importance in a number of disease states, including cardiovascular disorder, chronic inflammation and arthritis and maturity onset diabetes (Poyner 1992a) (for details see Wimalawansa 1997). However, the true clinical importance of CGRP awaits a deeper understanding of the physiology of the peptide in man.

CGRP can be used to relieve acute and chronic coronary arterial spasms. It can relax human coronary arteries directly, without mediation of endothelium-derived growth factors. Unlike other vasodilators, CGRP markedly increases both renal blood flow and glomerular filtration rate and excretion of sodium and chloride in humans (Wimalawansa 1997). Administration of CGRP8-37 has been shown to minimise ischemic damage to the brain after subarachnoid hemorrhage and this could imply an important therapeutic role for CGRP in reversing cerebral vasospasm. Though there is still controversy, administration of CGRP by intracisternal or selective arterial delivery may have a place in therapeutic management of subarachnoid hemorrhage (Wimalawansa 1997).

Since CGRP affects cell proliferation and differentiation and has additional neurotrophic effects, interest has been focused on possible involvement in nerve regeneration. There is increasing evidence that the down-regulation of CGRP synthesis in regenerating sensory neurones is due to the lack of a peripheral tropic factor, possibly NGF or leukaemia inhibitory factor (Wimalawansa 1997).

In summary, CGRP possesses a wide variety of biological effects in the brain as well as in peripheral tissues including very potent vasodilator actions, pro-inflammatory and nociceptive effects, direct inotropic and chronotropic activities, modulation of locomotor behaviours, etc. Despite its unclear therapeutic potential, an analogue of CGRP could be a useful medical tool. These various effects are mediated by the activation of specific plasma membrane receptors. Thus, more information about CGRP receptors seem to be important in developing novel drugs.

1.7 Cell lines

1.7.1 L6 cell line

The L6 cell line was derived from rat thigh muscle and adapted to culture conditions by Yaffe (1968). The L6 cell line is a clonal skeletal muscle cell line that has been widely used to study the biochemical and electrophysiological mechanisms of skeletal muscle at a cellular level. L6 cell development can be classified into two stages: 1) mononuclear myoblasts (myogenic precursor cells) which divide and increase their number exponentially, and 2) multinucleate myotubes, which are formed by fusion of the myoblasts. The myoblasts spontaneously fuse when the culture dish becomes confluent and the myotubes are electrically excitable (Kidokoro 1973; Kidokoro 1975). The L6 cell line at Aston was obtained from the European Culture Collection. Unfortunately, their properties alter after approximately 25 passages. These changes are often unpredictable but can reduce or abolish CGRP receptor expression.

The L6 cell line expresses a wide variety of receptors. Thus it is a useful working model for research. The receptors including a CGRP1 receptor that couples to adenylate cyclase (Poyner 1992a) and also receptors for insulin (Klip et al. 1984). The effect of CGRP on some clones of L6 cells is to confer insulin resistance (Kreutter et al. 1989) but it has recently been reported that this effect may not be mediated by the adenylate cyclase-coupled receptor mentioned above (Kreutter et al. 1993). The L6 cells at Aston do not respond to CGRP by producing insulin resistance (K. James 1993; unpublished observations) suggesting that there may be significant differences between the European and American lines. L6 cells express functional V1-vasopressin receptors coupled to stimulation of inositol phospholipid metabolism (Wakelam et al. 1987). β-adrenoceptors are also present on L6 cells and like CGRP they couple to adenylate cyclase (Pittman et al. 1984).

1.7.2 SK-N-MC cell line

The human SK-N-MC cell line was originally prepared from a human tumour diagnosed as a neuroblastoma (Biedler et al. 1973). The cells were composed of small fibroblast-like cells with little cytoplasm. These cells did not resemble normal human fibroblasts that are larger, more flattened and stretched out. In monolayer culture, they form disoriented growth patterns. Population-doubling time for SK-N-MC cells was 32hr.

The SK-N-MC cell line can express many receptors (Zhen et al. 1998) so that it is a useful working model. CGRP receptors are found on these cells (Van Valen et al. 1990). These receptors bind CGRP8-37 with a pKd value in excess of 7,

consistent with them belonging to CGRP1 receptor subtype (Semark et al. 1992; Muff et al. 1992; Longmore et al. 1994; Zimmerman et al. 1995). Poyner et al. (1998) reported that SK-N-MC cells showed a similar sensitivity to CGRP analogues as did L6 cells. [Ala11,18]-CGRP8-37, 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 hα-CGRP8-37.

1.7.3 Col 29 cell line

The Col 29 (Colony 29) cell line was first derived from human colonel epithelium and expresses CGRP receptors linked to adenylate cyclase (Kirkland, 1985; Poyner et al., 1993). Like many epithelial cells *in vivo*, colonic enterocytes are polarized with functionally distinct apical and basolateral membrance. The two membranes show different biochemical activities. For example, a single class of high-affinity epidermal growth factor receptor were detected in the basolateral compartment, whereas the apical compartment of polarized cells, and cells cultured on the plastic, displayed two classes of receptor affinity (Damstrup et al. 1999).

CGRP receptors are also preferentially located on the basolateral surface (Cox et al. 1994). However, these receptors were found to be distinct compared to CGRP receptors found on the other tissues both in their sensitivity to CGRP8-37 and agonist order of potency. The receptors have also been investigated, using cAMP accumulation instead of the electrogenic ion transport analysis methods carried out by Cox (Poyner et al. 1998). The cells showed different responses to CGRP antagonists and agonists compared to the SK-N-MC cells. hα-CGRP19-37, AC187, rat amylin 8-37 and hα-[Tyr°]-CGRP28-37 cannot antagonize the

action of CGRP on Col 29 cell line at concentrations of up to 3 μM. Of the antagonists tested, only CGRP8-37 could inhibit responses to CGRP and it had a pA2 of about 6.5, much lower than on the SK-N-MC cells (Poyner et al. 1998).

1.8 Aims of the work

1.8.1 Structure-activity studies on CGRP8-37

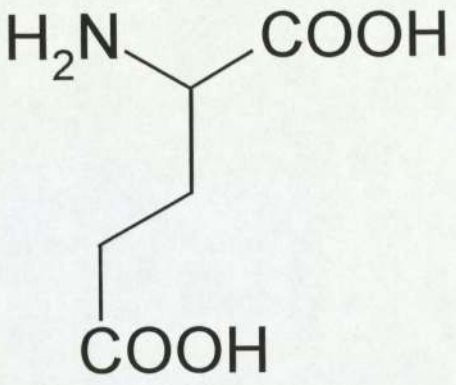
The main aim of this work was to investigate the structure-activity relationship of CGRP and its receptors. As discussed before, the structure of CGRP can be divided into several parts, including the N-terminus, the α-helix, and the C-terminus. The N-terminus is thought to be responsible for biological activity. Usually, the analogues without the N-terminus act as antagonists, e.g. CGRP8-37. The present study focussed mainly on this antagonist, particularly the role of its α-helix.

In 1988, Lynch and Kaiser carried out research on the amphipathic α-helix structure (See Diagram 3, 4). The reason why they chose this part of peptide is that an amphipathic α-helix was important in several peptides for its function (Moe et al. 1983; Moe & Kaiser 1985; Green et al. 1987). Thus two CGRP analogues CGRM-1, CGRM-2 were synthesized. The first model, CGRM-1, which had been substituted with residues generating an idealized amphipathic α -helix in the region between residues 8 and 25, was found to have no agonist activity. The second model, CGRM-2, had an idealized amphipathic α-helix between residues 8 and 18 equivalent to approximately three turns of a α-helix. The peptide was an agonist with a potency one-fourth that of the native hormone on rat vas deferens assay. CGRM-2 showed a much lower potency in an

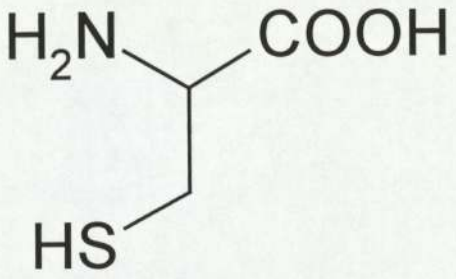
adenylate cyclase assay. Thus they concluded that there was an amphipathic α-helix in rat αCGRP between residues 8 and 18. This concept has now been expanded to all the forms of CGRP. A β-turn localised somewhere around residues 17 and 21 was hypothesised to terminate the α-helix (Lynch & Kaiser 1988; Breeze et al. 1991; Hubbard et al. 1991). Two conflicting structures have been proposed: a β-turn around position 18-21 (Wisskirchen et al. 1999) or a γ-turn around residues 19-21 (Boulanger et al. 1995). Although it is hard to say which one is near the truth, both of them suggest that α-helix is terminated around position 18.

After the α-helix had been established as an important part of the peptide, more detailed work was carried out. Wisskirchen et al. (1999) showed that substitution of proline (which disrupts α-helices) at position 16 abolished antagonism presumably by disrupting the biologically important α-helix, whilst the substitution was accepted in the putative bend region at position 19. Thus an 8-18 amphipathic α-helix structure was confirmed. At the same time, Boulanger et al. (1996) used two-dimensional NMR and CD, followed by molecular modelling to investigate the structure of CGRP8-37. They reported that alanine substitutions at positions 17 and 20 of CGRP8-37 produced good antagonists to CGRP1 receptor, but not when position 21 was substituted. Comparing those three structures of peptide by NMR, they concluded that residue 21 (serine) stabilised an important conformation of the C-terminus of CGRP.

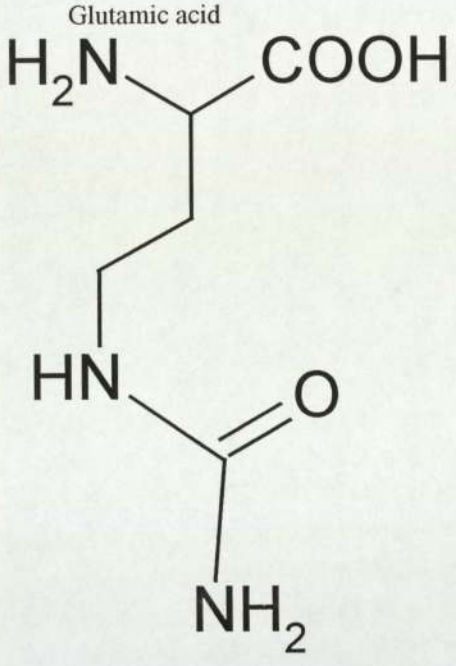
Amylin and calcitonin derivatives have also been investigated of their cross-reactions on the CGRP receptors. The results showed that AC187 (a salmon calcitonin derivative), CGRP8-37, and amylin8-37, all showed antagonist activity on CGRP1 receptor.



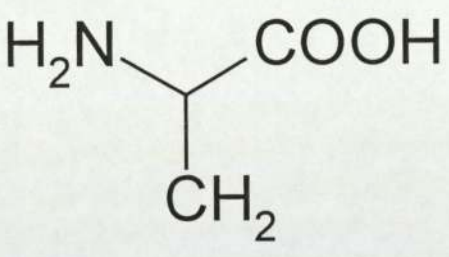
Glutamic acid



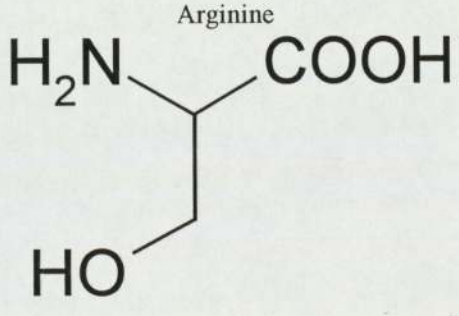
Cysteine



Arginine



Alanine



Serine

Diagram 7 Structure of some amino acids

These all contain an amphipathic α-helix, which has two conserved basic residues corresponding to Arg11 and Arg18 in CGRP. It has been concluded that this structure was critical for high affinity interactions with the CGRP receptors (Mimeault et al. 1992). Those two arginine residues have been substituted by alanine, either individually or collectively (Howitt & Poyner 1997). The data suggested that arginine 11 was more important in the interaction of the receptor with the ligand than Arg18. However, neither substitution produced a particularly large change in affinity (less than 10-fold; Howitt & Poyner 1997). By contrast, replacement of both arginines reduced affinity by over a 100-fold. The authors concluded that role of two arginines was to maintain the amphipathic structure of the α-helix. The question still remains whether this structure interacts directly with the receptor, whether it interacts with other regions of the peptide that are actually involved in receptor contacts or whether the hydrophilic face of the helix interacts with the solvent (Wisskirchem et al. 1999). This study was specifically designed to investigate the role of the positive charge at positions 11 and 18. The arginines have been replaced with negatively charged glutamic acids or hydrophilic but uncharged serines. The results show that arginine18 and arginine11 have different roles. It is possible arg 18 may interact directly with a negative charge on the receptor, where arg11 may simply interact with the solvent.

