Characterisation of calcitonin gene-related peptide (CGRP) and amylin receptors.

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The aims of this study were to examine the binding characteristics of the rat CGRP receptor and to further the classification of CGRP and amylin receptors in guinea-pig tissue preparations.

Binding characteristics of CGRP were investigated on rat splenic, cerebellar and liver membrane preparations. Human-α-CGRP, rat-α-CGRP and the CGRP receptor analogues Tyr°-$CGRP_{(28-37)}$ and [Cys $(ACM)_{2,7}$]-human CGRP and the CGRP receptor antagonist $CGRP_{(8-37)}$ were utilised in competitive radioligand binding experiments to identify possible CGRP receptor subtypes in these tissues. There appeared to be no significant differences between the rat CGRP receptors examined. A panel of monoclonal antibodies (Mabs) raised against CGRP were employed to investigate the structure-activity relationships of CGRP and its receptor. No differences between the tissue receptors were observed using this panel of Mabs.

The effects of human-α, human-β, rat-α-CGRP, human and rat amylin and $adrenomedullin_{(13-52)}$ were examined on the spontaneously beating right atria and on electrically evoked twitch contractions of isolated guinea-pig ileum, vas deferens and left atria. All of the peptides caused concentration-dependent inhibition of twitch amplitude in the ileum and vas deferens. CGRP produced positive inotropic effects in the right and left atria and positive chronotropic effects in the right atria. A variety of CGRP receptor antagonists and putative amylin receptor antagonists were used to antagonise these effects. $CGRP_{(8-37)}$ is currently used as a basis for CGRP receptor classification (Dennis, *et al.*, 1989). Based upon results obtained using $CGRP_{(8-37)}$ it has been shown that the guinea-pig ileum contains mainly $CGRP_1$ receptors and the vas deferens contain $CGRP_2$ receptors. Amylin was shown to act at receptors distinct from those for CGRP and it is postulated that amylin has its own receptors in these preparations. Experiments using $CGRP_{(19-37)}$ and Tyr°-$CGRP_{(28-37)}$ indicate that human and rat CGRP act at distinct receptors in guinea-pig ileum and vas deferens. The amylin receptor antagonist $amylin_{(8-37)}$ and the putative antagonist AC187 provide evidence to suggest human and rat amylin also act at receptors able to distinguish between the two types of amylin.

Key words: CGRP, amylin, receptor classification, guinea-pig.

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Chapter 1

General Introduction

1.1 CGRP and related peptides

Calcitonin gene-related peptide (CGRP), calcitonin (CT), amylin and adrenomedullin (AM) have been reported as belonging to a 'super family' of peptides which show substantial sequence homology (figure 1.2). In particular most of these peptides share a common hexapeptide disulphide bridge at their N-terminal end, with the exception of CT which has a heptapeptide di-sulphide bridge (Cooper *et al.*, 1987; Kitamura *et al.*, 1993; Rosenfeld *et al.*, 1983; Amara *et al.*, 1985; Steenbergh *et al.*, 1985; Morris *et al.*, 1984; Westermark *et al.*, 1987a).

1.1.1 CGRP and Calcitonin

CGRP and calcitonin (CT) are encoded by a single CGRP/CT gene complex, found on chromosome 11 (Breimer *et al.*, 1988), whose primary transcript is alternatively spliced to produce both CT and CGRP mRNA. These mRNAs are translated to produce large precursor peptides that are subsequently cleaved to produce the 37 amino acid CGRP and the 32 amino acid CT. Processing of the primary transcript occurs in a tissue specific manner such that CT mRNA is formed almost exclusively in the thyroid C cells and CGRP mRNA in the nervous system (Amara *et al.*, 1982; Rosenfeld *et al.*, 1983). Both peptides have an overall positive charge and CGRP has a carboxy phenylalanine amide (figure 1.2)

The existence of CGRP was predicted by mRNA analysis and subsequently demonstrated using antibodies raised against a synthetic peptide corresponding to the predicted C-terminal of CGRP. Four types of CGRP are commonly available; rat-α-CGRP, rat-β-CGRP, human-α-CGRP and human-β-CGRP which are highly conserved (Morris *et al.*, 1984; Amara *et al.*, 1985; Steenbergh *et al.*, 1985). CT was first identified as a hypercalcaemic factor, secreted by the parafollicular cells of the thyroid gland, in response to elevated serum levels of calcium (Copp *et al.*, 1962). CT receptors are commonly defined using human and salmon CT, which show species-related differences. Salmon CT (sCT) shows greater structural homology to human-α-CGRP (31.2%) than human CT (hCT) (18.8%) (see figure 1.2) and is usually 2-3

orders of magnitude more potent in its effects on CT receptors (Houssami *et al.*, 1994). CGRP has been suggested to contain an α-helical segment which is positioned in the region between amino acid residues 8-18. Modelling studies suggest a turn exists immediately after the helix in residues 17-21 and another in residues 29-34 (Lynch & Kaiser, 1988; Hubbard *et al.*, 1991).

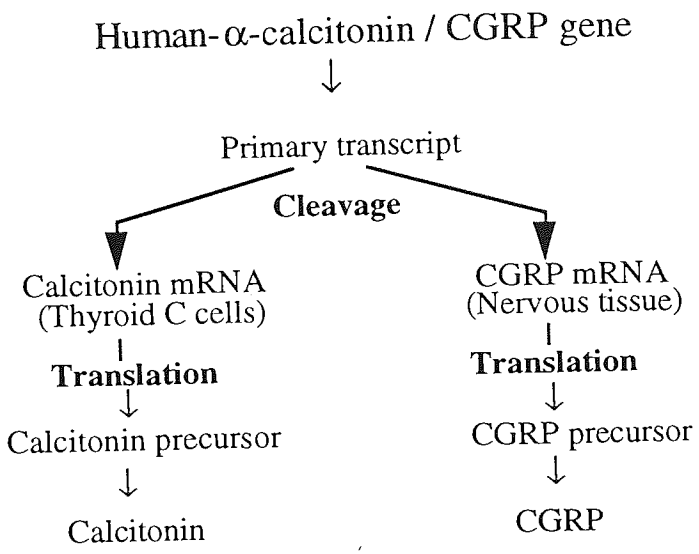


Figure 1.1 *Tissue specific alternative processing of the human calcitonin gene to produce CGRP and calcitonin.*

1.1.2 Amylin

Amylin forms the major constituent of the 'amyloid' extracellular deposits found in the islets of Langerhans of non-insulin dependent diabetic (NIDDM) patients (Cooper *et al.*, 1987). These deposits have also been found in some benign insulinomas and in the normal pancreas of ageing humans (Cooper *et al.*, 1987). Sequence analysis of both rat and human amylin shows that they are approximately 46-49% homologous to human-α-CGRP (figure 1.2) (Cooper *et al.*, 1987; Westermark *et al.*, 1987b; Leffert *et al.*, 1989). The gene which encodes for amylin, in humans, is located on the short arm of chromosome 12 (Mosselman *et al.*, 1988; Nishi *et al.*, 1989) which is believed

to be an evolutionary homologue of chromosome 11. As chromosome 11 contains the gene encoding CGRP and CT, this may explain the substantial sequence homology between CGRP and amylin. Amylin also possesses structural similarities with CGRP as it is reported that amylin has turns between amino acid residues 19-23 and in residues 29-34 (CGRP has turns between 17-21 and 29-34) (Hubbard *et al.*, 1988). Amylin is co-localised with insulin in pancreatic islets and co-secreted in response to stimuli for insulin secretion, such as hyperglycaemia (Fehmann *et al.*, 1990).

1.1.3 Adrenomedullin

Adrenomedullin (AM) is a novel vasorelaxant peptide which has been isolated from human pheochromocytoma. It consists of 52 amino acid residues with an N-terminal di-sulphide bridge and a C-terminal amide group (Kitamura *et al.*, 1993). AM has 27% sequence homology with human-α-CGRP and has been reported to share some of its physiological effects, such as the production of potent and lasting hypotension in rats *in vivo* (Kitamura *et al.*, 1993). AM-like immunoreactivity and mRNA have been demonstrated to exist in human adrenal medulla, lung, kidney and circulating in the plasma (Eguchi *et al.*, 1994).

Human-α-CGRP

NH_2 - A C **D** T A T C V T H R L A G L L S R S G G V V K N N F V P T N V G S K A F - $CONH_2$ (100%)

Human-β-CGRP

NH_2 - A C N T A T C V T H R L A G L L S R S G G **M** V K **S** N F V P T N V G S K A F - $CONH_2$ (91.9%)

Rat-α-CGRP

NH_2 - **S** C N T A T C V T H R L A G L L S R S G G V V K **D** N F V P T N V G S **E** A F - $CONH_2$ (89.2%)

Rat-β-CGRP

NH_2 - **S** C N T A T C V T H R L A G L L S R S G G V V K **D** N F V P T N V G S K A F - $CONH_2$ (91.9%)

Chicken CGRP

NH_2 - A C N T A T C V T H R L A **D F** L S R S G G V **G** K N N F V P T N V G S K A F - $CONH_2$ (89.2%)

Human amylin

NH_2 - **K** C N T A T C A T **Q** R L A **N F** L **V H S S** N N **F G A I L S S** T N V G S N **T Y** - $CONH_2$ (45.95%)

Rat amylin

NH_2 - **K** C N T A T C **A** T **Q** R L A **N F** L **V** R **S S N N L G P V L P** P T N V G S **N T Y** - $CONH_2$ (48.7%)

Human calcitonin

NH_2 - **C G N L S** T C **M L G T Y T Q D F N K F H T F P Q** - - - - - **T A I G V G A P** - $CONH_2$ (18.8%)

Salmon calcitonin

NH_2 - **C S N L S** T C **V L G K L S Q D L H K L Q T Y P R** - - - - - **T N T G S G T P** - $CONH_2$ (31.2%)

Adrenomedullin

NH_2 - **Y R Q S M N N F Q G L R S F G** C **R F G T** C **T V Q K L A H Q I Y Q F T D K D K D N V A P R S K I S P Q G** Y - $CONH_2$ (27%)

Figure 1.2 *Sequence comparisons of CGRP and related peptides. Amino acids differing from those in human-α-CGRP are highlighted. The underlined cysteines in positions 2 and 7 indicate the location of the disulphide bridge. The numbers in brackets indicate the percentage of sequence homology with human-α-CGRP. - - - = alignment of amino acids sequences for direct comparison with human-α-CGRP.*

Single amino acid code is used: 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 CGRP, Calcitonin and Amylin receptor interactions

Historically, the first study to identify specific CGRP and CT receptors was that of Goltzman & Mitchell (1985), who found distinct binding sites for the two peptides in brain and peripheral tissue. CGRP was found to be unable to stimulate adenylate cyclase via activation of CGRP receptors in rat spinal cord or pituitary gland, but could cause production of cyclic adenosine 3, 5'-monophosphate (cAMP) in CT target tissues, such as rat kidney. It was suggested that this occurred by interaction with CT receptors implicating that receptor/peptide cross-reactivity may exist (Goltzman & Mitchell, 1985).

Data regarding CGRP, CT and amylin receptor classification is conflicting, with some studies reporting distinct receptor types for each peptide and others reporting cross-reactivity. In the rat and human cerebellar cortex, CGRP and CT have been shown to possess their own specific binding sites with little interaction between receptors (Tschopp *et al.*, 1985; Sexton *et al.*, 1986). In rat nucleus accumbens, binding studies using sCT, amylin and CGRP, have shown that receptor subtypes exist with differing specificity's and binding profiles (Sexton *et al.*, 1988; Veale *et al.*, 1994; Beaumont *et al.*, 1993). This discovery suggested a number of receptors were present, that were subsequently classified upon the basis of the ligands they recognised. These receptor subtypes were named C1, C2 and C3 where C1 receptors showed high affinity binding for sCT, C2 for CGRP and C3 for amylin, sCT and CGRP (Sexton *et al.*, 1988) (see table 1.1). Although the C1 and C2 receptor nomenclature has now largely been incorporated into the current CT and CGRP receptor classification system, the C3 nomenclature still persists. This binding site shows some similarities to a peripheral amylin receptor (Beaumont *et al.*, 1995) and will bind amylin and its analogues with high affinity (see also section 1.2.2) causing speculation that it may be an amylin receptor.

Receptor subtype	Relative order of potency	Current classification
C1	sCT > rat-α-CGRP > rat amylin	Calcitonin receptors
C2	Rat-α-CGRP > rat amylin > sCT	$CGRP_1$ & $CGRP_2$ receptors
C3	Rat amylin ≥ sCT > rat-α-CGRP	$CGRP_3$ or amylin receptor

Table 1.1 *Relative orders of potency for sCT, rat amylin and rat-α-CGRP, at the C1, C2 and C3 binding sites in rat nucleus accumbens. Data based upon IC_{50} values (nM) obtained from Sexton et al (1988), Veale et al (1994) and Beaumont et al (1993).*

In rat skeletal muscle, CGRP and amylin are reported to act via an amylin receptor to alter glycogen metabolism (Beaumont *et al.*, 1995). In rat pancreas and guinea-pig atria, rat-α-CGRP and amylin have been reported to act, at least in part, via a common CGRP receptor type (Silvestre *et al.*, 1993; Guiliani *et al.*, 1992). In rat lung, amylin showed a binding affinity 10 times greater than that observed for rat-β-CGRP and 150 times greater than that for rat-α-CGRP, indicating that amylin has its own receptors, distinct from the CGRP receptor present in this preparation (Bhogal *et al.*, 1992, 1993).

1.2.1 CGRP receptor classification and characterisation

1.2.1.1 CGRP receptor classification

CGRP has been reported to possess a widely distributed receptor population, which shows high affinity CGRP binding (Poyner, 1992 for review).

It is currently accepted by many workers that CGRP receptors can be classed into 2 main types; $CGRP_1$ and $CGRP_2$. A third CGRP receptor type has however been suggested, $CGRP_3$, which corresponds to the C3 binding site in the rat nucleus accumbens (table 1.1, section 1.2). The putative $CGRP_3$ receptor binds sCT with a

14-30% higher affinity than rat-α-CGRP (Sexton *et al.*, 1988; Beaumont *et al.*, 1993; Veale *et al.*, 1994). $CGRP_1$ receptors are selectively antagonised by the CGRP receptor antagonist, $CGRP_{(8\text{-}37)}$, whilst $CGRP_2$ receptors show a high affinity for the linear CGRP agonist, acetoamidomethylcysteine$_{2,7}$-CGRP ([Cys $(ACM)_{2,7}$]-human CGRP). $CGRP_1$ receptors are typified by those found on guinea-pig atria, whereas $CGRP_2$ receptors are typified by those on rat vas deferens (Dennis *et al.*, 1990; Mimeault *et al.*, 1991; Quirion *et al.*, 1992).