1.8.2 Effect of protein modifying reagents on the CGRP receptor

The structure-activity relationship of the CGRP receptor has been examined in the present work. CRLR and RAMPs are widely accepted forming a CGRP receptor (see above). It is thought that RAMP1 presents CRLR as a terminally glycosylated, mature glycoprotein (Fraser et al. 1999). It has also been noticed

that deglycosylation can reduce the size of CRLR in SDS-PAGE assay. Detailed study of CRLR revealed that there might be glycosylation points at residues 66, 118 and 123 of human CRLR and at positions 66, 118, 123, 128 and 129 of rat CRLR. In a study carried out by Hao et al. (1999), glycosylation was found to be important for the calcitonin (another member of calcitonin receptor super family) receptor in binding the ligand. In that experiment, the glycosylation site at Asn 1 was found to be not so important as those sites at Asns 2, 3 and 4. It is important to discover the role of glycosylation in the function of CRLR Thus tunicamycin, which is an inhibitor of N-glycosylation, was used to pre-treat cells. The results show that tunicamycin does affect the cells' responses to CGRP, consistent with the hypothesis that glycosylation is important in CGRP receptor function.

The sequence of CRLR (Dia 7) shows that it contains many cysteines potentially with free -SH groups. The family II of GPCRs contain 5 well conserved cysteines, some of which are probably involved in disulphide bridge formation. Previous work has shown that dithiothreitol, which can reduce disulphides to free -SH groups, decreases the binding of [125I]-iodohistidyl-CGRP (H. Snook, unpublished final year project). Thus NEM (N-ethyl-maleimide), which can irreversibly alkylate free sulfhydryls (Zoeller et al. 1997), was used in this experiment. The results show that NEM treatment does increase radioligand binding. However, opposed to this effect, NEM seems to decrease the cAMP accumulation mediated by CGRP.

10 20 30 40 50

MEKKCTLYFLVLLPFFMILVTAELEESPEDSIQLGVTRNKIMTAQYECYQ

60 70 80 90

KIMQDPIQQAEGVYC*NRTWDGWL***C**WNDVAAGTESMQL***C**PD

100 110 120 130

YFQDFDPSEKVTKI***C**DQDGNWFRHPASN*RTWTN*YTQ***C**N*VN*

140 150 160 170 180

THEKVKTALNLFYLTHGHGLSIASLLISLGIFFYFKSLSCQRITLHKNLF

190 200 210 220 230

FSFVCNSVVTHHLTAVANNQALVATNPVSCKVSQFIHLYLMGCNYFWMLC

240 250 260 270 280

EGIYLHTLIVVAVFAEKQHLMWYYFLGWGFPLIPACIHAIARSLYYNDNC

290 300 310 320 330

WISSDTHLLYIIHGPICAALLVNLFFLLNIVRVLITKLKVTHQAESNLYM

340 350 360 370 380

KAVRATLIVPLLGIEFVLIPWRPEGKIAEEVYDYIMHILMHFQGLLVSTI

390 400 410 420 430

FCFFNGEVQAILRRNWNQYKIQFGNSFSNSEALRSASYTVSTISDGPGYS

440 450 460

HDCPSEHLNGKSIHDIENVLLKPENLYNN

Diagram 9. Sequence of Rat Calcitonin Receptor-Like Receptor. * Glycosylation site; ***C*** conserved cysteines; C other cysteines. Copyright from GPCRDB.

Chapter 2. Experimental procedures

2.1 Cell culture

L6 cells were grown up in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% foetal calf serum (FCS) and glutamine. SK-N-MC cells were incubated in DMEM/F12 medium supplemented with 10% foetal calf serum and Col 29 cells in DMEM with 10% foetal calf serum. All the media were supplemented with 1% antibiotic-antimycotic solution (Streptomycin/Penicillin/Amphotercin B) and for L6 cells only, by addition of 1mM of glutamine that was filter-sterilised via a 0.22μm-filter before addition. Cell lines were grown in 75cm cell culture flasks in a humid atmosphere containing 5% CO_2 at 37°C and passaged with 0.1% trypsin when confluent. Cells were sub-cultured at 4-7 days intervals when confluence was reached and seeded into culture plates (24 wells) applicable to the assay to be performed. In the experiments, cells can be used until 35 passagings. After that period, cells usually lost the ability to express CGRP receptors. Alternatively, cells were seeded onto 9cm Petri Dishes and grown up to produce membranes for radioligand binding.

2.2 Biochemical assays

2.2.1 Adenosine 3';5'-cyclic monophosphate (cAMP) accumulation

The protocol described is based on the procedure of Gilman (1970). The assay relies on competition between exogenous radiolabelled cAMP and endogenous

cAMP produced by the cells for the binding sites of a cAMP-dependent protein kinase.

Cells were grown to confluence as monolayers in 24 well plates (16mm well diameter) and pre-treated with antagonists for 5 mins. For the SK-N-MC and Col29 cells, the incubating medium was replaced by assay buffer (Krebs with 1mM IBMX, 0.1% BSA) 20 to 30 minutes before being treated with antagonists. IBMX (Isobutylmethylxanthine) is a phosphodesterase inhibitor, which was necessary to prevent cAMP from being hydrolysed. However, it was not necessary for L6 cells, since they did not express phosphodiesterase (enzyme that causes cAMP hydrolysed). Then, the cells were pre-treated with antagonists for 5 mins where appropiate and stimulated by agonists (usually CGRP) for a further 5 mins. Incubations of all cells were terminated by putting the cells on ice, and replacing the medium in each well with 0.5ml of ice-cold buffer (20mM HEPES, 5mM EDTA pH7.7). Then the plates were put in a boiling water bath for 5 min. 50μl aliquots were taken into Eppendorf tubes for assay of cyclic AMP. The sample was mixed with 50μl 20nM [^{3}H]-cyclic AMP (in buffer:20 mM HEPES, 5 mM EDTA) and incubated for 2-24 hours. Free [^{3}H]-cAMP was removed by addition of 100 μl of 5% (w/v) charcoal suspension made up in 20 mM HEPES, 5 mM EDTA, 0.1 % BSA, pH 7.7. Only protein-bound [3H]-cAMP was remained in the tubes, following to pellet the charcoal. 185μl of the supernant was removed into 5ml Scintilation vial inserts. 4ml of 'Hisafe 3' Scintillation cocktail (EG&G) was added to each vial and the vials were then counted for 5 mins in a Beckman TRI-CARB 1600 TR Liquid Scintillation Analyzer using a ^{3}H detection programme to record CPM, which is the index of radioactivity.

The production of cAMP was measured by the standard curve. cAMP solutions of 10^{-10}M to 10^{-6}M were made up and 50µl these were assayed as described above. A standard curve was constructed, relating moles cAMP to CPM (See figure 1).

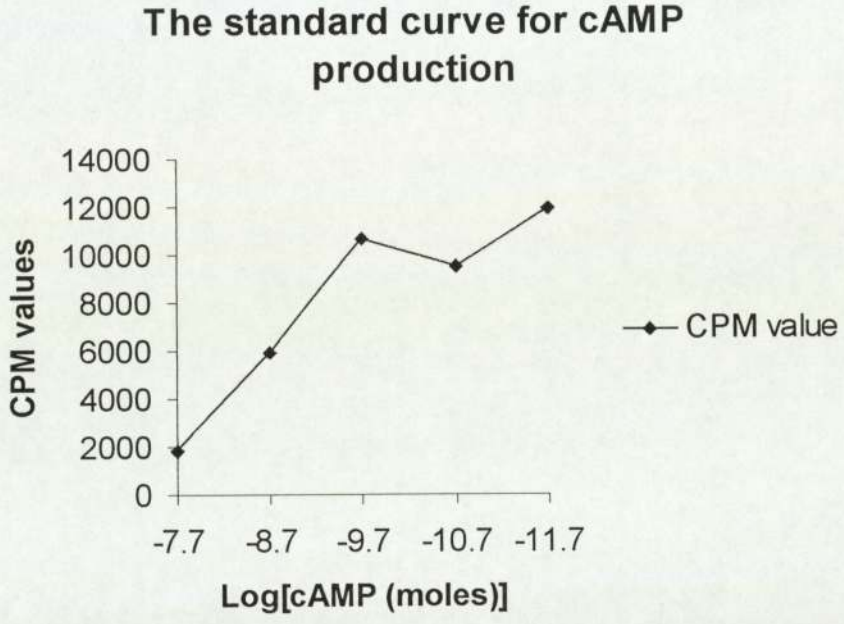


Figure 1. cAMP assay: standard curve

2.2.2 [I^{125}]-Iodohistidyl-CGRP binding

Cells were seeded into 9cm Peris Dishes and grown up to confluence. Membranes were prepared by adding 15 ml of ice cold buffer, containing 20mM HEPES, 0.1% bovine serum albumin, 5mM EDTA, to each 9cm Peris dish and cells were harvested with a rubber policeman. The cells were homogenized with 6 up and down strokes of a pestle in a glass homogeniser and centrifuged at 35,000rpm for 30 mins. The pellet was resuspended again in assay buffer

(HEPES 20mM, EDTA 5mM) at a concentration of 4 mg/ml and aliquoted into 0.5 ml portions. These were stored at -70°C.

For subsequent binding assays, the membranes were thawed quickly prior to experiments and resuspended at a dilution of 1 in 40 in assay buffer containing 5mM EDTA, 20mM HEPES (pH7.5), 0.3% BSA (bovine serum albumin) 1mM $MgCl_2$ or other buffers as indicated in the text. Microcentrifuge tubes were set up with 0.5ml of membranes, 0.1nM [I^{125}]-iodohistidyl human-α-CGRP and competing ligand and incubated for 30 mins normally at room temperature. In time-course studies, points at 0, 5, 15, 30, 60, 120 min were chosen to detect the bound ligand. Then, the tubes were taken out to centrifuge at 4°C at 14,000 rpm for 5 min. The tubes were rinsed by water twice, dried and bound radioactivity measured by a γ counting machine ("Wallac" 1282 compugamma; Universal gamma counter).

2.2.3 Protein quantification

The quantity of protein for radioligand binding was measured by the BioRad method. It includes two steps: A. the construction of standard curve. B. the assay of protein quantity.

Construction of standard curve:

BSA (bovine serum albumin) solutions of 0, 0.01, 0.03, 0.1, 0.3, 1 mg/ml were made up in distilled water. 0.5 ml of each of these solutions was mixed with 0.5 ml of 0.1 M NaOH. This was left for 30 mins. 0.4 ml of the above solution was mixed with 2.8 ml distilled water and 0.8 ml of Biorad Reagent and left for 5

mins. Finally UV-VIS absorbance was read at 595 nm. The blank sample was made up of 0.2 ml Biorad Reagent and 0.8 ml of water. A typical standard curve is shown in Figure 2.

Assay of protein:

0.5 ml of membrane homogenate (made up as for radioligand binding as above) was mixed with 0.5 ml NaOH and left for 30 mins. 0.4 ml of the above solution was processed as for the protein standards. The average membrane protein concentration was 0.094±0.066 mg/ml. (SD)

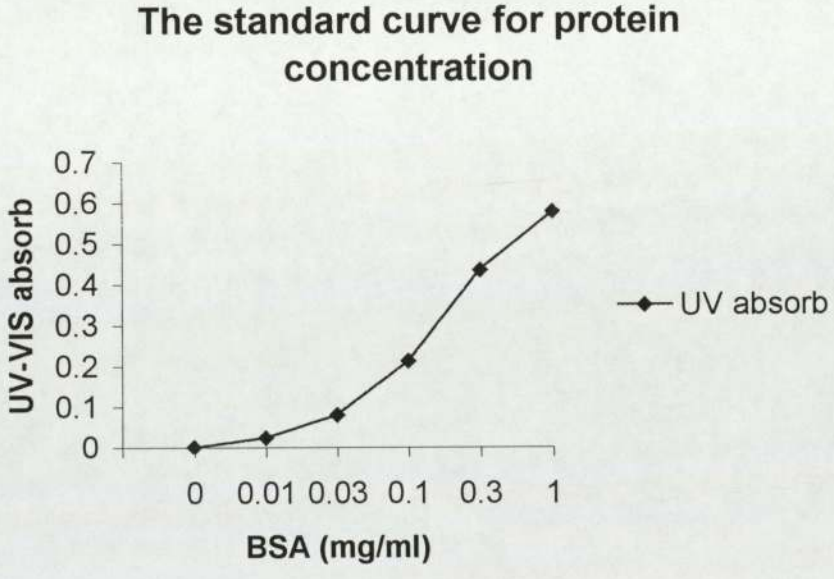


Figure 2. Protein assay: standard curve

2.2.4 Peptide synthesis

The peptides were synthesized in a stepwise manner in a 0.1 mMol scale on an Applied Biosystem Model 431A peptide synthesizer on solid support using FMOC chemistry. Deprotection of the side chains and cleavage of the peptide from the resin were performed using trifluoroacetic acid with thioanisole and ethandithiol as free radical scavengers. Analysis and purification were by HPLC. All these steps were performed by Dr. D. Poyner.