At the present time CGRP receptor classification is based upon studies using 2 different animal species, namely rat and guinea-pig. Few, if any, studies have been performed which compare CGRP receptors in tissues from the same species. Thus the problem of inter-species variation between receptors, which could complicate receptor classification, has not been fully addressed.

1.2.1.2 CGRP receptor characterisation

The reported structural requirements for CGRP receptor-ligand interactions show some degree of inconsistency. The lack of effectiveness of the linear analogue [Cys $(ACM)_{2,7}$]-human CGRP in guinea-pig atria for example, suggests that the disulphide bridge is necessary for CGRP binding at this receptor (Dennis *et al.*, 1990). However, in rat whole brain and cortex, [Cys $(ACM)_{2,7}$]-human CGRP shows high affinity binding (Sexton *et al.*, 1986; Seifert *et al.*, 1988; Dennis *et al.*, 1989).

Many studies have reported the need for the CGRP molecule to be intact before any biological activity can be achieved (Zaidi *et al.*, 1990) but partial modification of the middle and C-terminal regions does not always greatly alter biological activity (Tippins *et al.*, 1986). Other studies have shown that disruption of the α-helical section of CGRP, found between amino acid residues 8-18, by insertion of a proline residue at 16 prevented binding in rat vas deferens (Wisskirchen *et al.*, 1994). It has been suggested that the primary role of the C-terminal is interaction with the CGRP receptor. The C-terminal phenylalanine amide (see figure 1.2), has been shown to be involved in human-α-CGRP receptor binding on L6 myocyte cells and to contribute

to the potency and specificity of such receptor-ligand interactions (O'Connell *et al.*, 1993). In rat liver membranes, the C-terminus was reported to be essential for receptor binding but subsequent receptor activation was not observed without an intact di-sulphide bridge. In general, it would appear that the C-terminal of CGRP is important for successful receptor binding but that the middle and N-terminal regions are required for successful receptor activation.

Recently, the 'orphan' receptor clone, RDC1, has been shown to have the characteristics of a CGRP receptor. This is a typical G-protein coupled receptor and should facilitate CGRP receptor pharmacology (Kapan & Clark, 1995)

1.2.1.3 CGRP receptor antagonists

Binding studies utilising CGRP receptor antagonists such as $CGRP_{(8\text{-}37)}$, have yielded Kd values that show little variation. This occurs regardless of the tissue from which the receptor was examined (see table 1.2) and implies CGRP receptor homogeneity. In contrast, functional studies performed on a variety of tissues have produced varied pA_2 values ranging between 6 and 9 (see table 1.2). The tissue to tissue differences in pA_2 values obtained with the same antagonist suggest receptor heterogeneity and thus functional studies may be a more reliable method of CGRP receptor classification than receptor binding assays.

The current basis for CGRP receptor classification is from results obtained using $CGRP_{(8\text{-}37)}$. The first report of the antagonistic effects of this fragment was by Chiba *et al* (1989) who showed $CGRP_{(8\text{-}37)}$ capable of antagonising human-α-CGRP-induced cAMP production in rat liver membranes. The cardiac effects of CGRP and amylin in the rat and guinea-pig have been shown to be antagonised by $CGRP_{(8\text{-}37)}$, thereby suggesting that either CGRP and amylin act via the same receptor in the rat and guinea-pig heart, or that $CGRP_{(8\text{-}37)}$ is not a selective antagonist for CGRP receptors in these preparations (Gardiner *et al.*, 1991; Guiliani *et al.*, 1992). In rat soleus muscle $CGRP_{(8\text{-}37)}$ has also been shown to antagonise ^{14}C-glycogen

accumulation by amylin and CGRP which are reported to be mediated via an amylin receptor (Beaumont *et al.*, 1995).

The binding affinity of $CGRP_{(8-37)}$ has also been reported to be sensitive to factors such as temperature and buffer composition, especially Mg^{2+} and NaCl content. Replacement of buffer Mg^{2+} with EDTA or increased NaCl (100mM) or Mg^{2+} (5mM) content, reduced binding by approximately 50-60%. The optimal buffer concentration of Mg^{2+} for CGRP has been shown to be 1mM (Poyner *et al.*, 1992).

Animal species	Reference	Tissue type	pA_2 value	pK_D value
Guinea-pig	**Dennis *et al* (1990)**	Right atrium	**7.61**	**9.0**
		Left atrium	**6.98**	
		Ileum	**6.77**	
		Vas deferens	**6.77**	**9.3**
	Mimeault *et al* (1991)	Right atrium	**7.22**	
	Maggi *et al* (1991)	Left atrium	**6.89**	
Rat	**Mimeault *et al* (1991)**	Vas deferens	**6.24**	
	Poyner *et al* (1992)	L6 myocytes	**8.83**	**9.2**
	Maggi *et al* (1991)	Vas deferens	**6.55**	
	Foulkes *et al* (1991)	Mesenteric arterial rings	**8.50**	

Table 1.2 *Comparison of pA_2 and pK_D values obtained for $CGRP_{(8\text{-}37)}$. pA_2 = negative logarithm of [antagonist] causing a 2-fold shift in EC_{50} value for CGRP. pK_D = negative logarithm of dissociation constant for $CGRP_{(8\text{-}37)}$.*

Other CGRP fragments have been reported to antagonise the actions of CGRP at its receptors. Tyr°-$CGRP_{(28\text{-}37)}$ has been reported to antagonise the human-α-CGRP-induced reduction in tension of the opossum internal anal sphincter. $CGRP_{(8\text{-}37)}$ also antagonised the human-α-CGRP-induced effect in this preparation but with a higher

potency than Tyr°-$CGRP_{(28-37)}$ (pA_2 values of 7.8 and 7.3 respectively; Chadker & Rattan, 1991). Tyr°-$CGRP_{(28-37)}$ has also been shown to antagonise human-α-CGRP-induced cAMP production in SK-N-MC neuroblastoma cells with a pA_2 value of 6.7 (Van Valen *et al.*, 1990).

$CGRP_{(19-37)}$, $CGRP_{(23-37)}$ and $CGRP_{(11-37)}$ have been reported to possess antagonistic ability against human-α-CGRP-induced effects in the guinea-pig left atrium, with pA_2 values of 5.4, 4.8 and 6.2 respectively, indicating that short, middle and C-terminal fragments of CGRP can act as competitive antagonists in this preparation (Mimeault *et al.*, 1991; Rovero *et al.*, 1992) but with differing potencies as $CGRP_{(19-37)}$ and $CGRP_{(23-37)}$ have low pA_2 values.

1.2.2 Amylin receptor classification

As previously explained (section 1.1), amylin shows considerable sequence homology to CGRP (46%) and because of this relationship, many reports have concluded that amylin acts via CGRP receptors. Amylin has however been reported to possess its own distinct receptors as well as cross-reacting at CGRP and sCT receptors (Beaumont *et al.*, 1993; Rink *et al.*, 1993; Veale *et al.*, 1994; Bhogal *et al.*, 1992; Sexton *et al.*, 1988; Guiliani *et al.*, 1992). Amylin binding sites in the rat nucleus accumbens have been found to show a high affinity for the putative amylin receptor antagonist acetyl-[Asn^{30},Tyr^{32}]-$sCT_{(8-32)}$ (AC187) (K_i of 79pM). These CNS sites seem to be similar to the amylin receptor identified on rat soleus muscle (Beaumont *et al.*, 1995). Amylin and CGRP-induced muscle glycogen metabolism is reported to occur via an amylin receptor in rat skeletal muscle, an effect antagonised by AC187 (Beaumont *et al.*, 1995; Young *et al.*, 1994). Another amylin receptor antagonist, $amylin_{(8-37)}$ has been shown to antagonise both amylin-induced responses on rat soleus muscle (Deems *et al.*, 1991) and amylin-induced inhibition of insulin secretion from isolated rat pancreatic islet cells (Wang *et al.*, 1993). Thus there is evidence to suggest the existence of independent amylin receptors.

1.2.3 Adrenomedullin receptor classification

The existence of specific receptors for AM has been proposed as AM-specific binding sites have been demonstrated on cultured, rat aortic vascular smooth muscle cells (VSMC). The cAMP-induced formation by AM and CGRP on these cells was antagonised by $CGRP_{(8-37)}$, indicating that CGRP and AM may interact with the same receptors on VSMC (Eguchi *et al.*, 1994). On human SK-N-MC neuroblastoma cells, both AM and CGRP responses were antagonised by $CGRP_{(8-37)}$, implying that AM may act via a $CGRP_1$ receptor on this preparation (Zimmermann *et al.*, 1995). It has been shown that CGRP and AM stimulated cAMP production in rat aortic vascular smooth muscle cells and that AM was 10 times more potent in its effects than CGRP (Eguchi *et al.*, 1994). Specific binding sites for AM have been identified on rat heart and lung membranes. A separate second set of AM binding sites identified on rat lung tissue seem to correspond to sites previously thought to be amylin receptors (Owji *et al.*, 1995).

AM has also been shown to increase $[Ca^{2+}]_i$ on vascular endothelial cells by activation of phospholipase C and inositol 1,4,5 trisphosphate formation leading to nitric oxide release, demonstrating that AM can act via 2 separate signal transduction pathways (Shimekaka *et al.*, 1995) i.e. elevation of cAMP and $[Ca^{2+}]_i$. The 'orphan' receptor clone GLOd has been identified as encoding an AM receptor, showing that AM has receptors distinct from those for CGRP (Kapas *et al.*, 1995).

1.2.4 Calcitonin receptor classification

High affinity binding sites for CT have been demonstrated in bone, kidney, testes, spermatozoa, placenta, lung and widely in the central nervous system (CNS) (Fischer *et al.*, 1981; Chausmer *et al.*, 1980; Silvestroni *et al.*, 1987; Nicholson *et al.*, 1988; Fouchereau-Peron *et al.*, 1981; Henke *et al.*, 1983). The best documented second messenger effect of CT at cellular level, is cAMP stimulation (Gorn *et al.*, 1992). CT receptors have been cloned from human, rat and porcine tissues. They belong to a

family of G-protein coupled receptors and can elevate both cAMP and Ca^{2+} and all have a low affinity for CGRP (Abrandt *et al.*, 1993; Kuestner *et al.*, 1994; Zolnierowitz *et al.*, 1994).

1.3 General physiology of CGRP

1.3.1 CGRP in the cardiovascular system

1.3.1.1 CGRP in the heart

Cardiovascular control has historically been attributed to adrenergic (sympathetic) and cholinergic (parasympathetic) neurotransmitters. Cardiac and vascular tissues are also innervated by peptidergic neurones, which may mediate non-adrenergic, non-cholinergic (NANC) functions. CGRP has been reported to be a neurotransmitter of the NANC neurones in guinea-pig, rat and human atrial preparations (Goto *et al.*, 1992; Lundberg *et al.*, 1985; Saito *et al.*, 1986; Miyauchi *et al.*, 1987; Franco-Cereceda *et al.*, 1987).

CGRP-like immunoreactive nerves are densely distributed in the sino-atrial node and atria of the guinea-pig heart but are sparse in the pericardium of the ventricles (Ishikawa *et al.*, 1988; Miyauchi *et al.*, 1987). [^{125}I]-α-human CGRP binding has been shown to be high in the atria but low in the ventricles of the rat (Sigrist *et al.*, 1986). The CGRP binding sites, reported on rat cardiac myocytes, have been shown to exist in multiple affinity states and undergo time-dependent conversions from low to high affinity (Chatterjee *et al.*, 1991).

CGRP released from capsaicin-sensitive NANC neurones in the heart has been reported to produce positive inotropic and chronotropic effects (Franco-Cereceda & Lundberg, 1985; Marshall *et al.*, 1986; Saito *et al.*, 1987; Miyauchi *et al.*, 1988). 5-hydroxytryptamine- (5-HT) and bradykinin-induced positive inotropic effects on the guinea-pig heart, are reported to be mediated via CGRP release (Tramontana *et al.*, 1993; Bernoussi & Rioux, 1989). Positive inotropic responses induced by CGRP in

guinea-pig and rat hearts have been shown to be associated with an increase in cAMP, comparable to that produced by isoprenaline (Ishikawa *et al.*, 1987; Mantelli *et al.*, 1992; Wang & Fiscus, 1989). The cAMP increase has been reported to increase the slow inward calcium current via L-type calcium channel phosphorylation (Nakajima *et al.*, 1991) leading to an increase in the amplitude and duration of the cardiac action potential plateau. In guinea-pig atria, CGRP has been reported to produce a shortening of the time taken to reach peak force and an acceleration of relaxation in isometric contraction (Ishikawa *et al.*, 1988). In guinea-pig and frog atrial cells, increased cAMP levels induced by CGRP, lead to an increase in the delayed rectifier potassium current and faster repolarisation of the heart by shortening the cardiac action potential (Ohmura *et al.*, 1990; Ono *et al.*, 1991). The effects of CGRP upon the calcium and potassium ion channels are thought to primarily account for CGRP's inotropic and chronotropic effects.

Local release of CGRP from NANC neurones in the heart is thought to occur via a type of local reflex, such as myocardial ischaemia. Indeed release of CGRP can be prompted by a reduction in pH and an increase in lactic acid produced in response to ischaemia (Franco-Cereceda *et al.*, 1993). At high concentrations (> 0.1μM), in rat atrial cells, CGRP activates a muscarinic-gated potassium current. Patch-clamp studies have suggested that this occurs via a GTP-binding protein, either Gi or Go (Kim, 1991). Activation of the muscarinic-gated potassium current stabilises membrane potential at the potassium equilibrium potential (-90mV) and therefore opposes action potential generation. This effect of CGRP seems to oppose its positive inotropic and chronotropic actions but as it only occurs at high CGRP concentrations, it may provide a cardio-protective effect which becomes important when CGRP-containing nerves are strongly activated, such as during myocardial ischaemia.

1.3.1.2 CGRP in the vasculature

CGRP has been reported to be a potent vasodilator and produce vasodilation in hamster, rabbit, human, bovine, porcine and rat at femtomole doses (Brain *et al.*,

1985, Greenberg *et al.*, 1987; Shoji *et al.*, 1987; Franco-Cereceda *et al.*, 1989, 1991; Coupe *et al.*, 1990; Yanagisawa *et al.*, 1988; Holman *et al.*, 1986; Marshall *et al.*, 1986). CGRP has little or no effect on human coronary resistance vessels when they are at rest or in non-ischaemic conditions but causes vasodilation when they are atheromatous or show irregular angiograms (Ludman *et al.*, 1993). However, vessels with areas of atheroma showed decreased numbers of CGRP binding sites compared to normal vessels (266 ± 10 grains/1000μm^2 tissue and 158 ± 35 grains/1000μm^2 tissue respectively) (Coupe *et al.*, 1990). In human epicardial coronary arteries, CGRP has been reported to produce a marked relaxant effect (Lundberg *et al.*, 1985; McEwan *et al.*, 1986). In the guinea-pig heart, ischaemic conditions produced an increase in coronary flow and an increase in CGRP levels in the coronary circulation (Franco-Cereceda *et al.*, 1989). In the coronary arteries, specific CGRP receptors have been reported, which are linked to adenylate cyclase activation (Shoji *et al.*, 1987; Sano *et al.*, 1989). The relaxant effect of CGRP on coronary vessels has been reported to occur via an increase in cAMP and by a decrease in intracellular calcium concentration, in a manner similar to isoprenaline (Edvinsson *et al.*, 1985; Kubota *et al.*, 1985; Kageyama *et al.*, 1993; Zschauer *et al.*, 1992). However, CGRP's relaxant effects in the rabbit ophthalmic artery have been shown to be produced by activation of ATP-sensitive potassium ion channels, an effect not seen in porcine coronary arteries (Nelson *et al.*, 1990; Kageyama *et al.*, 1993).

In the rat mesenteric artery, CGRP-like immunoreactivity has been demonstrated in varicose perivascular nerve fibres in the adventitia and adventitial medial border (Holzer *et al.*, 1988). CGRP has been reported to be the mediator of NANC neurone-induced vasodilation in the perfused rat mesenteric arterial bed (Han *et al.*, 1990, Kawasaki *et al.*, 1988). Pre-synaptic CGRP receptors regulate CGRP release by a negative feedback mechanism (Nuki *et al.*, 1994).

The CGRP-induced relaxant effects on the vasculature have been reported to be both endothelium-dependent and independent, depending upon species and blood vessel type (see table 1.3).

Species	Tissue	Endothelium-dependent	Endothelium-independent	Reference
Rat	Proximal coronary artery	√		Preito *et al.*, 1991
	Distal coronary artery		√	Preito *et al.*, 1991
	Abdominal aorta	√		Brain *et al.*, 1985 Grace *et al.*, 1987 Wang *et al.*, 1991a
	Thoracic aorta	√		Brain *et al.*, 1985 Grace *et al.*, 1987
Rabbit	Ophthalmic artery	√		Zschauer *et al.*, 1992
	Mesentery		√	Nelson *et al.*, 1992
Cat	Cerebral artery		√	Edvinsson *et al.*, 1985
Human	Cerebral artery		√	Marshall, 1992
Guinea-pig	Pulmonary artery		√	Maggi *et al.*, 1990

Table 1.3 *Summary of the role of the vascular endothelium in some of the relaxant effects of CGRP on the vasculature.*

The relaxant effects of CGRP, in the vasculature, appear to be mediated via a number of different mechanisms: (a) CGRP has been reported to activate ATP-sensitive potassium ion channels and cause hyperpolarisation of arterial smooth muscle (Nelson *et al.*, 1990; Zschauer *et al.*, 1992), (b) via an endothelium-dependent

mechanism involving activation of CGRP receptors on endothelial cells. Activation of such receptor induces nitric oxide synthase activation, producing nitric oxide and subsequently cGMP (Wang *et al.*, 1991; Gray & Marshall, 1992). This is unusual as nitric oxide synthase activation occurs via Ca^{2+}, not cAMP pathways, (c) Via a non-endothelium dependent mechanism, where the CGRP receptor is located on the smooth muscle itself. Activation of this receptor causes production of cAMP leading, via activation of protein kinase A, to the phosphorylation and subsequent inactivation of the myosin light chain kinase (Grace *et al.*, 1987).

The CGRP-induced elevations in cAMP and cGMP levels, in rat aorta, have been reported to be inhibited by nitric oxide inhibitors such as N_{ω}-nitro-L-arginine (L-NNA) (Hoa *et al.*, 1994; Fiscus *et al.*, 1991) suggesting that nitric oxide is responsible for CGRP-induced vascular relaxation. Some care may be needed in the interpretation of the actions of CGRP on the vasculature as reports exist which suggest that it may be capable of cross-reacting at AM receptors (see section 1.2.3).

1.3.2 CGRP in the gastrointestinal system

CGRP has been shown to have marked effects on gastrointestinal (GI) motor function and transit through the GI tract, following its administration directly into the systemic circulation or cerebrospinal fluid (Fargeas *et al.*, 1985). CGRP has also been reported to have a role in the inhibition of gastric emptying, primarily by actions upon the sympathoadrenal axis, causing release of noradrenaline which acts via β-receptors to inhibit gastric smooth muscle. The vagus nerve is also involved in the effects of CGRP on gastric emptying as subdiaphragmatic vagotomy reduces the response to CGRP (Tache *et al.*, 1984; Raybould, 1992). The CGRP-induced inhibition of gastric emptying seen in dogs, was antagonised by intravenous injection of $CGRP_{(8\text{-}37)}$ (0.5μg) implying that in this species, at least, CGRP acts at its own receptors to produce this effect (Plourde *et al.*, 1993). CGRP has also been shown to reduce

gastric acid output and promote enzyme secretions from isolated pancreatic acini (Seifert *et al.*, 1985; Tache *et al.*, 1984; Tache, 1992; Hughes *et al.*, 1984).

CGRP receptors have been identified in the GI tract by receptor autoradiography. They were located in the stomach myenteric plexus and external muscular coat, and in the ileal myenteric plexus (Gates *et al.*, 1989). CGRP binding sites have also been shown to exist in the hypothalamus and brainstem, areas involved in regulation of autonomic nervous system outflow to the GI tract (Rosenfeld *et al.*, 1983).

The effects of exogenous CGRP on smooth muscle function in the GI tract of the rat and guinea-pig have been reported to be exerted directly on smooth muscle cells in the stomach, small intestine and colon, via cAMP production (Katsoulis & Conlon, 1989; Chijiiwa *et al.*, 1992; Maton *et al.*, 1988) and indirectly via the activation of NANC neurones in the duodenum and oesophagus (Raybould, 1992). CGRP has been shown to produce relaxant effects in the rat duodenum (Maggi *et al.*, 1987; Palmer *et al.*, 1986) but contractile and relaxant effects in the guinea-pig ileum (Bartho *et al.*, 1987/1991; Mulholland *et al.*, 1990; Schworer *et al.*, 1991; Holzer *et al.*, 1989). In the guinea-pig small intestine, CGRP-like immunoreactivity is widely distributed in the myenteric and submucosal plexi, especially in the connective tissue and among the crypts of Leiberkühn (Furness *et al.*, 1985; Feher *et al.*, 1986). In the rat, CGRP containing neurones supply the myenteric plexus and circular muscle (Ekblad *et al.*, 1988).

In the guinea-pig ileum, the predominant action of CGRP is relaxant and the affect appears to occur on the longitudinal smooth muscle directly, however, contractile activity via acetylcholine (ACh) release from cholinergic nerves also has been reported (Bartho *et al.*, 1991). On the circular muscle, CGRP produced a contractile effect via ACh release from cholinergic neurones (Mulholland & Jaffer, 1990; Holzer *et al.*, 1989; Schworer *et al.*, 1991).

In the guinea-pig distal colon, CGRP has been located within the myenteric neurones projecting to the submucosal ganglia or the mucosa, and in nerve fibres within the mucosa and circular smooth muscle themselves (Ekblad *et al.*, 1988). CGRP has been

shown to stimulate chloride secretion directly by activation of these myenteric neurones (M^{c}Culloch & Cooke, 1989).

CGRP has been shown to be co-localised with other peptides within the rat colon. In the perivascular nerve fibres CGRP and substance P (SP) have been identified, others contain CGRP and vasoactive intestinal polypeptide (VIP) and in the submucous plexi CGRP positive cells contain somatostatin (SOM) (Ekblad *et al.*, 1988). It has been suggested that antisecretory effects of CGRP, seen at low concentrations, in rat colon are actually mediated by SOM (Cox *et al.*, 1989). These results highlight a species difference in the effects of CGRP on mucosal ion transport in the rat and guinea-pig colon.

1.3.3 The actions of CGRP on internal viscera

1.3.3.1 The kidney and ureter

Capsaicin-sensitive primary afferents in the rat and guinea-pig kidney and ureters, release CGRP and tachykinins to produce their effects (Maggi *et al.*, 1992a; Amann *et al.*, 1988). A dense plexus of CGRP-containing nerves have been located in the ureter (Su *et al.*, 1986) and depolarising stimuli promote CGRP release in this preparation (Santicioli *et al.*, 1988; Maggi *et al.*, 1992b). CGRP release produced inhibition of uretal smooth muscle activity, a mechanism which may be activated to prevent back-flow of urine or ascending infections of the urinary tract (Maggi *et al.*, 1992c). The inhibitory effects of CGRP on the guinea-pig ureter are reported to be mediated by specific CGRP receptors and the subsequent opening of glibenclamide-sensitive ATP-potassium channels (Maggi *et al.*, 1994; Santicioli & Maggi, 1994). Thiorphan (10μM), an inhibitor of endopeptidase 24:11, potentiated the inhibitory effects of CGRP on the guinea-pig ureter (Maggi & Giuliani, 1994).

In the kidney, CGRP has been shown to affect the glomerular filtration rate, renal blood flow and renin secretion (Kurtz *et al.*, 1988; Gnaedinger *et al.*, 1989; Dipette *et al.*, 1989; Castellucci *et al.*, 1993). In rat kidney membranes, CGRP has been reported to interact with calcitonin receptors and cause the accumulation of cAMP (Goltzman

& Mitchell, 1985). CGRP also induces cAMP accumulation in human kidney medulla and rat glomeruli (Edwards & Trizna, 1990; Geppetti *et al.*, 1989). CGRP binding sites have been identified in porcine medullary membranes, which are also coupled to adenylate cyclase (Aiyar *et al.*, 1991).

1.3.3.2 The lung

CGRP-like immunoreactivity has been found in sensory nerves in the lungs of rats, guinea-pigs and humans (Lundberg *et al.*, 1985; Cadieux *et al.*, 1986) and it has been previously suggested that CGRP may be involved in the regulation of airway function. Autoradiography has demonstrated CGRP binding to occur around airway smooth muscle and around epithelium and endothelium of large blood vessels in rat and human lungs (Carstairs, 1987; Bhogal *et al.*, 1993) and this distribution indicates that CGRP may exert local regulatory effects on airway and pulmonary smooth muscle. CGRP-specific binding sites have been found in the guinea-pig lung, which are of low and high affinity (Umeda & Arisawa, 1989). *In vitro*, CGRP has been shown to be a potent constrictor of human airway smooth muscle (Palmer *et al.*, 1987). The classification of CGRP binding sites in the lung is complicated by the presence of AM and amylin receptors which may be activated by CGRP (Owji *et al.*, 1995).

1.3.3.3 The liver and pancreas

In the rat liver, CGRP binding sites are suggested to be coupled to GTP-binding proteins and stimulate adenylate cyclase when activated. CGRP when bound to its receptors in the liver, appears to be protected from degradation (Stangl *et al.*, 1993; Yamaguchi *et al.*, 1988; Morishita *et al.*, 1990). In rat hepatocytes CGRP receptors have been identified which are also coupled to adenylate cyclase and stimulate cAMP accumulation when bound . These receptors have been suggested to function as high affinity amylin binding sites by involving a common protein capable of existing in a variety of affinity states (Houslay *et al.*, 1994).

In the pancreas, CGRP fibres originating from sensory neurones, are distributed in the endocrine and exocrine parenchyma. Binding sites for CGRP have been located in the exocrine parenchyma and CGRP interaction at these receptors stimulates amylase secretion via a cAMP-mediated pathway (Seifert *et al.*, 1985). In pancreatic islet cells, CGRP inhibited basal and stimulated insulin secretion in the mouse. In the rat, CGRP stimulated basal insulin and glucagon secretion but inhibited stimulated insulin and glucagon secretion. In the pig, CGRP inhibited basal insulin secretion but potentiated stimulated glucagon secretion (Petterson *et al.*, 1986; Ahren *et al.*, 1987). These results show that CGRP exerts species-specific effects on pancreatic islet cells.

1.3.4 CGRP and the immune response

Intradermal injection of CGRP (0.025-2.5pM) into humans, rats, hamsters and rabbits, produced an increase in cutaneous blood flow (Brain *et al.*, 1985). CGRP has been shown to be co-localised with SP in sensory C-fibres in many organs including the skin (Gibbins *et al.*, 1985). In the rabbit, CGRP released by electrical or chemical stimuli (capsaicin) (Holzer, 1991), has been reported to potentiate the effects of mediators of inflammation such as platelet activating factor (PAF), interleukin-1 (IL-1), SP, bradykinin and histamine and thus potentiate oedema formation and inflammatory cell accumulation (Brain & Williams, 1985; Buckley *et al.*, 1991a, 1991b). SP, acting via neurokinin-1 receptors on endothelial cells, can cause leakage of vascular plasma protein or activate mast cells to release histamine (Brain & Williams, 1989). CGRP has also been shown to potentiate neutrophil accumulation induced by IL-1 and C5a and to directly activate neutrophil granulocytes (Buckley *et al.*, 1991a & b; Richter *et al.*, 1992). Pre-treatment with CGRP, in hamster and human preparations, has been reported to have an anti-inflammatory effect (Raud *et al.*, 1991). In the rat, CGRP-induced effects are variable, with some studies showing CGRP to produce inhibition of oedema and others not (Raud *et al.*, 1991; Newbold & Brain, 1993; Escott & Brain, 1993). These results show CGRP to have species-

specific actions in its effects as a mediator of inflammation. CGRP binding has been demonstrated on mouse T-cells and on rat T and B-cells. Functional effects of CGRP on lymphocytes and monocytes include the ability to modulate responses to T-cell mitogens, inhibition of interferon-γ-induced hydrogen peroxide production and antigen presentation by mouse and human macrophages (McGillis *et al.*, 1991; Nong *et al.*, 1989). Monocyte macrophages have been shown to possess specific receptors for CGRP. CGRP has been shown to suppress proliferation and differentiation of macrophages into osteoclast-like cells, suggesting CGRP is important in bone metabolism (Owan & Ibaraki, 1994) an effect shared with CT and amylin.