2.3 Tunicamycin treatment

Cells were treated with 10 μg/ml of tunicamycin for 24 hrs (SK-N-MC, L6 cells), or 48 hrs (Col 29 cells). This was added to the DMEM/FCS in which the cells were growing. Control cells were treated with an equivalent quantity of DMSO. They were then harvested for radioligand binding assays or used in cAMP accumulation assays as described above.

2.4 N-ethylmaliemide (NEM) treatment

Cells were pre-incubated with 100 μM N-ethylmaliemide for 30 mins. This was added to the DMEM/FCS in which the cells were growing. The medium was changed by the assay buffer to remove the NEM and used for either radioligand binding or cAMP accumulation assays.

2.5 Statistical analysis

Statistical analysis was performed using a Student's unpaired t-test. Results were deemed to be significantly different if p was less than or equal to 0.05. For radioligand binding experiments, initially it was attempted to fit the data by a non-linear regression assay using the EBDA-LIGAND package for calculating the nH (Hill coeffcient) and the IC50 values. The binding curves were extremely shallow and proved very difficult to fit. A particular problem was in constraining the non-specific binding to the values estimated experimentally. It proved very difficult to achieve this objectively. Eventually, it was decided that the most practical way of estimating IC50 values was by eye.

Concentration-response curves for cAMP were normalized by expressing stimulation as a percentage of that obtained in presence of 10^{-6} M CGRP (or the highest concentration used in each experiment). Thus for each curve stimulation of cAMP production is expressed as a percentage of the maximum. Individual curves were analysed to obtain pEC50 values in the presence and absence of antagonists. These values were used to calculate apparent pKb values from the equation $pKb = \log_{10}\{(\text{dose ratio} - 1)/[\text{antagonist}]\}$. The individual pKb values were finally averaged. Although multiple antagonist concentrations were not used (so Schild plots could not be constructed), the parent antagonist CGRP8-37 behaves as a simple competitive antagonist. Accordingly it was assumed that other antagonists would also show competitive behaviour.

The values of parameters quoted in this study represent the mean ± standard deviation.

2.6 Materials

The L6 cell line was obtained from the European culture Collection. The Col 29 cell line was provided by Dr. S. Kirkland of London University and the SK-N-MC was from Prof. S. Nahorski of Leicester University.

[11Glu] and [18Glu] CGRP8-37 were made in house at Aston by a peptide synthesiser. [11,18Ser]CGRP8-37 was a gift from Dr. J. Longmore (Merck Sharpe & Dhome). Human α CGRP was purchased from Calbiochem and CGRP8-37 was from Bachem. [^{3}H]-adenosine 3';5'-cyclic phosphate (29Ci/mmol) and [I^{125}]-iodohistidyl-α-CGRP (2000Ci/mmol) were bought from Amersham Pharmacia Biotech Co.

All the mediums were from Life Technologies. Other bio-chemicals were obtained from Sigma (St. Louis, USA). The Calcitonin Gene-Related Peptide (CGRP) form used in the present work was human α-CGRP, except where otherwise stated.

Table 1. The components of Krebs physiological saline solution

Name	Weight(g)/liter
NaCl	6.92
KCl	0.354
$MgSO_4 \bullet 7H_2O$	0.29
KH_2PO_4	0.162
$NaHCO_3$	2.1

D-Glucose	2.1
$CaCl_2$	2.5 mls/L of a 1M solution

NaCl: FSA Laboratory Supplies Bat. 12727279

KCl: BDH Laboratory Supplies Lot, 312TA 387838

$MgCl_2$: BDH Laboratory Supplies Lot 322 A621133

KH_2PO_4: BDH Chemicals Ltd. Production No. 10203

$MgSO_4 \bullet 7H_2O$: BDH Chemicals Ltd. Product No. 10151

$NaHCO_3$: HOPKIN &WILLIAMS 788230

D-Glucose: BDH Ltd. Poule England 10117

$CaCl_2$: BDH Laboratoy Supplies Prod. 190464 K

Bovine Serum Albumin: SIGMA Lot. 67H1101

Tunicamycin : SIGMA T-7765

Bacitracin: SIGMA B-0125

3-Isobutyl-1-methyxanthine (IBMX): SIGMA I-7018

N-ethyl-maleimide (NEM): SIGMA E-3876

Ethylenediaminetetra-acetic acid (EDTA): BDH Laboratory Supplies 1906630

HEPES : SIGMA 15H5715

Charcoal activated: SIGMA C-5260

Chapter 3. Results

3.1 L6 cell line

The responses of L6 cells to CGRP showed considerable variability. Sometimes, the EC50 was about 1nM, while it was close to 100nM at other times. There was a tendency for the sensitivity of the cells to CGRP to be optimum from 4 to 8 days after passaging, but the exact time was unpredictable. Different batches of CGRP were tried on the L6 cells. It was found that CGRP from Calbiochem was more potent than that from Sigma, while material synthesised in house was totally inactive. Clearly, care must be taken when using different batches of CGRP.

Initial experiments used CGRP8-37 made in house. Although previously active, the home-made CGRP8-37 did not antagonize the actions of CGRP at all. When changed to CGRP8-37 from Sigma, there was an improvement. As reported previously (Poyner et al. 1992b), CGRP worked as an agonist on L6 cells. It stimulated cells to produce cAMP with a pEC50 of 8.05±0.16 (n=10) (See figure 3, 4, 5). CGRP8-37 had antagonist activity and caused the concentration-response curve to shift rightward with an apparent pKd values of 6.68±0.10 (n=3) (See figure 3). In the present work, the rightward shift of the curve was most apparent at CGRP concentrations of 3×10^{-8}M to 10^{-7}M and it is arguable as to whether it truly was a parallel shift. However, as discussed above, these data are mainly from the using of the CGRP8-37 from Sigma, which was of dubious activity. This may be the reason for unexpected shape of the curve. Due to work

done previously, it is likely that CGRP8-37 is a good antagonist and should produce a parallel rightwards shift in the concentration-response curve (Poyner et al. 1992b). When CGRP8-37 from Bachem was used, an apparent pKd of 8.72±0.63 (n=3) was obtained in line with previous observations (Poyner et al. 1992b) (figure 6).

CGRP derivatives were prepared where negatively charged glutamic acid residues were used to replace the positively charged arginines at α-helix part of CGRP8-37. 1μM [11Glu]CGRP8-37 showed antagonist activity with an apparent pKd of 6.65±0.22 (n=3) (See figure4), while with 10μM [Glu18]CGRP8-37, no antagonist activity was seen: the apparent pKd value was too low to be calculated and must be less than 5.25 (See figure 5).

Table 2. pA2 values of CGRP fragments on L6 cells

	CGRP8-37 (Sigma)	CGRP8-37 (Bachem)	[11Glu]CGRP8-37	[18Glu]CGRP8-37
pA2	6.68±0.10	8.72±0.63	6.65±0.22	≤5.25

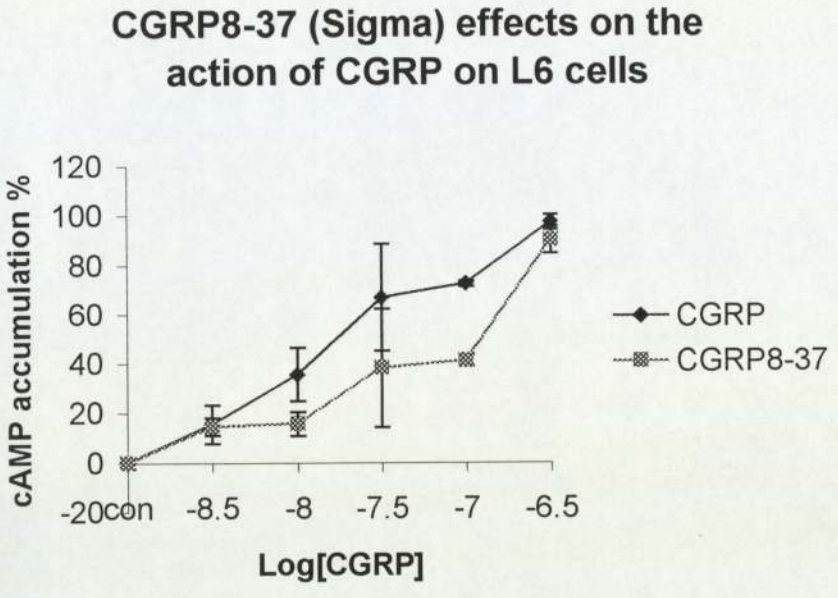


Figure 3. 1μM CGRP8-37 (Sigma) antagonising the action of CGRP on L6 cells. Each point represents the mean of 5-7 values±SD

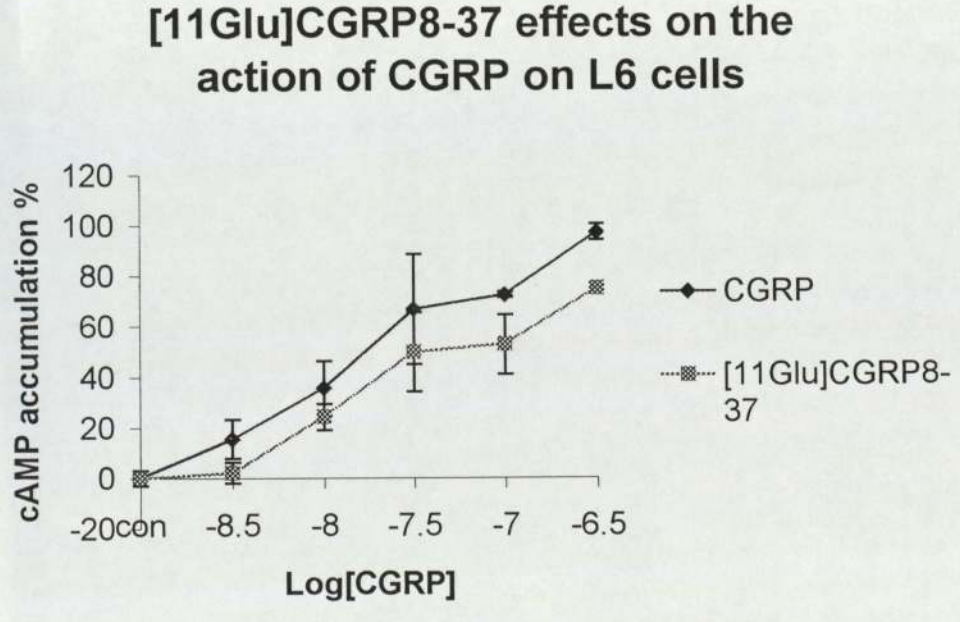


Figure 4. 10μM [11Glu]CGRP8-37 affecting the action of CGRP on L6 cells. Each point represents the mean of three values±SD.

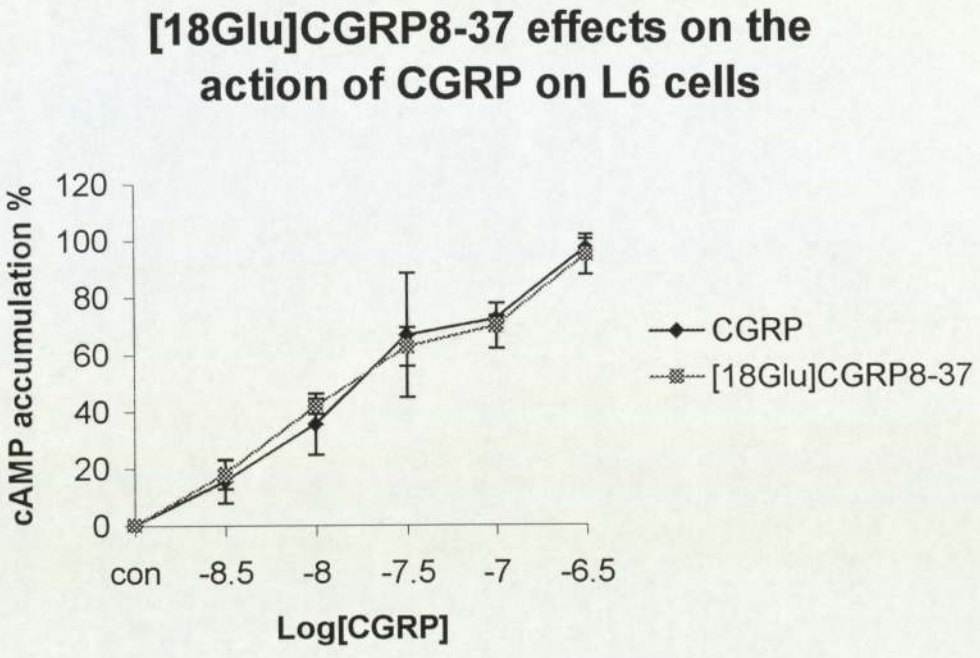


Figure 5. 10μM [18Glu]CGRP8-37 affecting the action of CGRP on L6 cells. Each point represents the mean of three values±SD

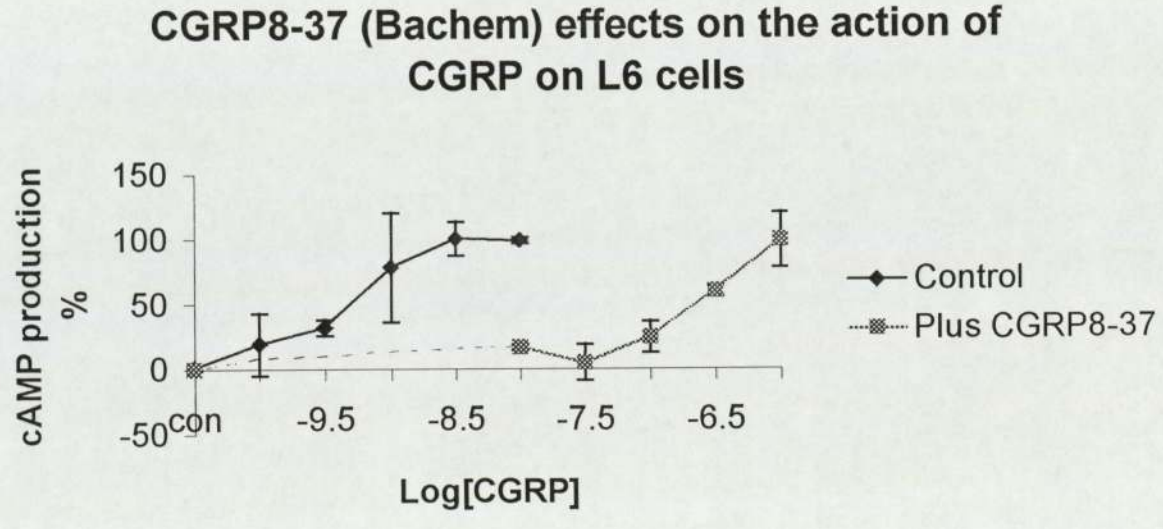


Figure 6. The antagonist action of CGRP8-37 1μM (Bachem) on L6 cells. Each point represents the mean of two valuess±S.D.

[11, 18 Ser] CGRP8-37 is a CGRP analogue, where the arginines at positions 11 & 18 have been replaced by serines, which have -OH groups. In the cAMP accumulation experiments, the new compound failed to inhibit the actions of CGRP at 1μM (See figure7). In individual experiments, at 10μM [11,18Ser]CGRP8-37 did cause a reproducible shift in the dose-response curve to CGRP (See figure 8), although the magnitude of this was variable. An average apparent pKd value of 6.32±0.60 (n=3) was calculated.

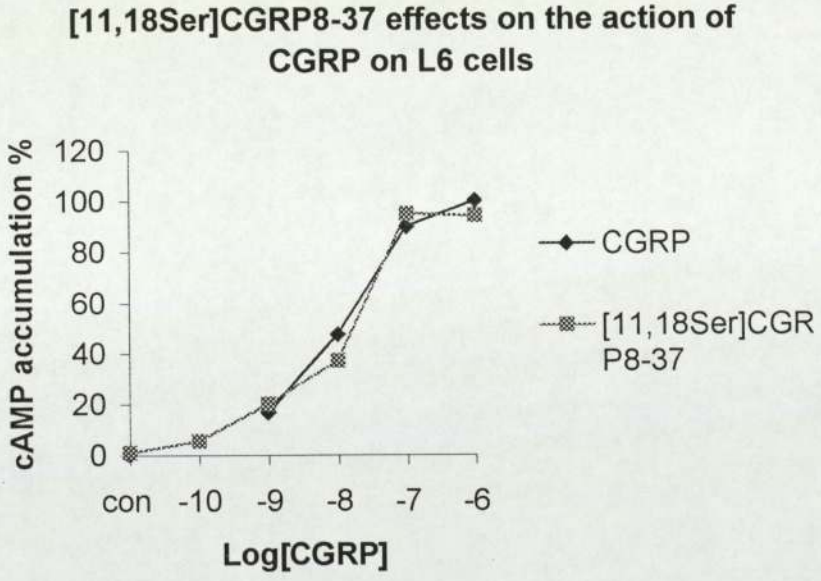


Figure 7 . [11,18Ser]CGRP8-37 (1μM) affecting the action of CGRP on L6 cells. Each point represents one value.

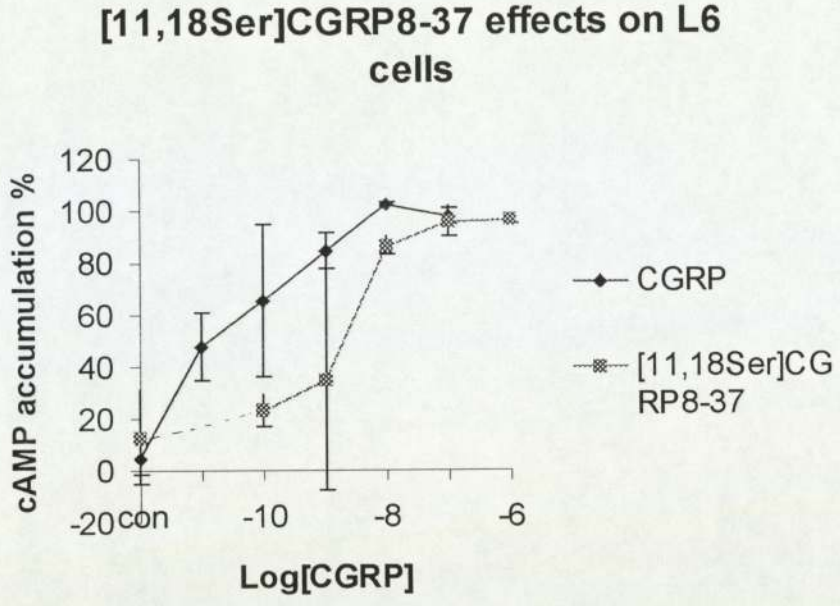


Figure 8. [11,18Ser]CGRP8-37 (10μM) antagonizing the action of CGRP on L6 cells. Each point represents the mean of three values ± SD.

3.2 SK-N-MC cell line

3.2.1 cAMP accumulation

The SK-N-MC cells express CGRP1 receptors and proved to have more robust expression of this receptor than L6 cells. The apparent pKd value for CGRP8-37 was about 7, consistent with the presence of a CGRP 1 receptor, even using CGRP8-37 from Sigma. Nonetheless, SK-N-MC cell line does change its properties for unknown reasons. During the one-year period of these experiments, the cell line has twice lost the ability to express CGRP receptors. It would be interesting to investigate the factors which lead to this effect.

The SK-N-MC cell line was shown to express CGRP1-like receptors in former studies (e.g. Longmore et al. 1994). In the present studies, it was confirmed that CGRP activated cAMP production with a pEC50 of 7.88±0.15 (n=8) (See figure 9, 10, 11). The action of the peptide can also be antagonised by CGRP8-37 with an apparent pKd value 6.99±0.51 (n=4) (Sigma, figure 9) or 7.54±0.68 (Bachem) (n=3) (See figure 10). The apparent pKd value from Bachem is more in line with previous work than that from Sigma. Just as with the L6 cell line, [Glu11] CGRP 8-37 showed antagonist activity with an apparent pKd value of 6.65±0.26 (n=4) (figure 11). [Glu18] CGRP8-37 lost antagonist activity on these cells even when the concentration reached as high as 10μM (figure 12). It must have a pKd in the region of 5 or less. The apparent pKd value for [11,18Ser]CGRP8-37 is 6.96±1.36 (n=3) (figure 13).

Table 3. pA2 values of CGRP fragments on SK-N-MC cells

	CGRP8-37 (Sigma)	CGRP8-37 (Bachem)	[11Glu]CGRP8-37	[18Glu]CGRP8-37
pA2	6.99±0.31	7.54±0.68	6.65±0.26	≤5.26

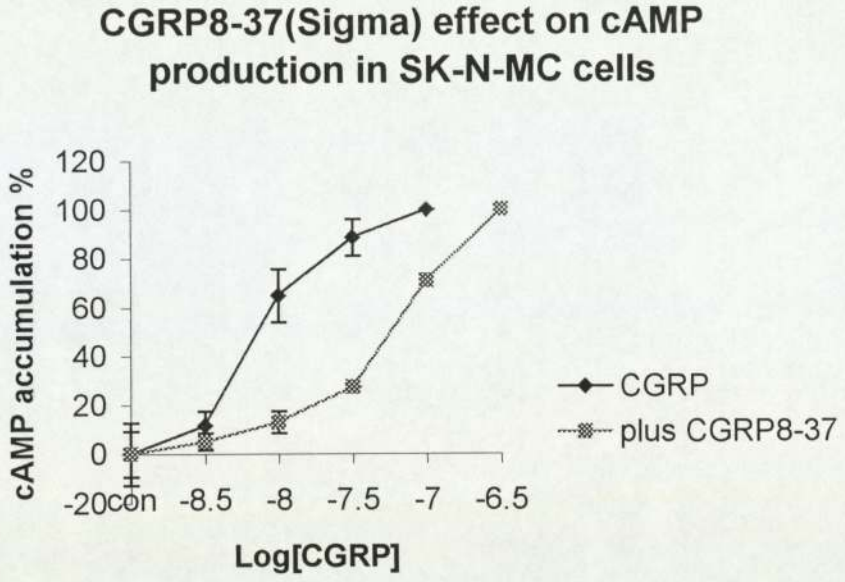


Figure 9. CGRP8-37 (1μM) (Sigma) affecting the action of CGRP on SK-N-MC cells. Each point represents the mean of three values ± SD.

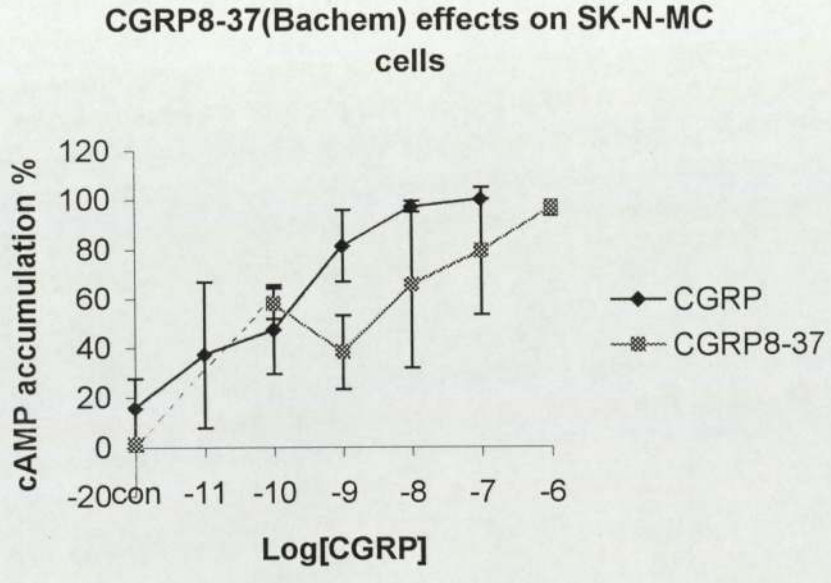


Figure 10. 1μM CGRP8-37 (Bachem) affecting on the cAMP production of CGRP on SK-N-MC cells. Each point represents the mean of three values±SD.

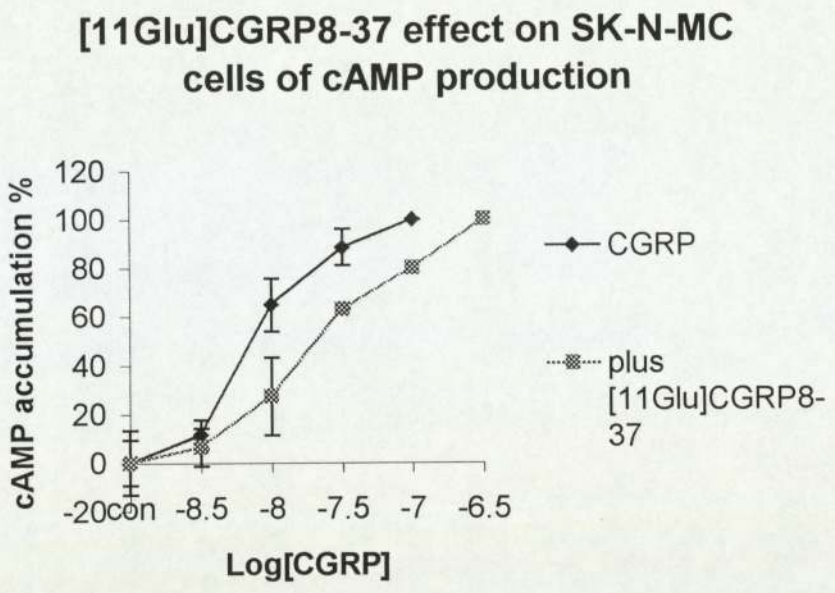


Figure 11. [11Glu]CGRP8-37 (1μM) affecting the action of CGRP on SK-N-MC cells. Each point represents the mean of three values ± SD.