1.3.5 CGRP in nervous tissue

1.3.5.1 The brain

Immunohistochemistry has shown CGRP to be located in neuronal structures of the rat, such as the spinal cord, trigeminal ganglion and spinal sensory ganglia (Rosenfeld *et al.*, 1983; Golzman & Mitchell, 1985; Kruger *et al.*, 1988). In humans, CGRP has been identified in the dorsal part of the spinal cord, with lower levels being found in the neocortex, cerebellum, pituitary and thyroid (Tschopp *et al.*, 1985). In the lower brainstem α- and β-CGRP-containing nerves have been located (Amara *et al.*, 1985). In sensory nerves, which contain both forms of CGRP, the concentration of α-CGRP is 3-6 times higher than that of β-CGRP (Mulderry *et al.*, 1988), CGRP has been found to be co-localised with other neurotransmitters in the brain. In the neurones of the parabrachial nucleus, CGRP and choleocystokinin (CCK) are found to co-exist (Inagaki *et al.*, 1984; Shimada *et al.*, 1985), in rat neurones and sensory ganglia, CGRP and SP are co-localised (Skofitsch & Jacobowitz, 1985a) and in the purkinje cells, CGRP and γ-amino butyric acid (GABA) co-exist (Kawai *et al.*, 1987). CGRP binding is widely distributed throughout the brain (Ishida-Yamamoto & Tohyama, 1989) and studies have reported the highest levels of CGRP binding in humans, to occur in the cerebellum, pituitary and thyroid (Chatterjee & Fischer, 1991; Henke *et*

al., 1987; Dotti-Sigrist *et al.*, 1988). CGRP binding has also been reported to be high in the nucleus accumbens and amygdala (Ishida-Yamamoto & Tohyama, 1989). Similarly, in the rat brain, high levels of CGRP binding have been demonstrated in the cerebellum and amygdala (Henke *et al.*, 1985; Inagaki *et al.*, 1986; Skotfitsch & Jacobowitz, 1985b). In the rat cerebellum, the CGRP binding sites have been reported to exist in multiple affinity states with 2 conformational forms of the receptor ternary complex state existing which allow the receptor to show low and high affinity binding (Chatterjee & Fischer, 1991) whilst still bound to a G-protein.

The localisation of immunoreactive CGRP-nerve fibres and CGRP binding sites is not always concomitant, for example, in the molecular layer of the cerebellar cortex, little immunoreactivity has been found but a dense population of binding sites have been identified. In the hypothalamus and thalamus, many CGRP-fibres have been identified but few CGRP binding sites (Ishida-Yamamoto & Tohyama, 1989). Intracerebroventricular (ICBV) injections of CGRP have been shown to decrease spontaneous motor activity and induce catalepsy in rats, effects related to dopaminergic neurones (Jolicoeur *et al.*, 1989). Along with CGRP binding sites found in the nucleus accumbens, these findings may suggest a modulatory role for CGRP on dopaminergic neurones innervating the basal ganglia. As the basal ganglia express 'normal' CGRP receptors as well as the C3 site (see section 1.2) it is not obvious which receptor CGRP interacts with in this tissue.

1.3.5.2 The peripheral nervous system

In peripheral tissues, CGRP has been shown to be widely distributed in the nervous and endocrine systems (Gibson *et al.*, 1984; Lee *et al.*, 1985; Tschopp *et al.*, 1985). CGRP has been shown to be co-localised with many other transmitters and neuropeptides. In the rat, CGRP co-exists with SP, galanin and VIP (Ju *et al.*, 1987). In cat dorsal root ganglia, CGRP is co-localised with SP, SOM and bombesin (Cameron *et al.*, 1988). Some pre-ganglionic nerves contain CGRP and corticotrophic

releasing factor (CRF) and enkephalin (Wanaka *et al.*, 1989; Matsyama *et al.*, 1987). CGRP is co-localised with ACh in the motor nucleus innervating the striated muscle (Takami *et al.*, 1985). Many CGRP neurones are preganglionic, both sympathetic and parasympathetic. Autonomic postganglionic neurones are influenced by both, sensory and preganglionic CGRP. Sympathetic ganglia generally lack CGRP but this seems to be species dependent. In the cat, CGRP cells in the paravertebral sympathetic ganglia are numerous but in the rat there are very few (Lindh *et al.*, 1988; Landis & Fredier, 1986).

The nicotinic ACh receptor mediates signal transduction at the post-synaptic membrane of the neuromuscular junction. This neurotransmitter-dependent ion channel is made up from 4 types of subunit; α,β,γ and δ. Binding of ACh to its receptor opens the ion channel, leading to depolarisation of the postsynaptic muscle cell (Stroud & Finer-Moore, 1985). CGRP has been shown to regulate ACh channel function via phosphorylation of the receptor in rat myotube cultures via cAMP-dependent kinase (Miles *et al.*, 1989). In cultured mouse muscle cells, CGRP has been shown to enhance the rate of ACh receptor desensitisation and decrease ion channel opening frequency (Mulle *et al.*, 1988). In contrast, a study by Eusebi *et al* (1988), showed that increased cAMP levels by CGRP regulates ACh channel conductance but did not accelerate ACh receptor desensitisation.

1.3.6 Actions of CGRP on cultured cell lines

CGRP receptors have been characterised in a number of immortalised cell lines: L6 skeletal myocytes, HCA-7 and Col-29 human GI epithelia, RINm5F, SK-N-MC human neuroblastoma and WE-68 Ewing's sarcoma cells.

On L6 myocytes, CGRP has been shown to possess high affinity specific receptors, linked to adenylate cyclase (K_d for rat-α-CGRP = 1nM). CT did not compete for the receptors on these cells thereby implying that the receptors are CGRP-specific (Kreutter *et al.*, 1989; Poyner *et al.*, 1992). CGRP receptors have been characterised

in HCA-7 and Col-29 cells where human-α-CGRP and human-β-CGRP produced prolonged increases in short circuit current (SCC). The human-α-CGRP-induced responses on the HCA-7 cells were antagonised by $CGRP_{(8-37)}$ (1μM) but not on the Col-29 cells, implying that the former cell line has $CGRP_1$ receptors and the latter $CGRP_2$ receptors (Cox & Tough, 1994). In RINm5F cells, CGRP and amylin have been shown to stimulate and inhibit insulin secretion in a concentration-dependent manner. At high concentrations (10nM-1μM) insulin secretion was stimulated probably via CGRP-induced adenylate cyclase activation but at lower concentrations (0.1nM-1nM) inhibition of insulin secretion was observed (Barakat *et al.*, 1994). Specific CGRP receptors have been identified on SK-N-MC and WE-68 cells. These receptors have been shown to be functionally coupled to cAMP accumulation (Van Valen *et al.*, 1989, 1990).

1.4 General physiology of amylin

Amylin evokes biological effects in a number of tissues including skeletal muscle, liver, pancreas, the cardiovascular system and the central nervous system (Morley & Flood, 1991; Chance *et al.*, 1991; Brain *et al.*, 1990).

Amylin has been shown to inhibit glucose-stimulated insulin secretion in rat pancreatic islets, an effect antagonised by $CGRP_{(8-37)}$ (10μM) (Ohsawa *et al.*, 1989; Wang *et al.*, 1991b,1993). Amylin also inhibited basal and submaximal insulin-stimulated glucose uptake in a manner similar to CGRP (Leighton & Cooper, 1988). CGRP and amylin produced insulin resistance in skeletal muscle and amylin also caused increased hepatic glucose output in the rat (Molina *et al.*, 1990). Amylin has been suggested to be involved in lactate mobilisation in response to glucose (Young *et al.*, 1994). Selective amylin receptors have been reported on rat skeletal muscle, which upon stimulation cause the inhibition of glycogen synthesis (Beaumont *et al.*, 1995). In skeletal muscle, amylin stimulated glycogen phosphorylase and inactivated

glycogen synthase in a cAMP-dependent manner and thereby stimulate glycogenolysis (Young *et al.*, 1991; Cooper *et al.*, 1988; Leighton & Foot, 1989). The metabolic actions of amylin in the liver could be regarded as promotion of the 'return arm' of the Cori cycle, as muscle glycogen provides a substrate for hepatic gluconeogenesis, glycogen production and fat synthesis (Rink *et al.*, 1993). Insulin has the opposing effect causing uptake of glucose into muscle and production of muscle glycogen.

Amylin has been shown to have hypocalcaemic effects in rats and rabbits, producing concentration-dependent reductions in bone resorption by osteoclasts, without altering osteoclast cell number or viability (Datta *et al.*, 1989; Zaidi *et al.*, 1987). In the cardiovascular system, amylin amide (10nM) produced vasodilation and systemic hypotension in rats and rabbits (Brain *et al.*, 1990; Gardiner *et al.*, 1991). As previously explained in section 1.2.2, amylin and CGRP have common binding sites, as well as possessing their own distinct receptors, consequently amylin and CGRP share some common actions. The main actions in peripheral tissues of bronchial dilation and decreased intestinal motility appear to be mediated via cAMP. When injected into the brain, amylin (6.4 nmol/kg) can also suppress food intake (Morley & Flood, 1991; Chance *et al.*, 1991; Krahn *et al.*, 1984).

1.5 Aims

At the present time the classification of CGRP receptors is complicated by reports of cross-reactivity of CGRP with receptors of other structurally related peptides such as amylin, calcitonin and adrenomedullin. The current classification of CGRP receptors is based largely on results using two animal species, the rat and guinea-pig and one receptor antagonist, $CGRP_{(8-37)}$. The literature regarding CGRP receptor classification shows that inter-species variations between receptors have been observed and that the specificity of action of $CGRP_{(8-37)}$ is under debate.

The initial aims of this project were to examine CGRP receptor classification and characterisation in one species using as wide a variety of CGRP receptor antagonists as is currently available. Receptor classification was investigated using radioligand binding studies on rat tissues which have previously been shown to possess CGRP receptor populations. The use of monoclonal antibodies raised against CGRP would provide an insight into the specific areas of the CGRP molecule necessary for successful ligand: receptor interactions in these tissues. The use of the endogenous ligand and its analogues would provide information regarding the affinities of such receptor agonists to be used in the design of subsequent *in vitro* functional assays. Due to technical difficulties arising during the course of these experiments, it was decided to examine CGRP receptor classification and the possible cross-reactivity of CGRP and amylin using a second animal species, which was not as well documented, the guinea-pig.

Tissue preparations from the guinea-pig were examined for their responsiveness to CGRP and amylin. The ileum, vas deferens and atria were shown to produce consistent responses to these agonists and were used to examine the effects of a number of CGRP and amylin fragments reported as having antagonistic properties in these and other tissue preparations. These CGRP and amylin fragments were also used to examine the amino acid residues necessary for successful ligand: receptor interactions and verify their antagonistic activity as reported in other studies. The previous identification of multiple receptors for CGRP raises the question as to whether the various forms of endogenous CGRP may have preferential affinities for the receptor subtypes. The comparison of data obtained for the three guinea-pig tissue preparations should indicate whether the CGRP receptors present in different organs of the same animal species have different pharmacology and whether or not the structurally related peptide amylin is capable of cross-reacting at these receptors.

Chapter 2

Methods

2.1 Radioligand Binding assay

2.1.1 Tissue preparation

Methods for tissue preparation were as previously described by Poyner *et al*.., (1992). Male Wistar rats obtained from Charles River, U.K., (150-250g), were sacrificed by a blow to the head and subsequent exsanguination. The spleen, liver and cerebellum were removed and placed on ice (approximately 4°C) to prevent tissue degradation. Wet tissue was weighed and homogenised (Fluid equipment (Adelphi) Ltd, London) in 20ml of ice cold 'EDTA-buffer' (4°C) of composition: 1mM EDTA, 20mM Tris in distilled water, pH 7.5. The tissue suspension was centrifuged at 19, 000g for 30 mins at 4° C (Beckman J2-21).

The supernatant was discarded and the tissue pellet re-suspended in 10ml ice cold 'BSA-buffer' of composition: 20mM Hepes, 1mM $MgCl_2$, 0.3% w/v BSA in distilled water, pH 7.4. This suspension was re-homogenised ensuring an even membrane distribution. The suspension was filtered using Propax open wove bandage BP type 1 (Smith & Nephew Medical Ltd, Hull) to remove any tissue clumps, and diluted to give a final tissue concentration of 1g tissue / 100ml BSA buffer.

2.1.2 Binding assays

5μl of 1nM [^{125}I]-α-human CGRP, (2000Ci/mmol) (Amersham, U.K) was added to microcentrifuge tubes to give a final concentration of 10pM (30,000cpm / tube) (previously estimated to produce 6717cpm total binding /mg tissue of which 5414 were specific) following the addition of 0.5ml of tissue suspension to each tube to start the incubation. Aliquots of unlabelled rat-alpha CGRP (0.1μM) ('cold ligand') were added to those tubes where an estimate of non-specific binding was required, as most reported Kd values for CGRP are not more than 1nM this concentration of unlabelled CGRP will effectively occupy 100/101% of the total receptor population (Poyner, 1992) All tubes contained 0.1mM, final concentration, of the protease inhibitors bacitracin and phenylmethylsulphonyl fluoride to reduce enzymatic breakdown of CGRP.

Incubations were terminated by centrifugation at 10,000g, at 25°C (Hermle Z229 microcentrifuge) after the reaction was deemed to have reached equilibrium where an equal number of associated and dissociated ligand-receptor complexes existed (experimentally determined to occur after 30 mins, see section 2.2.1.1). Total binding was determined by gamma counting at 25°C (LKB Wallac 1282-001).

Specific binding was estimated by subtraction of non-specific binding, defined using 0.1μM rat-α-CGRP, from total binding. The concentration of rat-α-CGRP used to estimate non-specific binding was derived from [^{125}I]-α-human CGRP displacement experiments using increasing concentrations of unlabelled ligand (data not shown).

2.1.2.1 Binding Kinetics

The association and dissociation of [^{125}I]-α-human CGRP with its receptor (termed "on" and "off" rates respectively) were experimentally determined. On rates were ascertained by measurement of specific binding at selected time points (between 0-330min), until steady state binding was deemed to have been achieved (see chapter 3, section 3.4.1). Off rates were determined by measurement of specific binding at selected time points (between 0-330min) following the initial addition of 0.1μM unlabelled rat-α-CGRP.

2.1.2.2 Competition studies

Receptor binding competition studies were executed using a variety of unlabelled CGRP analogues and fragments to contest [^{125}I]-α-human CGRP binding in rat tissue preparations. Increasing concentrations of unlabelled ligand were left in contact with the rat membranes for 15 mins; [^{125}I]-α-human CGRP was subsequently added and the reaction incubated at room temperature for a further 15 mins. Specific binding of [^{125}I]-α-human CGRP in the presence of the CGRP analogues and fragments was then determined as described in section 2.1.2.

2.1.2.3. Monoclonal antibody binding studies

Monoclonal antibody (Mabs) raised against CGRP were utilised in an attempt to investigate the structure-activity relationship of CGRP with its receptor in rat tissue preparations.

10µl of the antibody (0.1nM, from Shaw *et al.*, 1992) and 10pM [^{125}I]-α-human CGRP were pre-incubated for 60 mins, at 25°C, to ensure steady-state formation of [^{125}I]-α-human CGRP-antibody complexes. This mixture was incubated with the rat tissue preparations for 5 or 30 mins (see chapter 3, section 3.4.3.1). The reaction was terminated by centrifugation and the membranes counted to determine bound radiolabel by gamma counting.

Vehicle controls for the Mabs, using 10µl Dulbecco's modified Eagles medium (DMEM) were shown not to affect Mab binding ability.