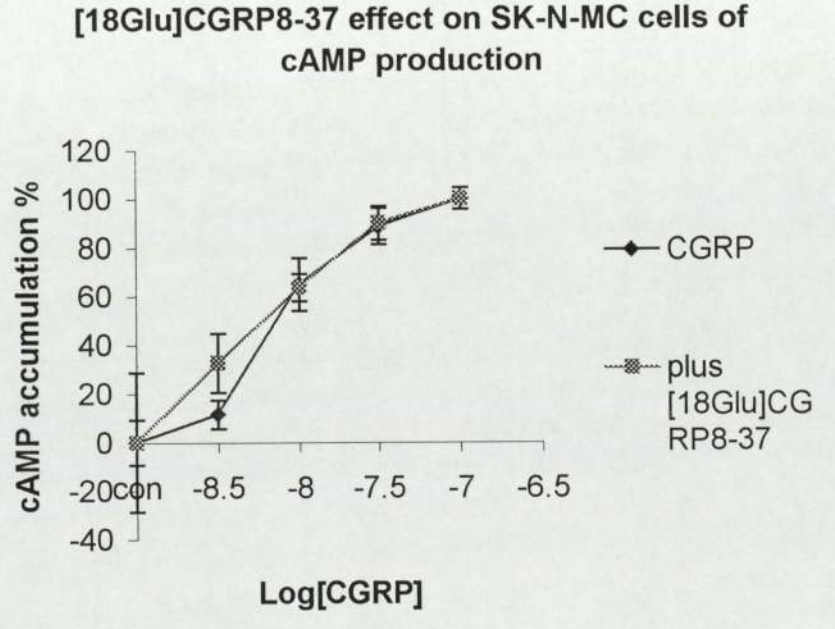


Figure 12. [18Glu]CGRP8-37 (10μM) affecting the action of CGRP on SK-N-MC cells. Each point represents the mean of three values ± SD.

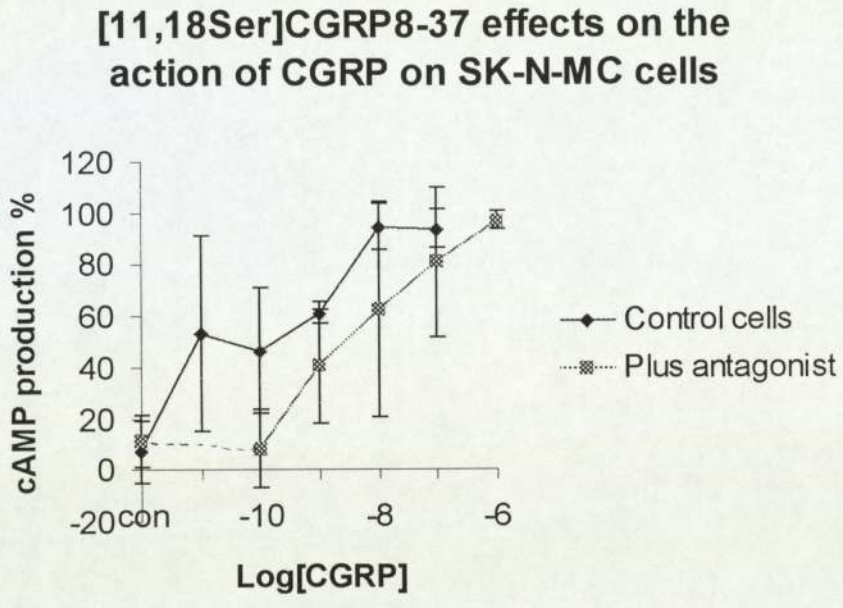


Figure 13. [11,18Ser]CGRP8-37 (2μM) affecting the action of CGRP on SK-N-MC cells. Each point represents the mean of three values ± SD.

3.2.2 [I^{125}]-Iodohistidyl-CGRP binding

In radioligand binding experiments, it proved impossible to obtain reproducible binding. It was thought this could be due to the different components of buffer, which might affect the binding. Thus several buffers were investigated in order to get optimum binding. Unfortunately, none gave particularly impressive results (see Table 4). These values represent typical results of experiments done 1 to 3 times. Especially , using assay buffer with bacitracin, there was little specific binding. The most serious problem with all buffers was the lack of stability, by 30 minutes binding was clearly declining (Figure 14). In addition, the affinity of CGRP was usually between 0.1 and 1 μM (Figure 15), some 100 fold lower than reported in many previous studies (e.g. Poyner et al. 1998; Semark et al. 1992;

Van Valen et al. 1989; Longmore et al. 1994). Binding appeared biphasic. Very high and very low affinity components both existed. This was not as reported previously (Van Valen 1989; Semark et al. 1992; Longmore et al. 1994; Poyner et al. 1998), where only high affinity components existed.

Table 4 Components of assay buffers

No. of buffer	Components (mM)	Peak CPM value
1	NaCl 100; bacitracin 0.4; HEPES (pH7.5) 20; 0.1% (w/v) BSA	Specific 1600 Total 1900
2	HEPES 20;$MgCl_2$ 5;0.1% (w/v) BSA	Specific 4700 Total 6700
3	HEPES 20;$MgCl_2$ 5; EDTA 1	Specific 1100 Total 3500
4	HEPES 20;$MgCl_2$ 1; 0.1% BSA	Specific 4000 Total 7000
5	HEPES 20;$MgCl_2$ 5;0.1% BSA;bacitracin 0.4	Specific 0 Total 200

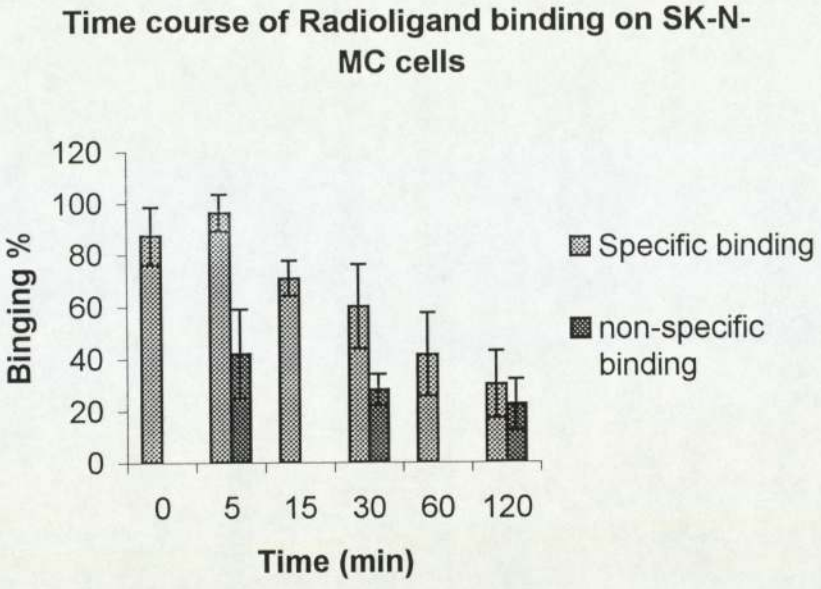


Figure 14. Time course of radioligand binding on SK-N-MC cell membrane. Each point represents mean of 3 values±S.D. (Buffer 2 used)

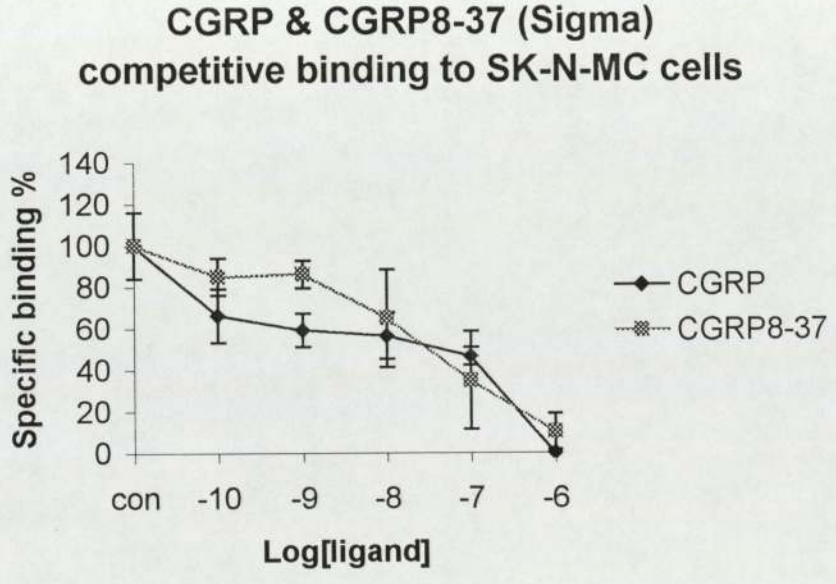


Figure 15. CGRP8-37 binding to SK-N-MC cell membrane. Each point represents the mean of six values ± SD.

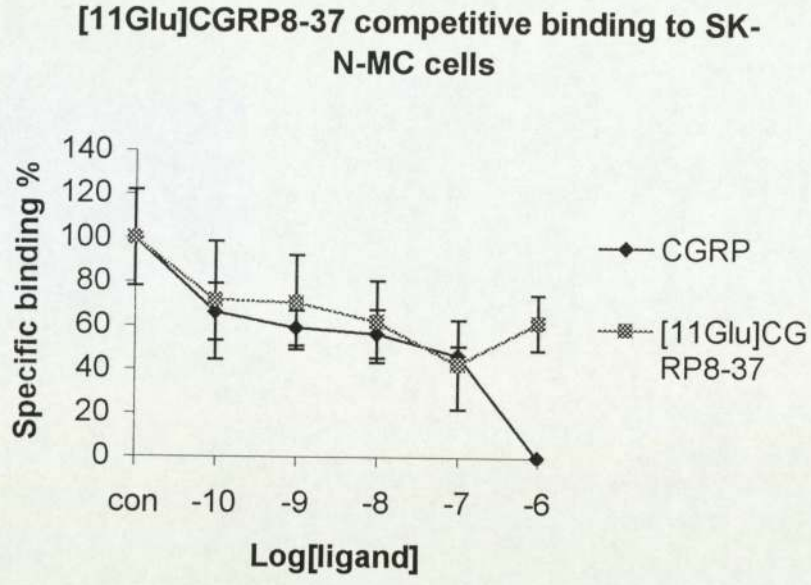


Figure 16. [11Glu]CGRP8-37 binding to SK-N-MC cell membrane. Each point represents the mean of six values ± SD.

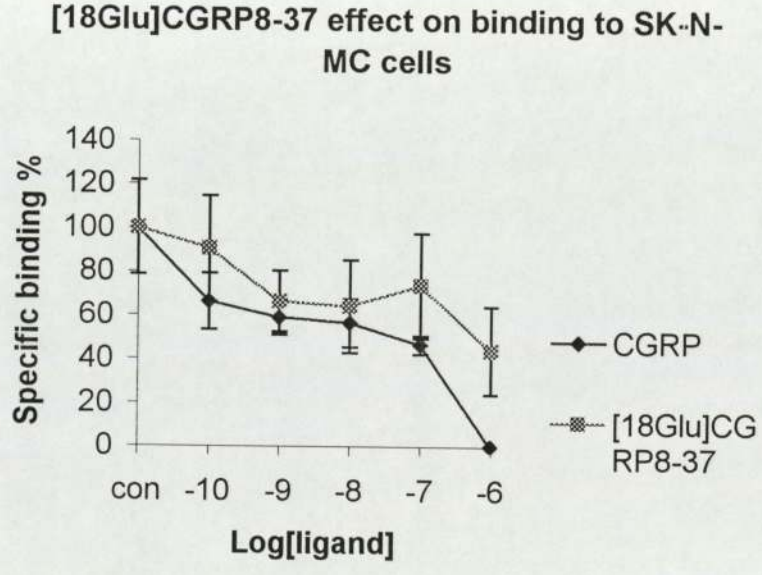


Figure 17 [18Glu]CGRP8-37 binding to SK-N-MC cell membrane. Each point represents the mean of six values ± SD.

Table 5. Comparison of radioligand-binding at different temperatures after 30 mins

Temperature	Specific binding (CPM)	Total binding (CPM)	Non-specific binding (CPM)
24°C	3249	6482	3233
4°C	4542	6714	2172

*Data representative of three experiments.

The time course of binding (figure 14) (using buffer 2, see table 4) at 24°C showed that the binding of ligand to receptor reached a peak at about 5 min and then declined. The peak CPM was about 4,000 with about 1,600 of non-specific binding. This corresponded to 5.45 fmoles(of CGRP bound)/mg membrane protein. Previous studies had reported that the binding was stable for much longer periods (e.g. Longmore et al. 1994). Addition of the protease inhibitors, bacitracin, PMSF, thiophan or leupeptin failed to improved stability (Data not shown). Additionally carrying out incubations at 4°C failed to improve stability (See table 5). The time-course of radioligand binding under 4°C was just like that under 24°C. No improvements were found on stability.

Based on all the results, an incubation time of 30 min at 24°C appeared to be optimum for binding. Displacement studies were carried out using different concentrations of CGRP and its antagonist CGRP8-37, [Glu11] CGRP8-37, [Glu18] CGRP8-37. The IC50 values were about 28nM (CGRP), 39nM (CGRP8-37), 41nM ([11Glu]CGRP8-37) and 617nM ([18Glu]CGRP8-37).

3.2.3 The effect of tunicamycin (TM)

After treatment with TM, the cells showed low sensitivity to CGRP in cAMP accumulation and slightly increased sensitivity to radioligand binding. The results are shown below.

3.2.3.1 [I^{125}]Iodohistidyl-CGRP binding

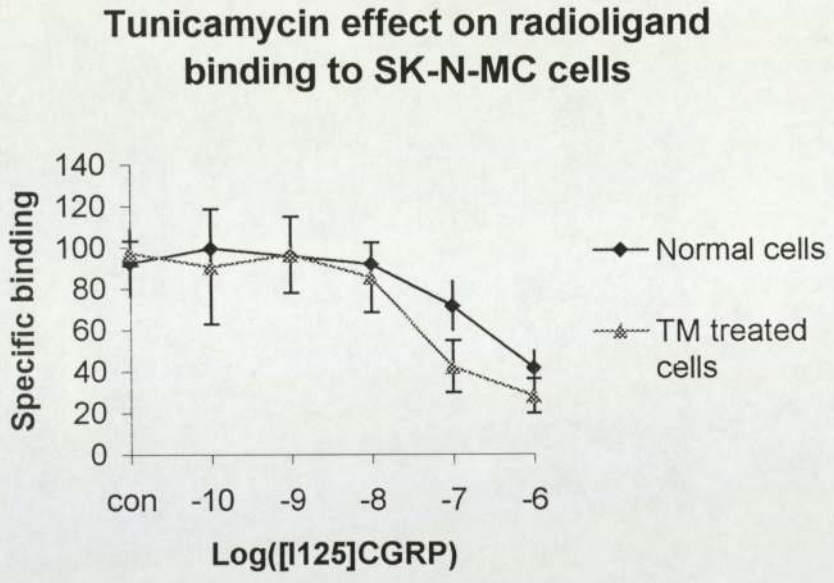


Figure 18. Tunicamycin effects on radioligand binding to SK-N-MC cells. Each point represents the mean of three values ± SD.

Tunicamycin caused a slight increase in the pIC50 for CGRP from 6.51 to 7.23, but seems to have little effect otherwise.

3.2.3.2 cAMP accumulation

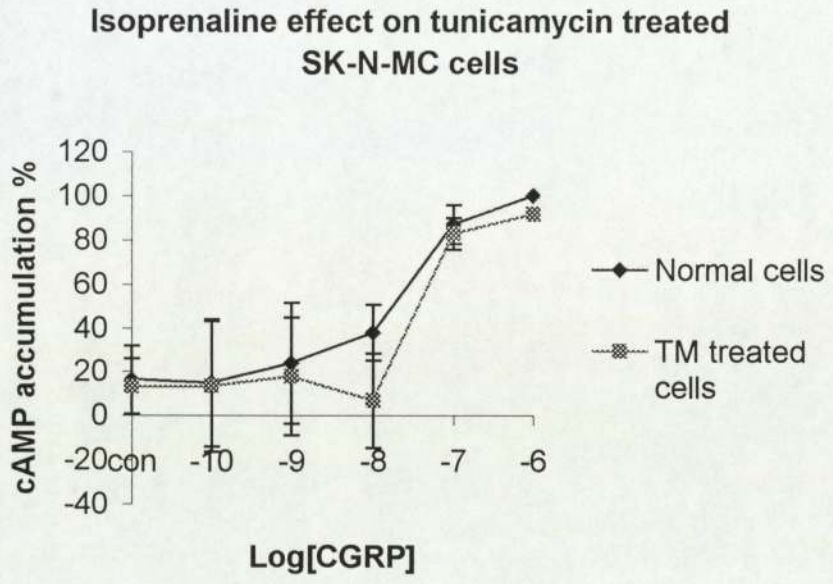


Figure 19. Tunicamycin effects on the Isoprenaline stimulation of cAMP production on SK-N-MC cells. Each point represents the mean of three values ± SD

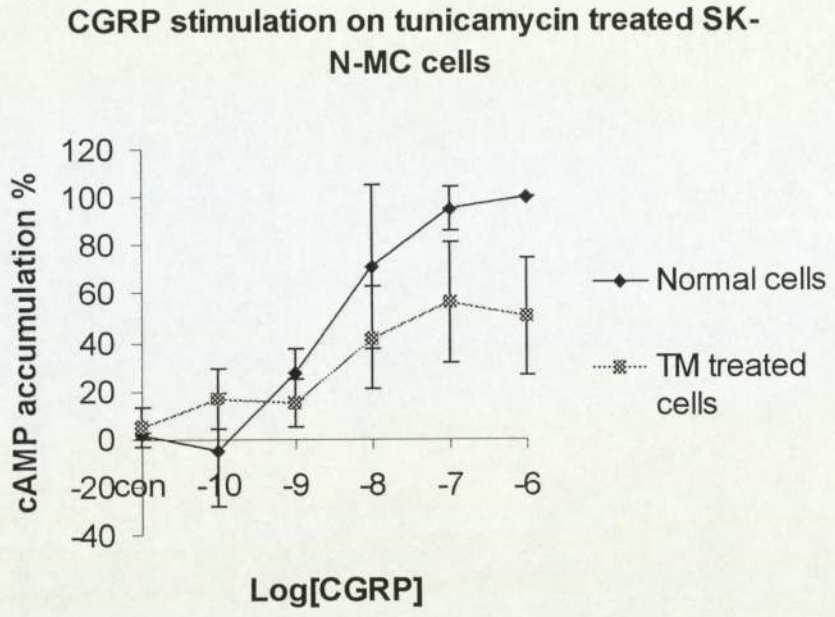


Figure 20. Tunicamycin effects on the CGRP stimulation of cAMP production on SK-N-MC cells. Each point represents the mean of three values ± SD

Cells were treated with tunicamycin for 24 h before exposure to Isoprenaline or CGRP. Compared with normal cells, tunicamycin reduced the response of cells to CGRP, but does nothing to the response to Isoprenaline (figure 20, 21).

3.2.4 The effect of NEM

3.2.4.1 cAMP accumulation

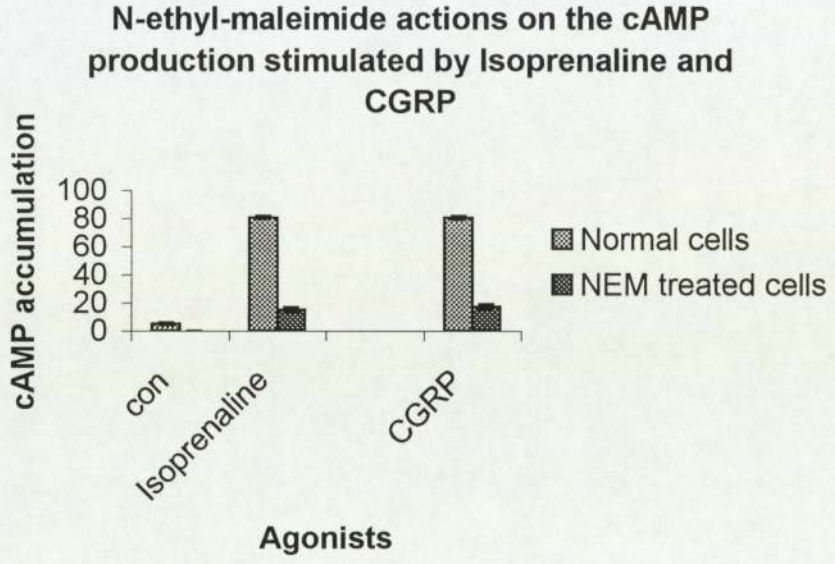


Figure 21. N-ethylmaleimide actions on the cAMP production stimulated by Isoprenaline and CGRP. Each bar represents the mean of 3 values±S.D.

NEM seems to block the cAMP production compared to the normal cells. In the experiment, isoprenaline and CGRP both stimulated the normal cells to produce cAMP, whilst neither of them did this on NEM treated cells (figure 21).

3.2.4.2 [I^{125}]Iodohistidyl-CGRP binding

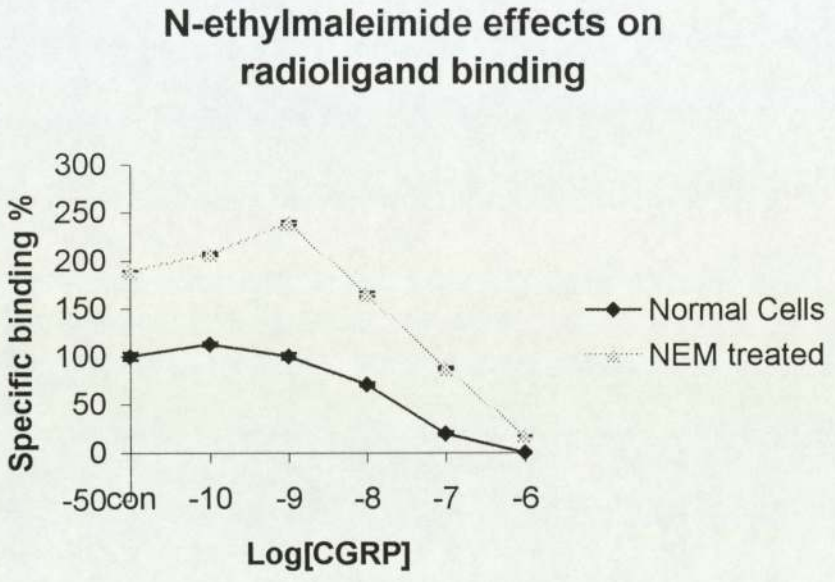


Figure 22. N-ethylmaleimide effects on radioligand binding to SK-N-MC cells. Each point represents the mean of 3 values ± SD.

NEM clearly can increase the maximal binding of CGRP in radioligand binding experiments. The IC50 of normal cells in binding is 84.8±3.3nM (n=3), Hill coefficient (nH) is 0.83±0.28 (figure 22). In NEM treated cells, the IC50 is 120.1± 7.5nM (n=3), nH = 0.57±0.32. As there is no significant change in IC_{50}, the NEM effect is probably mediated by a change in the apparent number of binding sites.

3.3 Col 29 cell line

3.3.1 cAMP accumulation

The Col 29 cell line expressed CGRP2 like receptors (Cox & Tough 1994; Poyner et al. 1998) as discussed in the introduction. However, the cells seem to be less sensitive to CGRP than L6 or SK-N-MC cells. As with the L6 cells, the Col 29 cells showed very variable responsiveness to CGRP, with marked variations between passages. It was not possible to establish a good correlation between sensitivity to CGRP and time after passaging. Col29 cells showed a lot of variation in EC50. Generally the total cAMP production on Col29 cells was less than that on L6 cells (See section 2.1 on page 53).