2.1.3 Definition of control responses

Controls were defined, for each experiment, as the mean of 3 separate determinations of the total binding of [^{125}I]-α-human CGRP to rat tissue preparations. This mean value was taken as 100% binding and results were normalised to this.

2.1.4 General precautions

Large peptides, such as CGRP, are known to adhere to plastic surfaces making exact determination of the amount of peptide in solution difficult. To overcome this and thus prevent losses in handling, all plasticware was coated in either dimethylsilane (BDH) or Sigmacoat (Sigma). Occasionally frozen tissue was used in the radioligand binding experiments as fresh tissue was not always available. Tissue was frozen following filtration (Section 2.1.1) at -20°C for upto 3 months. Preliminary studies on this frozen tissue showed that binding was not affected by the freezing process thus confirming receptor/tissue viability.

2.2 Isolated tissue preparations

Male, Duncan Hartley guinea-pigs (Charles River, U.K), 300-400g, were killed by cervical dislocation and subsequent exsanguination. The ileum (10cm), (defined as the region 10cm from the base of the small intestine), vas deferens and both atria were removed. All tissues were cleaned of excess fat and placed in Krebs buffer solution, gassed with 95% oxygen / 5% carbon dioxide made up in distilled water, of composition (mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $NaHCO_3$, 13.4; $MgSO_4.7H_2O$, 1.2; D-Glucose, 11.6.

The ileum (cut into 2cm lengths), vas deferens, right and left atria were mounted vertically, by loops of cotton thread, to an isometric force transducer (Dynamometer UF1). The lower end of the tissues being attached directly, by tissue puncture to a tissue hook (right atrium) or the lower hook of an electrical field stimulation electrode (ileum, vas deferens and left atrium) as described by Schworer *et al.*, 1991; Ellis & Burnstock, 1989; Sigrist *et al.*, 1986. A tension of 1g was placed on each tissue and the preparations were left to equilibrate for 60 mins, during which time the Krebs buffer was changed every 10-15 mins. The tissues were maintained in a 10ml jacketed organ bath in Krebs buffer at 37°C (at 34°C for ileal preparations to decrease spontaneous activity) and gassed with 95% oxygen / 5% carbon dioxide. Drugs were added as a bolus injection (1% volume of organ bath fluid volume) to the buffer in the organ bath using a Gilson pipette. The force transducer was connected to a flatbed chart recorder (Goerz SE 120) via an amplifier (Lectromed) calibrated so 1 gramme = 1cm.

The ileum preparations and left atria were electrically field stimulated using Scientific Research Instruments (SRI) stimulators (England) and the vas deferens using a Grass S48 stimulator (Quincy, Mass. USA). Electrical field stimulation was applied by paired platinum electrodes (in-house construction) placed parallel to the tissues.

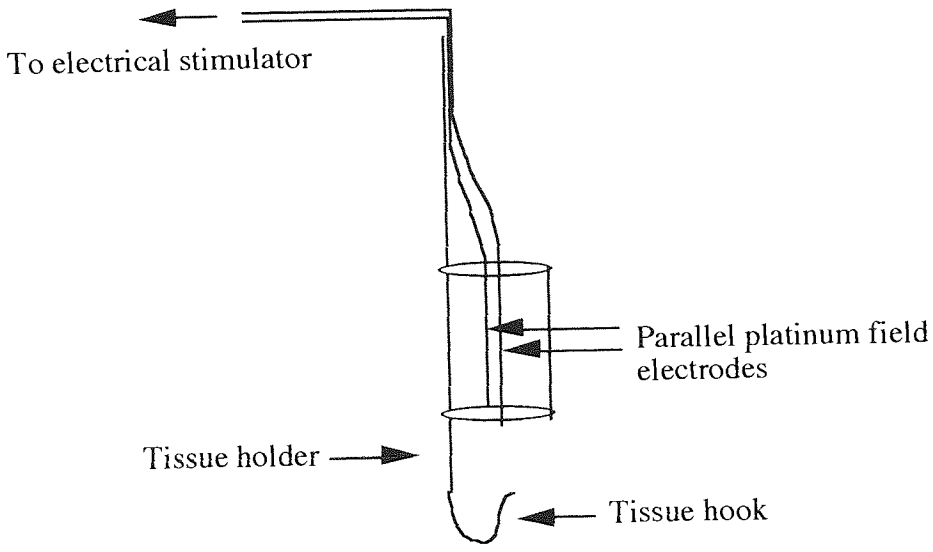


Diagram 2.1 *Representation of the tissue holder and field stimulators used with the guinea-pig ileal, right atrial and vas deferens preparations. Tissues were held between the field electrodes using cotton loops to the tissue hook (ileum and vas deferens) or by attachment to the tissue hook directly (left atrium).Cotton loops attached the tissues to a force transducer positioned above.*

The ileum was stimulated using parameters of 60 volts amplitude (shown to be supramaximal) for 1ms at a frequency of 0.15Hz (adapted from Schworer *et al.*, 1991). The left atrium was stimulated at 2Hz for 6ms at 80 volts amplitude (supramaximal) (adapted from Sigrist *et al.*, 1986). The vas deferens were stimulated with trains of rectangular pulses at 25 second intervals (0.04Hz). Each train was of 1s duration and consisted of 20 pulses of 0.5ms pulse width and 70 volts amplitude (supramaximal) (adapted from Ellis & Burnstock, 1989). The tissues were stimulated using the parameters detailed above, whilst cumulative concentration-response curves were constructed (see figures 4.1, 5.1 & 6.1 for examples of representative traces), subsequent to which the stimuli were removed and the tissues repeatedly washed at 10 minute intervals for 30-45 mins.

All results are expressed as % control, where control values are taken to be the twitch amplitude prior to the addition of test substance. All peptides were kept on ice

throughout experimental procedures to minimise enzymatic degradation and the effects of test substances upon the twitch contraction were investigated only when the contraction amplitude was constant. Effects of test substances, assessed within the 5 mins following their application, were used for calculation of results. Maximal responses were not always achieved with the concentrations of CGRP and amylin available.

2.3 Drugs.

All types of CGRP and its analogues; human-α-CGRP, rat-α-CGRP, human-β-CGRP, Tyr°-CGRP$_{(28-37)}$, Tyr°-CGRP, human CGRP$_{(8-37)}$, [Cys(ACM)$_{2,7}$]-human CGRP, rat amylin, human amylin and human amylin$_{(8-37)}$ were obtained from Sigma chemicals, Calbiochem or Peninsula laboratories and were dissolved in distilled water prior to further dilution using BSA buffer (see section 2.1.1). AC187 and CGRP$_{(19-37)}$ were synthesised in-house using a peptide synthesiser (Applied Biosystems, U.K) by D.R Poyner. Peptide purity was subsequently confirmed by high performance liquid chromatography (HPLC). [^{125}I]-α-human CGRP was obtained from Amersham International (Amersham, UK) and made up in BSA buffer. All CGRP solutions were stored as frozen aliquots and following 2-3 freeze-thaw cycles, were discarded.

The monoclonal antibodies were a gift from Derek Brown at Celltech, Slough.

All other chemicals used were from Sigma laboratories and were of reagent grade.

Abbreviations

EDTA, ethylenediaminetetraacetic acid; Tris, trizma base (reagent grade); HEPES, N-[2-Hydroxyethyl] piperazine-N-[2-ethanesulphonic acid]; BSA, Bovine serum albumin; AC187 (acetyl-Asn^{30}, Tyr^{32}]-salmon calcitonin$_{(8-32)}$; [Cys (ACM)$_{2,7}$]-human CGRP, acetoamidomethylcysteine$_{2,7}$-CGRP ; NANC, non-adrenergic, non-cholinergic; Mab, monoclonal antibody; DMEM, Dulbecco's modified Eagle's medium; rat CGRP, rat-α-CGRP; amylin$_{(8-37)}$, human amylin$_{(8-37)}$.

2.4 Analysis of results

2.4.1 Curve fitting

Concentration-response curves were fitted to a logistic equation incorporating Hill coefficients (1) using the fitting routine EBDA/LIGAND (Munson & Rodbard, 1980)

(1)

$$y = \frac{Rmax.\,[D]^n}{[D]^n + EC_{50}}$$

y = response, Rmax is the maximal response, [D] the drug concentration, ^{n} the Hill coefficient and EC_{50}, the concentration of drug required to produce 50% of the maximal response. A similar equation was used to analyse binding curves, to produce estimates of IC_{50} and non-specific binding.

2.4.2 Statistical analysis

For the Mab studies, analysis of results was by Student's t test for paired observations accepting significance at $P \leq 0.05$.

For all other studies, EC_{50} values and IC_{50} values from 'treatment' groups were compared against control using a one-way ANOVA followed by a Dunnetts test. The control was usually EC_{50} value of agonist alone and the EC_{50} values of the 'treatment' groups were agonist in the presence of antagonist. Further details can be found in the appropriate sections of chapters 4, 5 and 6.

Dunnett's test allows multiple comparisons of a common control against different experimental groups (as described in Roscoe, 1969). Briefly, the test statistic, Dunnett's t, is calculated from equation (2) and compared against the tabulated value, with significance being accepted at $P \leq 0.05$.

(2)

$$t = \frac{M1 - M2}{\sqrt{Msr\,(1/n1 + 1/n2)}} \qquad \text{degrees of freedom N-K}$$

Where M1 = control value, M2 = treatment value, n1, n2 = number of observations in control and treatment groups, Msr = residual mean squares (calculated from

ANOVA), N = total number of observations in all groups and K = number of experimental groups (Roscoe, 1969).

Estimated pA_2 values were calculated from means of dose-ratios using the equation

$$pA_2 = \log \frac{(\text{dose ratio}-1)}{[\text{antagonist}]}$$

Kd values were not calculated from the IC_{50} values obtained from the binding experiments as receptor occupancy was so low and $IC_{50} \approx$ Kd.

Chapter 3

CGRP receptor binding in rat spleen, liver and cerebellum.

3.1 Introduction

3.1.1 CGRP receptor binding

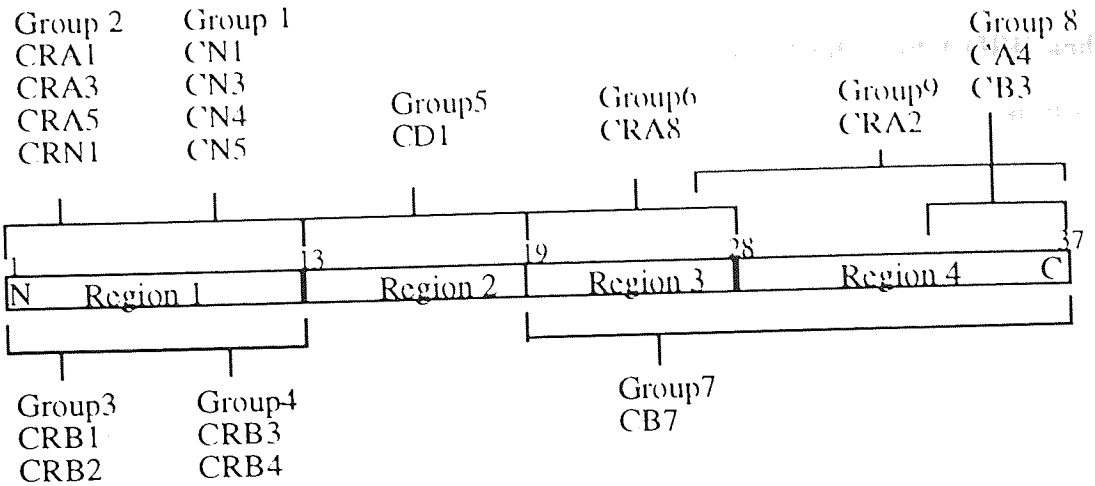
Specific high density binding sites for CGRP have been identified throughout the central nervous system, including the cerebellum and spinal cord, and in peripheral organs such as the liver, spleen and kidney (Tschopp *et al.*, 1985; Stangl *et al.*, 1993). In the rat brain, CGRP receptors have been reported to belong to a family of G-protein coupled receptors and more specifically, in the rat cerebellum the population of CGRP receptors have been shown to be homogeneous (Stangl *et al.*, 1991). In the rat, CGRP receptors found in the spleen, liver and pancreas, appear from some binding studies to belong to a single class of high affinity sites, while other studies have produced evidence to suggest that the CGRP receptors in these tissues are not of the same type (Van Rossum *et al.,* 1992; Yamaguchi *et al.*, 1988; Seifert *et al.*, 1985; Sigrist *et al.*, 1986; Stangl *et al.*, 1993). Differences in the Kd values obtained for CGRP, by different groups using the same tissue preparations (Yamaguchi *et al.*, 1988; Stangl *et al.*, 1993) makes the interpretation of binding affinities, and hence receptor heterogeneity, difficult. Results obtained from binding studies have not always correlated with results obtained with functional assays on the same tissues i.e. functional studies have highlighted tissue receptor heterogeneity giving pA_2 values of 7.6 and 6.77 for $CGRP_{(8\text{-}37)}$ on guinea-pig right atrium and vas deferens, which have not been reflected in binding assays producing pK_D values of 9.0 and 9.3 respectively (Stangl *et al.*, 1993; Chiba *et al.*, 1989; Dennis *et al.*, 1990). Further difficulties arise with receptor identification due to the co-localisation of CGRP with other structurally similar peptides e.g. in the rat brain, where CGRP is co-localised with salmon calcitonin (sCT)-like peptides, making the identification of the endogenous ligand more difficult. In the rat brain, the C3 binding site which recognises sCT and CGRP with high affinity, has been suggested to be an amylin receptor (Beaumont *et al.*, 1995).

The CGRP receptor has been reported to be G-protein coupled (Yamaguchi *et al.*,

1988; Van Rossum *et al.*, 1993) and the affinity of CGRP for its receptor has been shown to be dependent on the state of the receptor-G-protein coupling, which has itself been shown to exist in multiple agonist affinity states (Chatterjee & Fischer, 1991; Van Rossum *et al.*, 1993; Poyner *et al.*, 1992). CGRP is also likely to produce a high level of non-specific binding due to hydrophobic regions along the length of the peptide.

3.1.2 CGRP: antibody binding

An important extension to the number of analogues available for studying CGRP-receptor interactions was provided by Andrew *et al.*, (1990) with the development of a panel of 18 monoclonal antibodies (Mabs) raised against human CGRP. These antibodies form stable complexes with CGRP, effectively creating new CGRP derivatives. 7 of the antibodies were found to be specific for α-CGRP, 5 specific for β-CGRP and the remainder interacting with both. The monoclonal antibodies are able to define 9 different epitopes on the human CGRP molecule with each group recognising a different section of the peptide. These monoclonal antibodies thus provide a potential mechanism for identifying the sections of the human CGRP molecule important in receptor binding.