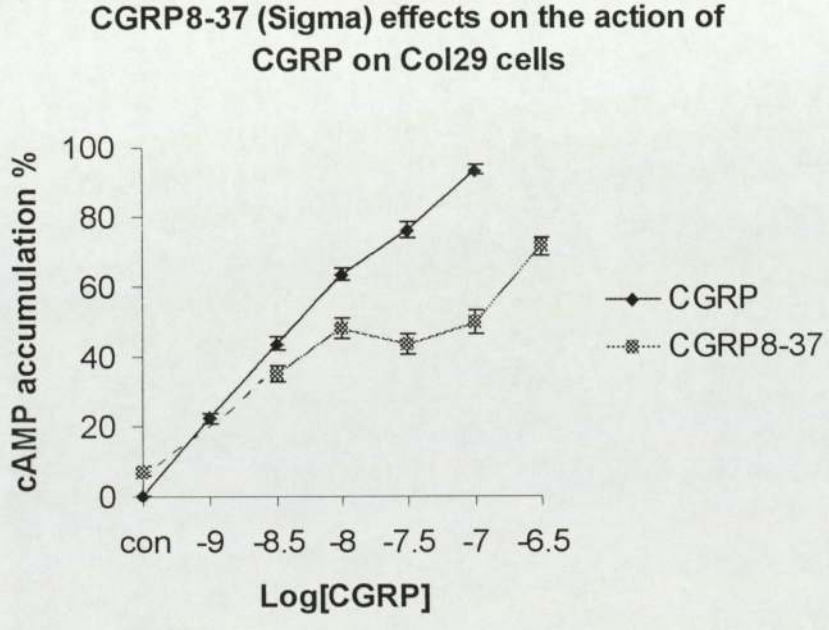


Figure 23. CGRP8-37 (10μM) affecting cAMP accumulation on Col 29 cells. Each point represents the mean of 3 values±SD.

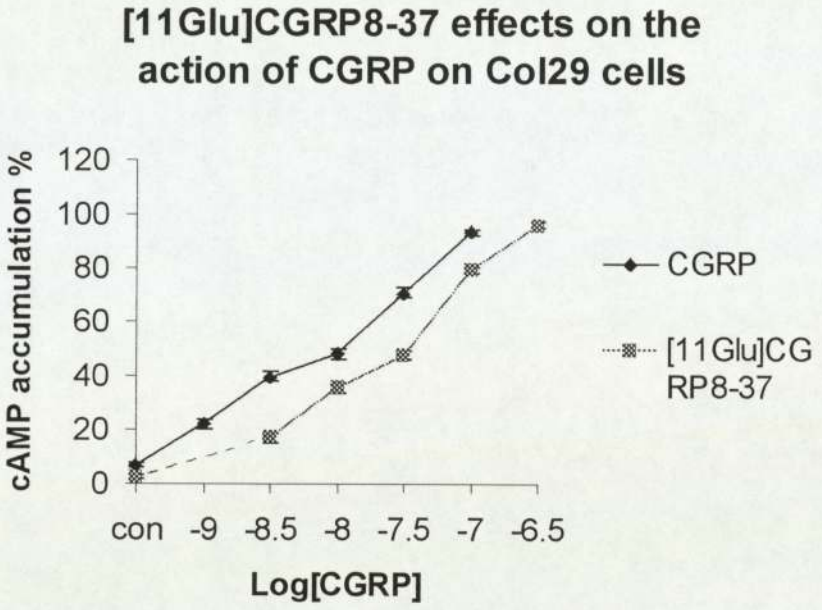


Figure 24. [11Glu] CGRP8-37 (10μM) affecting the cAMP accumulation on Col 29 cells. Each point represents the mean of 3 values±S.D.

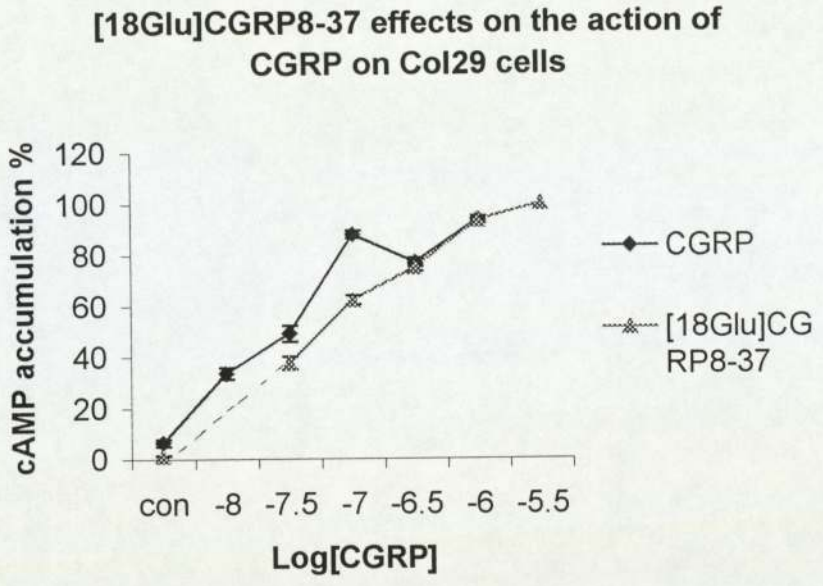


Figure 25. [18Glu]CGRP8-37 (10μM) affecting cAMP accumulation on Col 29, each point represents the mean of 4 values

Table 6. pA2 values of CGRP fragments on Col 29 cells

	CGRP8-37 (Sigma)	[11Glu]CGRP8-37	[18Glu]CGRP8-37
pA2	6.20±0.26	5.56±0.21	<5.25

Col 29 (Colony 29) cell line showed a CGRP2 like nature when treated with CGRP. The pEC50 of CGRP was 8.24±0.30 (n=4) (figure 24). CGRP8-37 caused the curve to shift rightwards with an apparent pKd value of 6.20±0.26 (Sigma) (figure 24). [11Glu]CGRP8-37 was a weak antagonist with an apparent pKd value of 5.6±0.21 (figure 25). [18Glu]CGRP8-37 lost the ability to antagonise CGRP just as happened on L6 and the SK-N-MC cell lines (figure 26). At 1μM [11,18Ser]CGRP8-37 had no significant antagonist properties

(figure 27). However these were apparent at 10μM, when it had an apparent pKd of 6.56±0.13 (n=3) (figure 28).

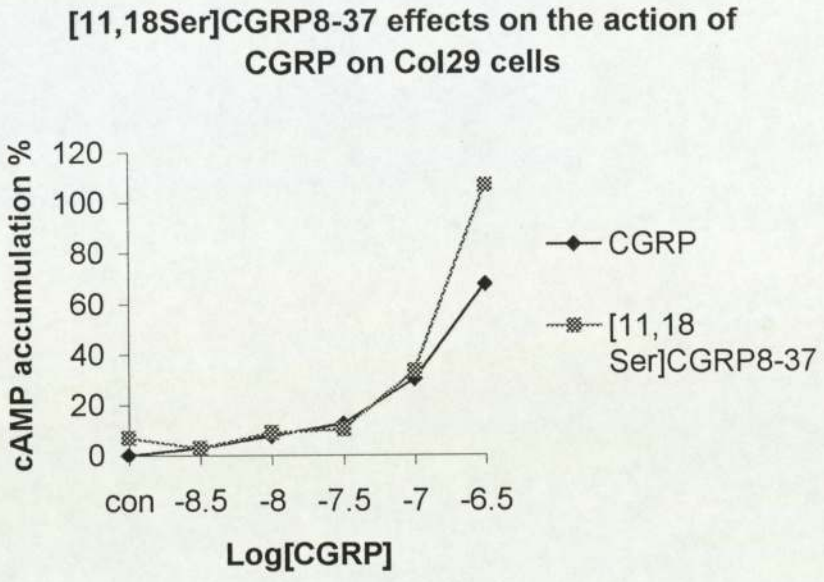


Figure 26. [11,18Ser]CGRP8-37 (1μM) affecting the action of CGRP on Col 29 cells. Each point represents the mean of three values, S.D. is less than 5%

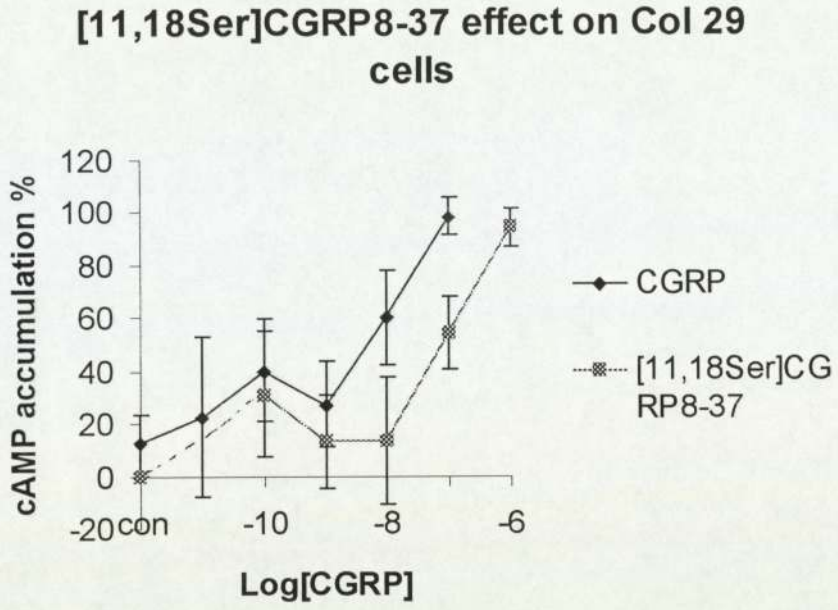


Figure 27. [11,18Ser]CGRP8-37 (10μM) affecting the action of CGRP on Col29 cells. Each point represents the mean of three values ± SD.

3.3.2 The effect of tunicamycin

After treatment with tunicamycin for 72 h, neither treated cells nor control cells responded to CGRP. When the period of treatment was reduced to 24 h, a response still could not be seen. Checking the cells under the microscope, most were rounded up and were probably dying. It is possible the cells were particularly sensitive to the DMSO vehicle.

3.4 cAMP production of individual cell line

From the data of figure 1 (page 54) and average CPM values in the experiments, it could be determined that under resting condition all cell line had a cAMP content of less than 10^{-13} moles/10^5 cells. On stimulation with supramaximal

CGRP, cAMP accumulation was 5×10^{-10}moles/10^5 cells for L6 cells, 3×10^{-10} moles/10^5 cells for SK-N-MC cells and 4×10^{-11}moles/10^5 cells for Col 29 cells.

Chapter 4. Discussion and suggestions for further work

4.1 Receptor classification

The provisional CGRP receptor classification was partly confirmed in the present work. It was thought that L6 and SK-N-MC both express CGRP1 receptor subtype, while Col 29 cells express the CGRP2 receptor subtype (Cox & Tough 1994). This was broadly confirmed by the responses with CGRP8-37, although batch-to-batch variation did confuse the issue. [11Glu] and [18Glu]CGRP8-37 act in the same manner on L6 and SK-N-MC cells. However, the apparent pKd value for [11Glu]CGRP8-37 was noticeably lower on Col 29 cells. It can be concluded that the CGRP receptor on L6 and SK-N-MC are different to the receptor on Col 29 cells. With [11,18Ser]CGRP8-37, there was no difference between the pKd of this compound acting at CGRP1 and CGRP2 receptor (See results section for apparent pKd values). The results hint that the difference between CGRP1 and CGRP2 receptor subtype may be very subtle and perhaps is determined by the charge distribution on the 8-18 α-helix.

During the early experiments, the CGRP receptor on L6 cells showed CGRP2 like properties, particularly with regard to CGRP8-37. In the above discussion, it was thought that the difference might be caused by the poor quality of CGRP from Sigma. However, another factor might also work. It is that CGRP1 and CGRP2 receptors share a common binding site but their pharmacology is

determined by accessory proteins. The L6 cells used in the early stages of this work might have failed to express some key components of the CGRP1 receptor type. Thus the receptor would appear as a CGRP2 receptor. Alternatively the L6 cells may have expressed some key component of CGRP2 receptor type, thus making it CGRP2-like. There are no reports of this type of behaviour of L6 cells and so the most likely explanation of the early results is the quality of the CGRP8-37 supplied by Sigma. However the above explanation cannot be excluded. This problem will ultimately be solved by the molecular characterisation of the CGRP2 receptor.

4.2 Radioligand binding

In the present work, it was decided to carry out the radioligand binding at room temperature for 30 mins. As seen in the results section, the amount bound reached its peak at 5 mins and then went down. After 120 mins, there was no significant difference between total binding and non-specific binding.

Two factors were considered when selecting the conditions for radioligand binding: incubation time and effects of proteolysis. The incubation time had to be long enough to allow equilibrium between the radioligand and the competing drug. On the other hand the effects of proteolysis or other factors leading to loss of binding needed to be minimised. Thirty minutes was chosen as a compromise. There was no advantage from working at 4°C.

The major problem with the radioligand binding experiment was that the affinity of CGRP binding was low. This was also seen when comparing these results with previously published experiments (Poyner et al. 1998; 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), where CGRP showed a high affinity to its receptor. There is no obvious explanation for this phenomenon. The same buffer as the former experiments was used (Poyner et al. 1998). However, in this experiments, bacitracin seemed to inhibit binding. This effect of bacitracin was also seen in separate experiments examining the binding of CCK (Poyner D. R., unpublished data). Although protease inhibitors seemed to make little difference to the binding in these experiments, it is possible that the SK-N-MC cells were expressing a novel protease that was resistant to the inhibitor used in this study. It would be useful to check the integrity of the receptor by SDS-PAGE and the ligand by HPLC to see if proteolysis was occurring.