Group 1, 2; human -α-CGRP specific
Group 3, 4, 7; human -β-CGRP specific

Diagram 3.1 *Epitope mapping of human CGRP. Diagram of the epitopes of human CGRP identified by a panel of monoclonal antibodies (Mabs) (adapted from Andrew, et al., 1990). Region 1 corresponding to amino acid residues 1-13, region 2 corresponding to residues 13-19, region 3 corresponding to residues 19-28 and region 4 corresponding to residues 28-37.*

In guinea-pig coronary artery rings these Mabs indicated that the active site of the CGRP molecule was located in the N-terminal region (Shaw *et al.*, 1992). This group suggested that the C-terminal and mid-portion regions might be involved in the structural conformation which the peptide must adopt for effective receptor binding. This proposal is concurrent with other information regarding the structure-activity relationship of CGRP and its receptor (see chapter 1, section 1.2.1.2).

3.2 Aims and objectives

This study was designed to investigate the binding characteristics of CGRP in rat tissues previously reported to contain high affinity CGRP binding sites; liver, spleen and cerebellum. Initially the objectives were to verify previous reports of the presence of CGRP receptors in the rat tissue preparations and to further examine such receptors using the Mabs. CGRP, its analogues and the CGRP fragment 8-37 were used to

examine ligand: receptor binding affinities for use in subsequent functional assays. If the three tissues showed differences in the binding affinities of CGRP and its analogues the information could be used subsequently for characterisation of other tissue receptors. A panel of 15 Mabs were used to examine the structure-activity relationship of CGRP with the receptors present in these tissue preparations. The results obtained from the Mab studies may provide information about the amino acid residues responsible for high affinity CGRP binding at the receptors present in the rat tissue preparations.

3.3 Methods and materials

The experimental protocol is described in chapter 2, section 2.1. The association and dissociation measurements began at time zero. This was experimentally achieved by termination of the experiment by centrifugation immediately following the addition of [^{125}I]-α-human CGRP for association and rat-α-CGRP for dissociation to the tissue preparation. Controls were defined using 10pM [^{125}I]-α-human CGRP incubated with the tissue suspension alone. Non-specific binding was defined with 0.1μM rat-α-CGRP. Statistical comparisons between the effects of Mab and its specific control used a paired Student's t-test ($P \leq 0.05$).

3.4 Results

3.4.1 Binding kinetics

The pattern of association and dissociation of [^{125}I]-α-human CGRP in rat liver, splenic and cerebellar membranes was examined, in order to establish a protocol for use in further binding studies.

The association rates for [^{125}I]-α-human CGRP in all 3 tissue preparations was shown to be rapid, with approximately 70% of binding occurring within 10 min and maximal binding occurring within 30 min (figure 3.1 for binding to liver membranes). After this time binding became unstable and began to deteriorate, probably due to

proteolysis of the receptor or ligand. The $t_{1/2}$ for association ($t_{1/2}$ ass., time to reach 50% of maximal binding) for liver and cerebellar membranes was estimated to be 1-2 min and 6 min for splenic membranes.

The dissociation rates for [^{125}I]-α-human CGRP in the 3 tissue preparations was shown to be gradual with $t_{1/2}$ for dissociation ± s.d (defined as $t_{1/2}$ dis.) of 20 min ± 8.7 for liver, 20 min ± 11.5 for cerebellar membranes (figure 3.1) and 50 min ± 24.66 for the splenic membranes.

Overall these results indicated that steady-state binding could be achieved after approximately 30 min incubation and this time point was therefore utilised in subsequent binding experiments.

Detailed kinetic analysis of equilibrium binding could not be accurately accomplished as the CGRP-receptor interaction was too rapid to measure reliably in a microcentrifuge binding assay where separation of bound ligand from free was achieved by centrifugation for 5 min (see chapter 2, section 2.1.2).

3.4.2 Binding inhibition by CGRP analogues

The binding affinities of a number of the currently available CGRP analogues and receptor antagonists were examined in the rat spleen, cerebellum and liver. The abilities of rat-α-CGRP, human-α-CGRP, [Cys (ACM)$_{2,7}$]-human CGRP, Tyr°-CGRP and CGRP$_{(8\text{-}37)}$ to displace [^{125}I]-α-human CGRP from these membrane preparations were investigated. IC_{50} values ± s.e.m were obtained for all the binding curves produced and these values were compared in order to highlight tissue CGRP receptor differences.

Rat-α-CGRP inhibited [^{125}I]-α-human CGRP binding in all 3 membrane preparations in a concentration-dependent manner (see figure 3.2 for binding in cerebellar membranes). No significant differences were found between the IC_{50} values for each tissue. Human-α-CGRP was subsequently tested to see if the 2 types of CGRP, which

seen in table 3.1 and figure 3.3, human-α-CGRP also inhibited [^{125}I]-α-human CGRP binding in a concentration-dependent manner, with no differences in the affinity of human-α-CGRP evident. There were no significant differences between the affinities of rat-α-CGRP and human-α-CGRP on any of the tissues.

The human-α-CGRP analogues [Cys (ACM)$_{2,7}$]-α-human CGRP and Tyr^{0}-CGRP also inhibited radioligand binding in all of the tissue preparations in a concentration-dependent manner with no significant differences between IC_{50} values obtained (figures 3.4 & 3.5 for examples of displacement curves).

The CGRP receptor antagonist CGRP$_{(8\text{-}37)}$ was the final fragment tested and like all of the other peptides, produced concentration-dependent inhibition of radioligand binding (see figure 3.6).

	IC_{50} values ± s.e.m (nM)		
	Liver	**Spleen**	**Cerebellum**
Rat-α-CGRP	3.3 ± 1.7 (4)	2.6 ± 1.7 (3)	0.8 ± 0.5 (4)
Human-α-CGRP	5.3 ± 5.2 (4)	5.9 ± 5.6 (4)	19.0 ± 18.0 (3)
[Cys(ACM)$_{2,7}$]-CGRP	7.4 ± 2.7 (3)	6.9 ± 4.2 (3)	17.0 ± 13.0 (4)
Tyr^{0}-CGRP	9.0 ± 4.0 (4)	2.4 ± 1.9 (3)	10.0 ± 7.6 (3)
CGRP$_{(8\text{-}37)}$	8.2 ± 4.0 (3)	1.0 ± 0.8 (3)	4.9 ± 1.9 (3)

Table 3.1 *Inhibition of [^{125}I]-α-human CGRP binding in rat liver, splenic and cerebellar membrane preparations by CGRP, CGRP analogues and CGRP fragments. IC_{50} values representing the concentration of unlabelled peptide displacing 50% of bound radiolabel are shown ± s.e.mean. Statistical analysis of results was by Student's t test for unpaired observation ($P \leq 0.05$). n values are denoted in brackets.*

Initial observations of the IC_{50} values obtained for the cerebellum show an approximately 24-fold difference between rat-α-CGRP and human-α-CGRP Other differences exist in this tissue, especially between the affinities of human-α-CGRP and [Cys $(ACM)_{2,7}$]-α-human CGRP but the large errors associated with these values precludes conclusive interpretation. Further work would be needed to obtain conclusive evidence to support these initial observations.

3.4.3 Binding inhibition by monoclonal antibodies (Mabs)

As mentioned in section 3.1.2, the panel of monoclonal antibodies raised against CGRP, when complexed with [^{125}I]-α-human CGRP, effectively provide a number of novel CGRP analogues available for investigation into CGRP binding characteristics and receptor classification.

3.4.3.1 Derivation of experimental protocol

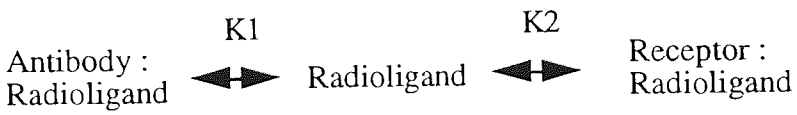


Diagram 3.2 *Representation of antibody: radioligand: receptor binding equilibrium where K1= association constant of antibody: radioligand complex & K2 = association constant of radioligand: receptor complex. When K2 > K1, radioligand receptor binding will be observed.*

The antibody: radioligand complex, upon addition to the tissue preparations will establish the equilibrium represented in diagram 3.2. As the affinity of the radioligand for the antibody is unknown, the use of two incubation periods (5 and 30 mins) in these experiments should help to show any possible inhibition of radioligand binding by the antibody.

If the radioligand has a higher affinity for the receptor than for the antibody then K2 > K1 and receptor:radioligand binding will be observed. However if the radioligand has

a higher affinity for the antibody than the receptor K1 > K2 and binding will be prevented.

If the radioligand has a higher affinity for the receptor than for the antibody but the dissociation of the pre-formed radioligand: antibody complex is slow, inhibition of binding might be seen using a 5 min incubation period but not using a 30 min incubation period.

If K1 > K2 and a stable radioligand: antibody complex is formed inhibition of radioligand binding will occur which will be seen using both the 30 min and 5 min incubation periods.

3.4.3.2 Results

The experiments using the 5 min incubation period showed that some of the Mabs formed low affinity complexes with [^{125}I]-α-human CGRP which produced inhibition of binding. Possible dissociation of the radioligand: antibody complex within 30 mins meant that significant inhibition of binding by these Mabs was only observed using a 5 min incubation (table 3.2). Although these results should be treated with caution, each of the 3 membrane preparations; liver, spleen and cerebellum, showed that small differences between CGRP receptor binding characteristics could be observed using this short incubation period. In the liver, CRB1, CRa5 and Ca4 inhibited binding. These Mabs correspond to epitopes 1 and 4, amino acids 1-13 and 32-37. In the spleen CB3 inhibited binding, a Mab corresponding to epitope 4, amino acids 32-37. In the cerebellum, CRB3, CRA8 and Ca4 produced inhibition of binding, these Mabs correspond to epitopes 1, 2 and 4, amino acids 1-13, 19-28 and 32-37.

		Liver		Spleen		Cerebellum	
	Epitope	5 minute	30 minute	5 minute	30 minute	5 minute	30 minute
CRB1	1	+	-	-	-	-	-
CRB3	1	-	-	-	-	+	-
CRB4	1	-	-	-	-	-	-
Cn1	1	-	+	-	+	+	+
Cn3	1	-	-	-	+	-	-
Cn4	1	-	-	-	-	-	-
Cn5	1	-	-	-	-	-	-
CB3	4	-	+	+	-	-	+
CB7	3 / 4	-	-	-	-	-	-
CRa2	4	-	-	+	+	-	+
CRa3	1	-	-	-	-	-	-
CRa5	1	+	-	-	+	+	+
CRa8	3	-	-	-	-	+	-
Ca4	4	+	-	+	+	+	-
CD1	2	+	+	+	+	+	+

Table 3.2 *Effects of the panel of monoclonal antibodies on specific [^{125}I]-α-human CGRP binding in rat liver, splenic and cerebellar membranes. + represents significant inhibition of radioligand binding compared to control. - indicates no significant inhibition of binding. Statistical analysis was by paired Student's t-test ($P \leq 0.05$). Controls were taken as binding of 10pM [^{125}I]-α-CGRP to membranes alone. Epitope 1 represents amino acid residues of CGRP, 1-13; 2 = amino acids 13-19; 3 = amino acids 19-28 and 4 = amino acids 28-37.*

The experiments using the 30 min incubation period highlighted some consistencies between the areas of the CGRP molecule required for receptor binding in the rat liver, spleen and cerebellum. In the liver, Cn1, CB3 and CD1 inhibited radioligand binding. In the spleen binding inhibition was seen with Cn1, Cn3, CRa2, CRa5, Ca4 and CD1, whereas in the cerebellum, Cn1, CB3, CRa2, CRa5 and CD1 caused inhibition of binding. In all of the tissues the Mabs causing significant inhibition of [^{125}I]-α-

human CGRP binding corresponded to epitopes 1, 2 and 4 (diagram 3.1) and amino acids 1-37.

		Liver	Spleen	Cerebellum
Amino acids	**Epitope**			
13-19	2	CD1	CD1	CD1
1-13	1	Cn1	Cn1	Cn1
27-37	4		CRa2	CRa2
1-13	1		CRa5	CRa5
19-37	3 / 4	CB3		CB3

Table 3.3 *Summary of the monoclonal antibodies producing significant inhibition of [^{125}I]-α-human CGRP in the rat liver, spleen and cerebellum using a 30 minute incubation period.*

The entire CGRP molecule appears to be required for successful CGRP receptor binding in rat liver, spleen and cerebellar membrane preparations in these experiments.

In the experiments using the 5 min incubation periods it would appear (Figures 3.7-3.9) that some of the Mabs potentiated [^{125}I]-α-human CGRP binding. This apparent observation was probably due to inconsistencies seen with experimental controls (see section 3.6 and table 3.4) and was not regarded as a real effect.

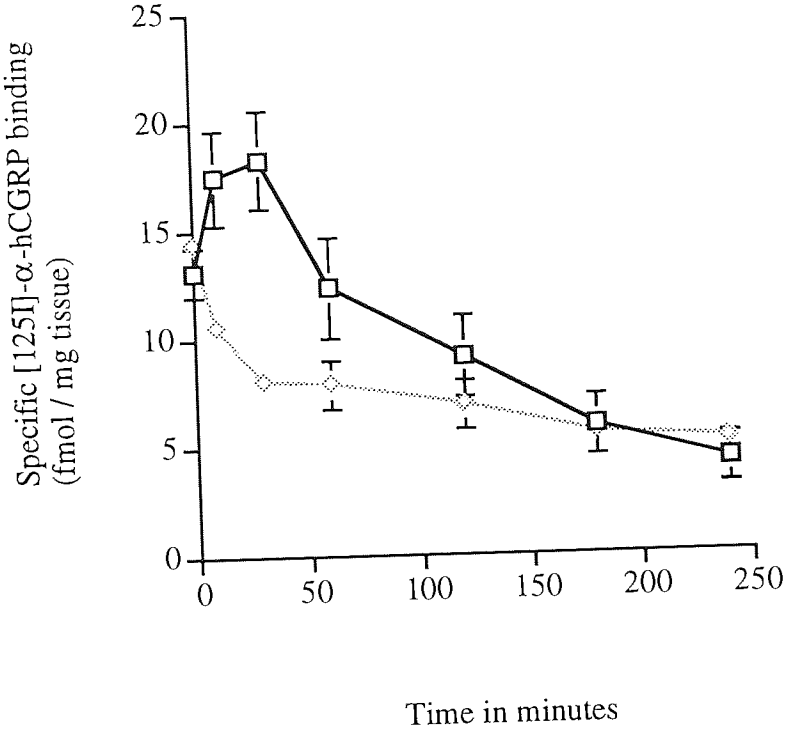


Figure 3.1 *Time course of association (□) and dissociation (◇) of 10pM [^{125}I]-α-human CGRP to rat liver membranes in 1mM $MgCl_2$, 20mM HEPES, 0.3% (w/v) protease-free BSA, pH 7.4 at 25°C. The dissociation of the radioligand was measured after the addition of 0.1μM unlabelled CGRP. Each point represents the mean of 6 separate experiments each performed in triplicate with s.e.mean shown by the vertical lines. At 100% total binding, the mean total d.p.m ± s.e.m per assay was 6717 ± 655 of which 1303 ± 138 was attributable to non-specific binding. These values are representative of those found in subsequent experiments illustrated in the following figures.*

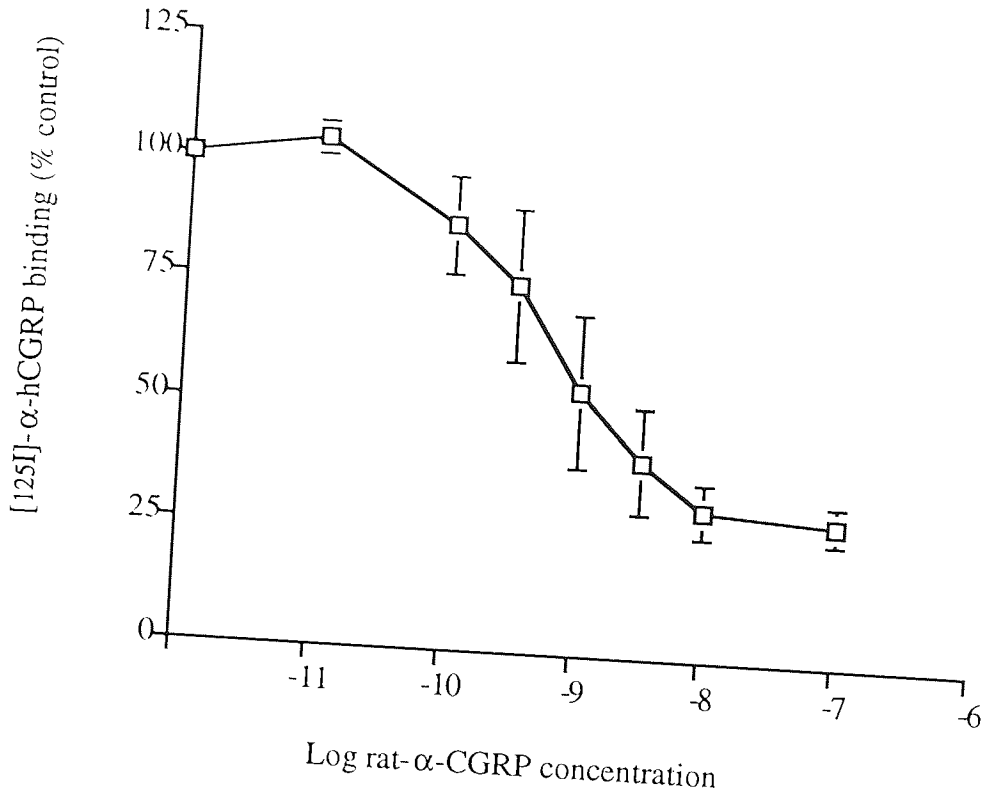


Figure 3.2 *Specifically bound 10pM [^{125}I]-α-human CGRP in rat cerebellar membranes in the presence of rat-α-CGRP in 1mM $MgCl_2$, 20mM HEPES, 0.3% w/v BSA buffer, pH 7.4 at 25°C. The points represent the mean of 4 separate experiments, each performed in triplicate. The s.e.mean is shown by the vertical lines. The calculated IC_{50} value ± s.e.m was 0.8 ± 0.5nM. Controls were taken as specific [^{125}I]-α-human CGRP binding in the absence of test substance.*

At 100% total binding, the mean total d.p.m ± s.e.m per assay was 2754 ± 1319 of which 1080 ± 586 was attributable to non-specific binding.

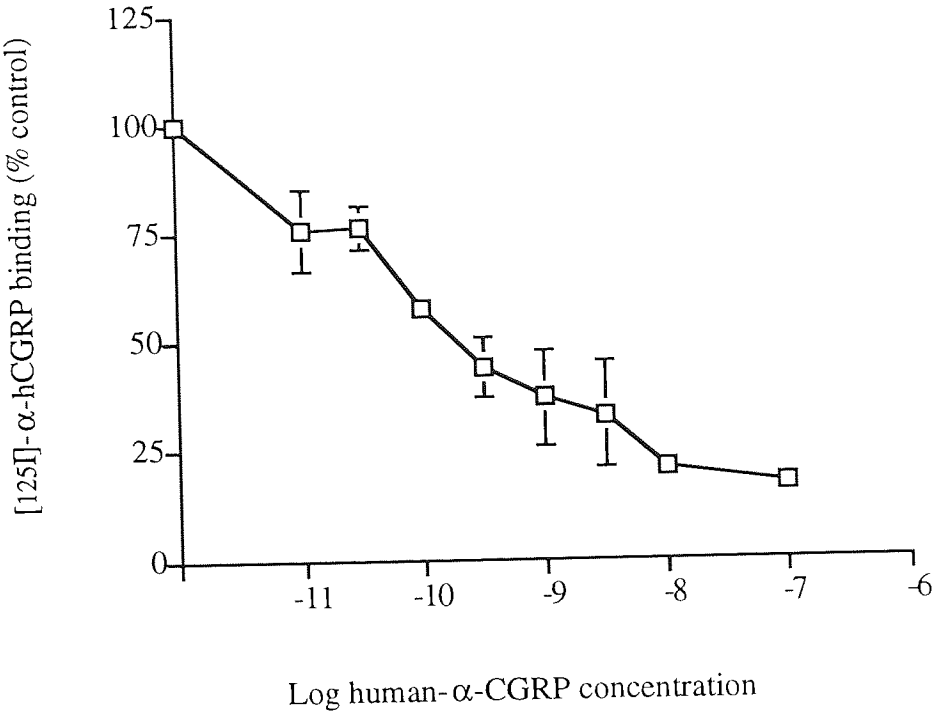


Figure 3.3 *Specifically bound 10pM [^{125}I]-α-human CGRP in rat liver membranes in the presence of human-α-CGRP in 1mM $MgCl_2$, 20mM HEPES, 0.3% w/v BSA buffer, pH 7.4 at 25°C. The points represent 4 separate experiments each performed in triplicate. The s.e.mean is shown by the vertical lines. The calculated IC_{50} value ± s.e.m was 19.0 ± 18.0nM. Controls were taken as specific [^{125}I]-α-human CGRP binding in the absence of test substance.*

At 100% total binding, the mean total d.p.m ± s.e.m per assay was 2756 ± 993 of which 477 ± 162 was attributable to non-specific binding.

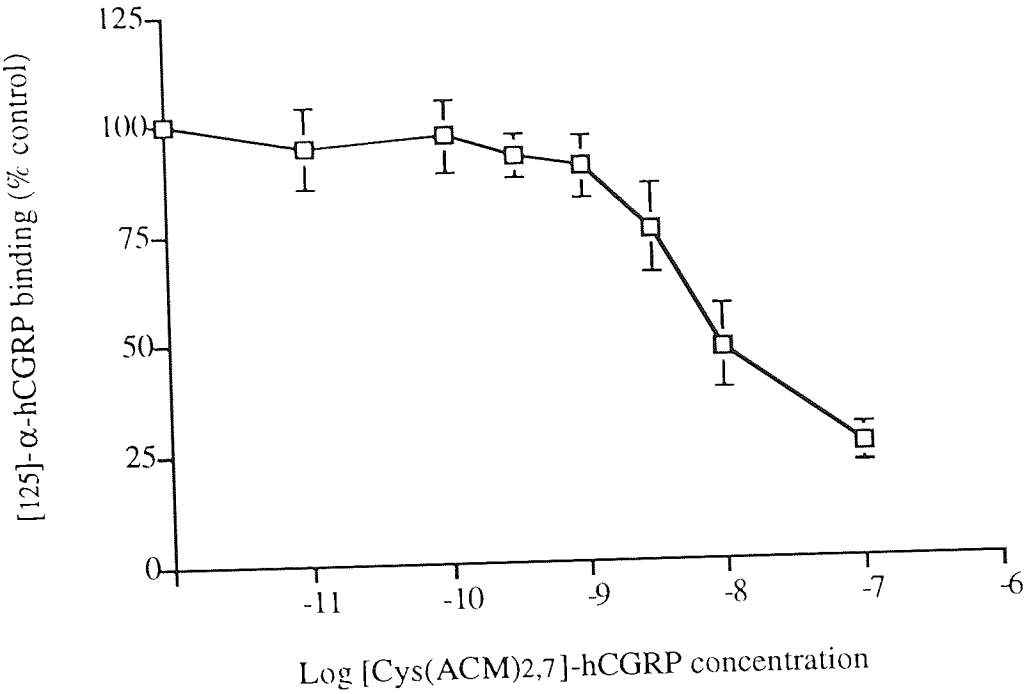


Figure 3.4 *Specifically bound 10pM [^{125}I]-α-human CGRP in rat liver membranes in the presence of [Cys (ACM)$_{2,7}$]-human CGRP in 1mM $MgCl_2$, 20mM HEPES, 0.3% w/v BSA buffer, pH 7.4 at 25°C. The points represent the mean of 4 separate experiments each performed in triplicate. The s.e.mean is shown by the vertical lines. The calculated IC_{50} value ± s.e.m was 7.4 ± 2.7nM. Controls were taken as specific [^{125}I]-α-human CGRP binding in the absence of test substance.*

At 100% total binding, the mean total d.p.m ± s e.m per assay was 2977 ± 1578 of which 710 ± 339 was attributable to non-specific binding.

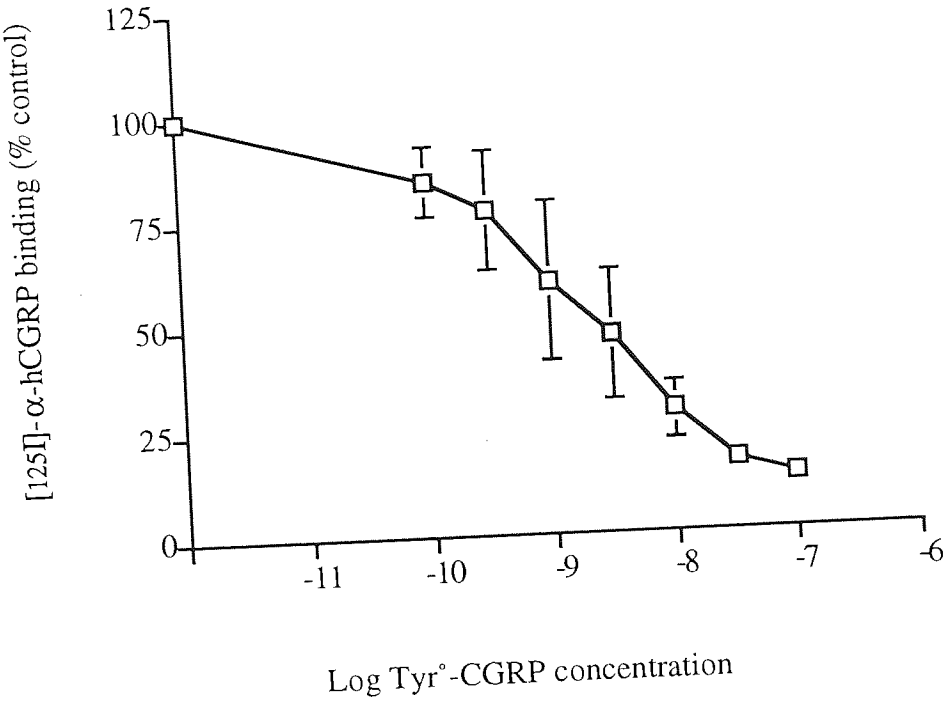


Figure 3.5 *Specifically bound 10pM [^{125}I]-α-human CGRP in rat splenic membranes in the presence of Tyr°-CGRP in 1mM $MgCl_2$, 20mM HEPES, 0.3% w/v BSA buffer, pH 7.4 at 25°C. The points represent the mean of 4 separate experiments each performed in triplicate. The s.e.mean is shown by the vertical lines. The calculated IC_{50} value ± s.e.m was 2.4 ± 1.9nM. Controls were taken as specific [^{125}I]-α-human CGRP binding in the absence of test substance.*

At 100% total binding, the mean total d.p.m ± s.e.m per assay was 2845 ± 1370 of which 402 ± 180 was attributable to non-specific binding.

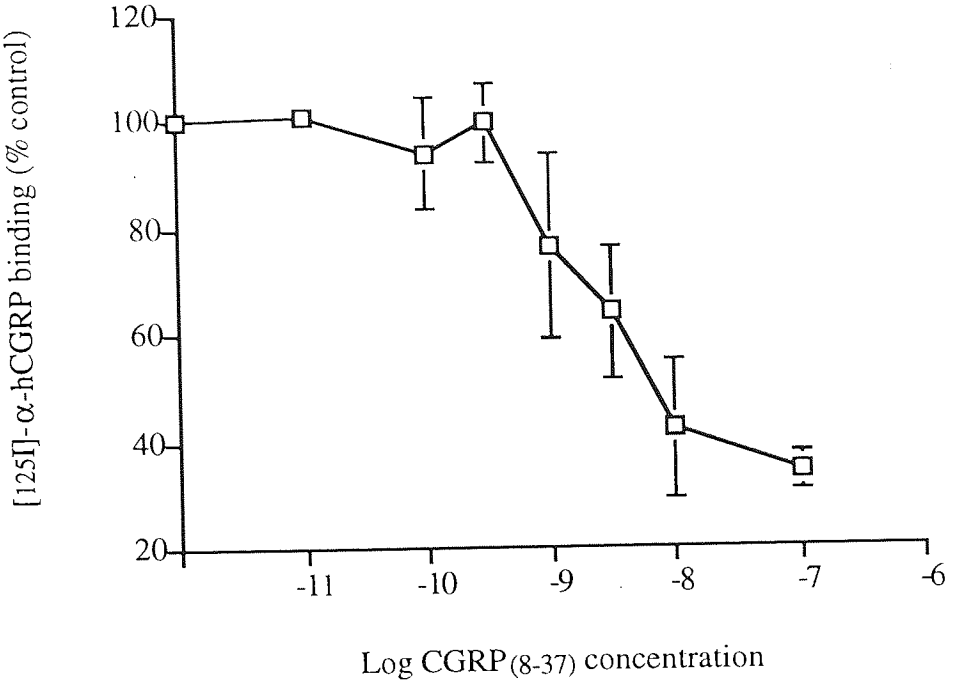


Figure 3.6 *Specifically bound 10pM [^{125}I]-α-human CGRP in rat liver membranes in the presence of CGRP$_{(8-37)}$ in 1mM $MgCl_2$, 20mM HEPES, 0.3% w/v BSA buffer, pH 7.4 at 25°C. The points represent the mean of 4 separate experiments each performed in triplicate. The s.e.mean is shown by the vertical lines. The calculated IC_{50} value ± s.e.m was 8.2 ± 4.0nM. Controls were taken as specific [^{125}I]-α-human CGRP binding in the absence of test substance.*

At 100% total binding, the mean total d.p.m ± s.e.m per assay was 3569 ± 1605 of which 1218 ± 527 was attributable to non-specific binding.