Despite the low affinity of CGRP in the present experiment, some observations could be made. In former experiments, $MgCl_2$ was thought to promote receptor G-protein coupling (Hulme et al. 1983; Semark et al. 1992). This was confirmed in the present study and the buffers with $MgCl_2$ showed the highest binding. 1mM $MgCl_2$ was more effective than 5mM $MgCl_2$. The other observation was that in the radioligand binding experiment, the potency of CGRP, CGRP8-37, [11Glu]CGRP8-37 and [18Glu]CGRP8-37 matched the same potency order as

that in cAMP accumulation. It seemed that doubly substituted glutamic acid peptide lost or partly lost its antagonist activity because it cannot bind to the receptor with a high affinity. Because of the low affinity of CGRP for its receptor in radioligand binding, it would be unwise to rely too much on the data, however, it is in line with what is really happening at the receptors as judged by functional assays and so it may be of some value.

4.3 CGRP analogues

As noted in the introduction, it is important to identify how CGRP interacts with both CGRP1 and CGRP2 receptor. In former experiments (Lynch & Kaiser 1988; Howitt & Poyner 1997), residues 8-18 were found to form a α-helix which was important in receptor-ligand binding. Within this part of the peptide, two positively charged amino acid arginines at position 11 and 18 appear particulary interesting, since they are conserved in most potent CGRP antagonists.

Arginines 11 and 18 were replaced by alanine in the previous work of Howitt & Poyner (1997). Although alanine is thought to promote α-helix formation, the double substituted peptide [11,18Ala]CGRP8-37 was found to be 300 fold less potent than the original one, while single substituted peptides [11Ala] and [18Ala]CGRP8-37 were 2 and 7 fold less potent, respectively. The results suggested that the overall hydrophobicity of the α-helix was an important factor in ligand binding, but did not rule out direct interactions between the positively

charged arginines and negatively charged groups on the receptor. Alternatively the arginines could be interacting with hydrophilic but uncharged groups on either the receptor or solvent. Thus the negatively charged amino acid glutamic acid was used to substitute for the arginine (For structure of the amino acids, see introduction). If the arginines were involved primarily in ionic interactions, these substitutions would lead to a marked loss of affinity. If they were involved in non-ionic interactions the substitutions should have much less affect.

The results are generally consistent with the hypothesis that arginines were involved primarily in ionic interactions. Both [11Glu]CGRP8-37 and [18Glu]CGRP8-37 were less potent than CGRP8-37 on L6, SK-N-MC and Col 29 cells. [18Glu]CGRP8-37 suffered the greatest loss of affinity and was unable to antagonize the action of CGRP at all at concentrations of 10μM. [11,18Ser]CGRP8-37 shows no antagonist ability at concentration of 1μM on all the cells. However, it is a weak antagonist at high concentration of 10μM so that [11,18Ser]CGRP8-37 is more potent than [18Glu]CGRP8-37 but weaker than [11Glu]CGRP8-37.

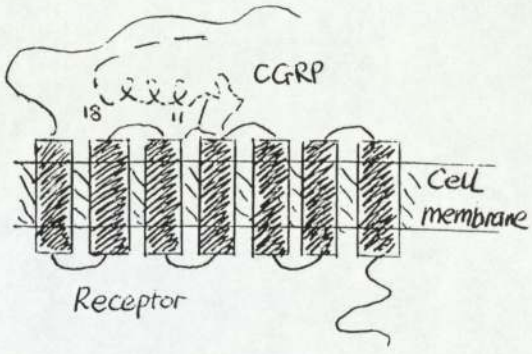
In the introduction section, it was said that the potent CGRP analogues would be very useful in CGRP receptor division. Although we did not find that one, it was confirmed that to develop a potent CGRP analogue, the positive charges at 11 and 18 must be conserved.

4.4 Hypothesis for receptor-ligand interaction

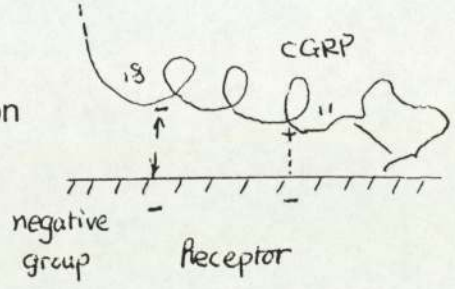
Based on the data from different substituted CGRP8-37 fragments, a hypothesis of how CGRP might interact with its receptor can be advanced. Whilst this is hypothetical, hopefully it will serve as a model in further work.

One important question is whether residues 8-18 interact with the receptor directly or just interact with C-terminus to help to maintain its conformation. I personally consider that the residues 8-18 interact with the receptor. Since the binding of the ligand with the receptor shows high specificity and affinity it seems that the peptide must make multiple contacts with the receptor. The positive charges must be of some importance as they are highly conserved. Based on the hypothesises: that residues 8-18 interact with the receptor directly, an idea of how CGRP interacts with the receptor can be put forward. We know that CGRP can be divided into three parts: the N-terminus, residues 8-18 (α-helix) and the C-terminus. The N-terminus is thought to be responsible for the biological activity of the peptide. Fragments without N-terminus show antagonist activity. There is poor tolerance of the extension of the N-terminus. Thus the N-terminus may bind in a conformationally restricted area, perhaps within the transmembrane helices, however, it contributes little to the overall binding energy between the peptides and the receptor. The residues 8-18 (with the C-terminus) play the major role of keeping the peptide and receptor together. They

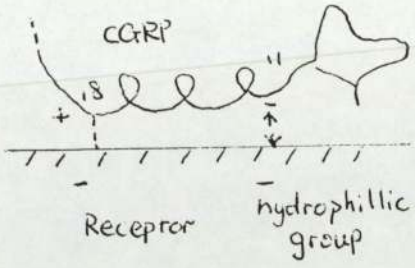
Normal receptor binds to CGRP, two arginines may interact with some negatively charged residues on the receptor



Arginine 18 may interact directly with a negatively charged residue on the receptor, thus negatively charged glutamic acid substitution leads to the strong ionic force repelling CGRP and receptor apart



Arginine 11 may interact with a hydrophilic residue or a negatively charged group a long distance away or both, thus the negatively charged glutamic acid substitution showed less effect on CGRP-receptor interactions



[11,18Ser]CGRP8-37 has two hydroxyl side chains on positions 11 and 18, -OH may help to form hydrogen bonds which promote CGRP binding to the receptor

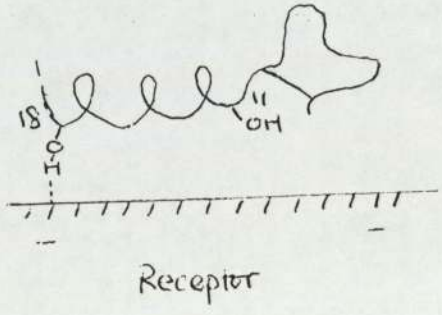


Diagram 9. Illustration of receptor-ligand binding

and 18. Arginine 11 and 18 must interact with some negatively charged groups on the receptor. However, they are not equally important. Arginine 18 is more important than arginine 11. It probably interacts largely with a negatively charged group on the receptor. Thus the substitution by glutamic acid is poorly tolerated, since the ionic repulsion will push the ligand and receptor apart. With arginine 11, there is a somewhat different story. It may interact only partially (if at all) with a negatively charged group on the receptor so that glutamic acid substitution does not affect the interaction so much.

This hypothesis has to be extended to cover the results involving the serine substitution. The hydroxyl group at serine can form a hydrogen bond, which can help the peptide and receptor stay together. [11,18Ser]CGRP8-37 appears to be intermediate in potency between [11Glu]CGRP8-37 and [18Glu]CGRP8-37. This is consistent with the suggestion that the counterparts to arginines 11 & 18 can form hydrogen bonds. However, these are obviously weaker than the ionic forces that normally maintain the interaction.

4.5 Receptor protein

In the present work, it is accepted that CRLR combined with RAMP1 is a CGRP receptor. In the work by McLachtie et al. (1998), CRLR in the presence of RAMP was found to be a terminally glycosylated protein. It is interesting that glycosylation also plays an important role in the binding of calcitonin to its

receptor (Hao et al. 1999). Thus it was interesting to test the role of glycosylation in CGRP receptor function. Tunicamycin was used to inhibit the formation of glycosylation. Tunicamycin was shown to affect cAMP accumulation although it had relatively little effect on radioligand binding. This was specific for CGRP since tunicamycin had no effects on isoprenaline actions.

It is difficult to say why glycosylation was important for cAMP accumulation, but did little to ligand binding to the receptor. In fact loss of glycosylation may even promote ligand binding as there was a small increase in apparent affinity for CGRP. A hypothesis may be that glycosylation will only affect coupling of the receptor to the G-protein and adenylate cyclase. Losing glycosylation does not impair ligand binding to the receptor. The main thing that glycosylation may do is to affect the conformation of receptor protein after it binds with the agonist so affecting the production of cAMP. However, further work must be done before a clear picture of the role of glycosylation can be concluded. The results of present work should not be taken too far, since we actually did not know whether tunicamycin really inhibits the glycosylation of the receptor or not. This would require visualisation of the receptor after SDS-PAGE to see if it had undergone a decrease in molecular weight, consistent with deglycosylation.

CRLR has many cysteines residues which may play a role in ligand-receptor interaction. NEM, which alkylates sulphydryl groups, was used in order to bind to free –SH groups. The result shows that binding to those free –SH can decrease

the cAMP accumulation stimulated by CGRP. However, NEM can also increase the radioligand binding. This phenomenon at first sight is a paradox. Simplistically, one might expect NEM to bind to –SH group and reduce binding, perhaps by steric hindrance That will result in the decrease of the receptor-peptide binding so decreasing the cAMP production. In fact although cAMP production was decreased, binding actually increased. Clearly NEM must be acting at two levels, to alter binding and signal transduction independently.

NEM will bind to –SH and will increase their hydrophobicity. Since CGRP contains numerous hydrophobic regions, it may be that this increase in hydrophobicity play a role in increasing ligand binding. Several explanations are possible as to why cAMP production is decreased and the response to both CGRP and isoprenaline in reduced. NEM may inhibit Gs or adenylate cyclase or stimulate Gi. Alternatively it may have separate actions on both the CGRP and β adrenoceptors to block G-protein activation. The actions of NEM and tunicamycin can be summarised in diagram 10, based on the scheme whereby an agonist first binds to the receptor and then activates it.

$$\mathbf{L(Ligand) + R(Receptor)} \longleftrightarrow \underset{\substack{\uparrow(+)\\ \text{NEM, TM}}}{\mathbf{LR}} \longleftrightarrow \underset{\substack{\uparrow(-)\\ \text{NEM, TM}}}{\mathbf{LR^*}}$$

Diagram 10. The effect of NEM and TM on ligand-receptor complexes. (*) shows active state of complex. (+) potentiates, (-) inhibits.

Both NEM and tunicamycin inhibit receptor activation (although they obviously have different mechanisms of action). NEM, perhaps also tunicamycin, can increase in radioligand binding, but because the steps leading to receptor activation are blocked, no cAMP can be produced.

4.6 Conclusion and suggestions for further work

In summary, it is clear that [11Glu]CGRP8-37 can work as a weak antagonist on both CGRP1 and CGRP2 receptor subtypes, while [18Glu]CGRP8-37 lost its antagonist ability at concentrations of up to 10μM. [11,18Ser]CGRP8-37 works as a weak antagonist on all the cells (L6, SK-N-MC, Col29) at high concentrations. Tunicamycin decreases cAMP accumulation, while NEM can decrease the cAMP accumulation, but also increases the radioligand binding. As discussed before it would be useful to use SDS-PAGE technique to detect whether the receptor protein is actually glycosylated and affected by the tunicamycin. Also, the role of –SH groups are interesting. It would be interesting to identify the –SH groups which are reacting with NEM as these might identify parts of the receptor involved in ligand binding.

It would be interesting to test the doubly substituted peptide fragment [11,18Glu]CGRP8-37 on the different cell lines. If the hypothesis of present work is right, it should not show antagonist activity. The other singly substituted peptide fragments, [11Ser]CGRP8-37 and [18Ser]CGRP8-37 are also

interesting. Using them, it can be decided which part of the receptor is more likely to form a hydrogen bond. Again, if the hypothesis is true, [18Ser]CGRP8-37 will show almost the same apparent pKd value as [11,18Ser]CGRP8-37, while [11Ser]CGRP8-37 would lost its ability to do any antagonist work. The mutation of receptor may be helpful to identify the binding site and will help to explain the details of receptor-peptide binding and actions.

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