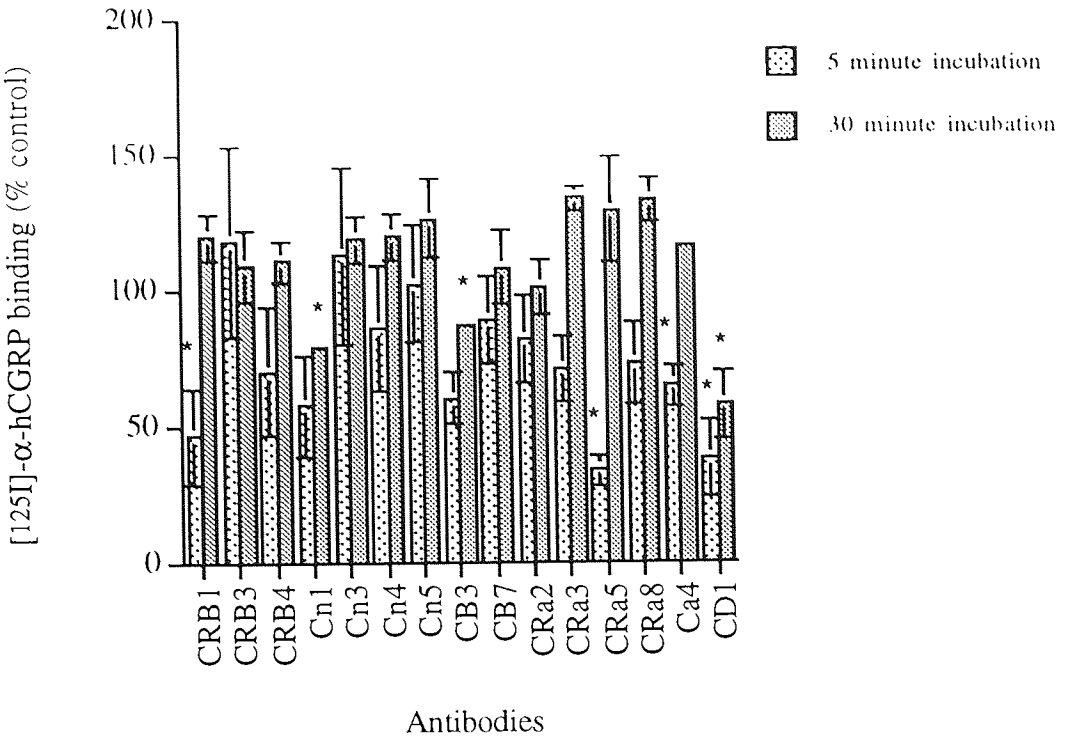


Figure 3.7 *Specific binding of [^{125}I]-α-human CGRP binding in rat liver membranes in the presence of a panel of Mabs using 5 and 30 minute incubation periods. Results expressed as % control, where control was taken as level of [^{125}I]-α-human CGRP binding in the absence of Mab. Each bar represents the mean of 3 separate experiments carried out in triplicate with s.e.mean shown by the vertical bars. * indicates statistically significant inhibition of radioligand binding in the presence of Mabs ($P \leq 0.05$). Statistical analysis was performed using a paired Student's t test.*

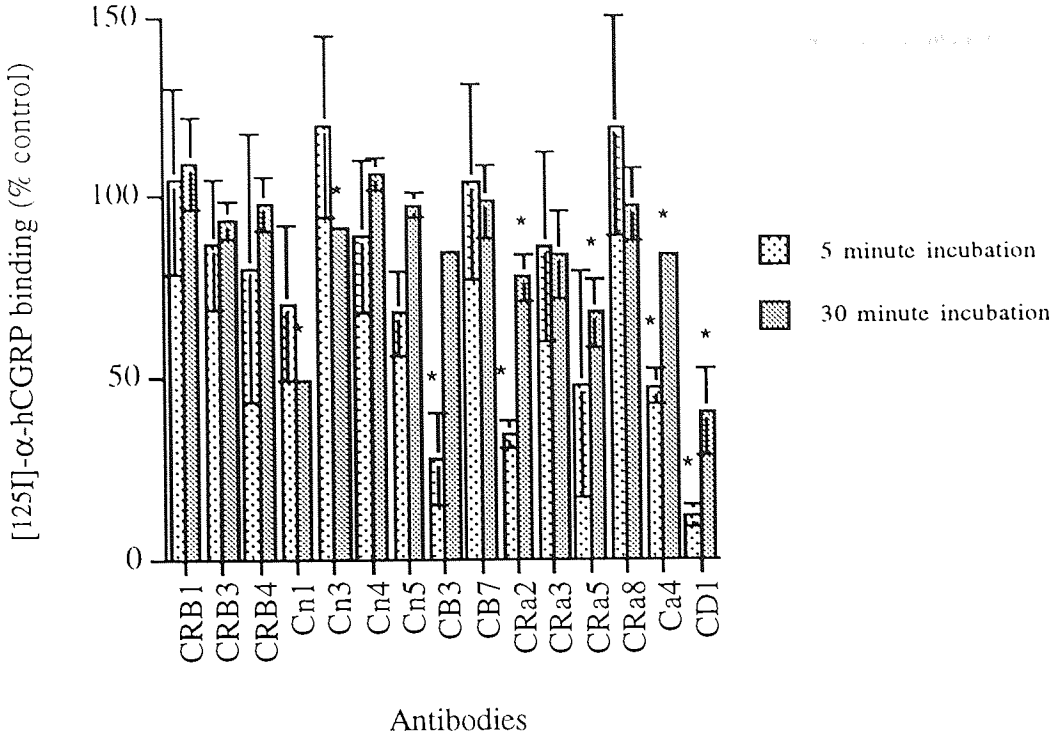


Figure 3.8 *Specific binding of [^{125}I]- α-human CGRP in rat splenic membranes in the presence of a panel of Mabs using 5 and 30 minute incubation periods. Results expressed as % control, where control was taken as level of [^{125}I]- α-human CGRP binding in the absence of Mab. Each bar represents the mean of 3 separate experiments carried out in triplicate with s.e.mean shown by the vertical bars. * indicates statistically significant inhibition of radioligand binding in the presence of Mabs ($P \leq 0.05$). Statistical analysis was performed using a paired Student's t test.*

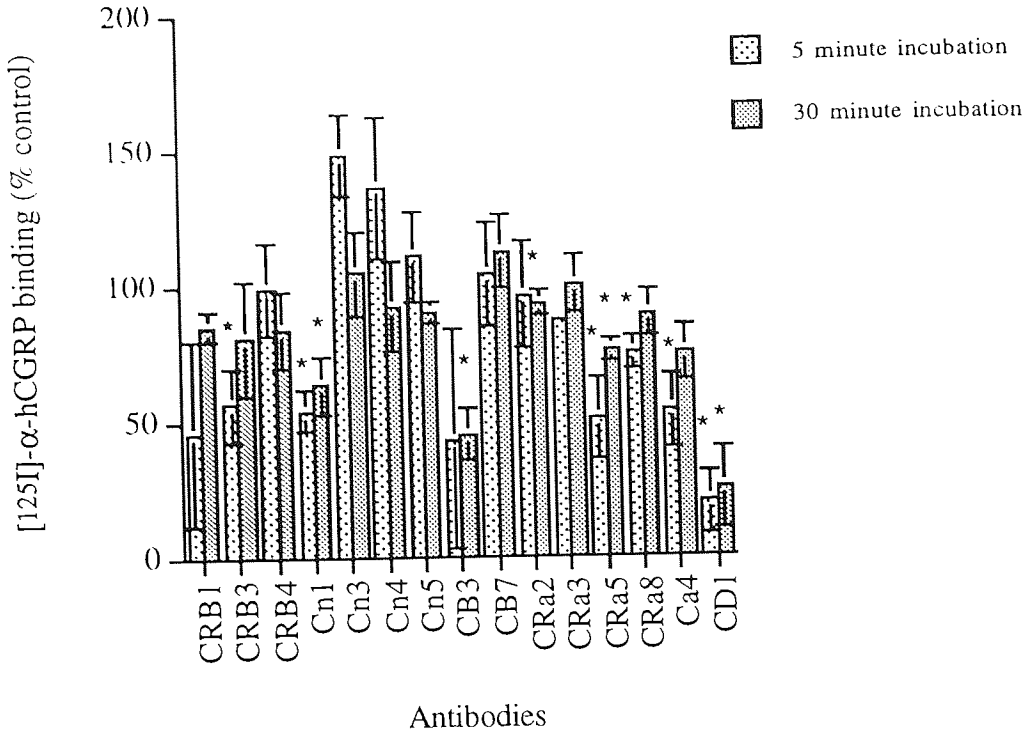


Figure 3.9 *Specific binding of [^{125}I]- α-human CGRP in rat cerebellar membranes in the presence of a panel of Mabs using 5 and 30 minute incubation periods. Results expressed as % control, where control was taken as level of [^{125}I]- α-human CGRP binding in the absence of Mab. Each bar represents the mean of 3 separate experiments carried out in triplicate with s.e.mean shown by the vertical bars. * indicates statistically significant inhibition of radioligand binding in the presence of Mabs ($P \leq 0.05$). Statistical analysis was performed using a paired Student's t test.*

3.5 Discussion and Conclusions

3.5.1 Binding inhibition by CGRP and analogues

In the rat liver, spleen and cerebellum [^{125}I]-α-human CGRP binding was shown to be rapid, with dissociation slow and gradual. This is as expected for high affinity binding sites and was seen to be stable for 30 mins. These observations are in agreement with previous studies on the CGRP receptor in rats. [^{125}I]-Tyr°-CGRP binding in rat liver membranes, has been shown to be rapid and reversible with equilibrium binding reached in 60 mins (Yamaguchi *et al.*, 1988). In the rat cerebellum, brainstem, liver and spleen, binding equilibrium was reached in 30 mins with rapid dissociation occurring subsequent to the addition of excess unlabelled ligand (Stangl *et al.*, 1993). In rat spleen [^{125}I]-α-human CGRP binding has been shown to be slower, reaching equilibrium, at 6°C, within 90 mins (Sigrist *et al.*, 1986).

The binding affinities of types of CGRP (rat-α-CGRP & human-α-CGRP), CGRP analogues ([Cys(ACM)$_{2,7}$]-human CGRP & Tyr°-CGRP) and a CGRP fragment (CGRP$_{(8-37)}$) were studied in rat liver, spleen and cerebellum. All of the peptides produced concentration-dependent inhibition of [^{125}I]-α-human CGRP binding in the rat liver, spleen and cerebellum. Due to low number of determinations and the large errors inherent in these studies, no statistically significant differences were observed between the binding affinities of the peptides used. Previous work showed a single class of binding sites existed in rat liver and spleen, (Stangl *et al.*, 1993). The estimated order of potency for the peptides tested in this study is:

Liver:

Rat-α-CGRP ≥ human-α-CGRP > [Cys(ACM)$_{2,7}$]-human CGRP > CGRP$_{(8-37)}$ >Tyr°-CGRP

Spleen:

CGRP$_{(8-37)}$ ≥ Tyr°-CGRP ≥ rat-α-CGRP > human-α-CGRP > [Cys(ACM)$_{2,7}$]-human CGRP

Cerebellum:

Rat-α-CGRP > $CGRP_{(8-37)}$ >Tyr^{0}-CGRP > [$Cys(ACM)_{2,7}$]-human CGRP > human-α-CGRP

The overall potency order was based upon IC_{50} values obtained between the CGRP analogues and fragments. The large experimental variation seen in these studies however precludes determination of a more conclusive potency order.

Initial observation of the IC_{50} values obtained for the cerebellum show an approximately 24-fold difference between rat-α-CGRP and human-α-CGRP. Other differences appear to exist, especially between human-α-CGRP and [Cys $(ACM)_{2,7}$]-human CGRP but the large errors associated with these values precludes conclusive interpretation. In the rat, [Cys $(ACM)_{2,7}$]-human CGRP has been shown to have a 10-fold lower affinity than human-α-CGRP in the cerebral cortex (Seifert *et al.*, 1988), an equal affinity in rat whole brain but a 50% lower affinity than human-α-CGRP in rat spleen (Dennis *et al.*, 1989). The former finding is in general agreement with the results presented in this thesis where [Cys $(ACM)_{2,7}$]-human CGRP > human-α-CGRP in the cerebellum but in the spleen [Cys $(ACM)_{2,7}$]-human CGRP < human-α-CGRP, a result similar to that seen by Dennis *et al* (1989).

Overall the results show that the rat membrane preparations, liver, spleen and cerebellum have differences in their affinities for the CGRP analogues and fragments tested but no conclusive interpretation of these results was possible due to the aforementioned experimental errors, thus the putative potency orders detailed here must be treated with some degree of caution. As can be seen in figures 3.2-3.6, the curves do not show 100% displacement of [^{125}I]-α-human CGRP therefore highlighting a problem with the radioligand binding. As [^{125}I]-α-human CGRP is an agonist, it may bind to a population of binding sites which may show multiple affinities (e.g. both G-protein and non-G-protein coupled). If [^{125}I]-α-human CGRP binds one of these sites with low affinity, the concentration of rat-α-CGRP used to define non-specific binding may not saturate such a site. This observation may also

imply that the concentration of CGRP used to define non-specific binding may not have been sufficient. 100nM CGRP has been previously shown to be a sufficient concentration to define non-specific binding in these tissue preparations and most reported Kd values for CGRP are not reported to be greater than 1nM (Poyner, unpublished results, Poyner, 1992).

3.5.2 Inhibition of binding by monoclonal antibodies (Mabs)

The effects of Mabs raised against CGRP, upon the binding of [^{125}I]-α-human CGRP in rat liver, splenic and cerebellar membrane preparations was investigated. As explained earlier (section 3.4.3) two incubation periods were used in these experiments; 5 and 30 mins, in order to observe inhibition of binding whatever the rate of dissociation of the pre-formed radioligand: antibody complex.

In general, the results which appear to be more reliable in examining the abilities of the Mabs at inhibiting radioligand binding, and which appear to fit with the theory regarding the antibody: radioligand: receptor equilibrium are:

(a) the results obtained using the 30 min incubation and (b) the results from the 5 min incubations where inhibition was not observed using the corresponding 30 min incubation.

This is because any inhibition of radioligand binding seen using the 30 min incubation period should also have be observed using the comparative 5 min incubation if stable radioligand: antibody complexes had been formed. Inhibition at 5 min, not seen at 30 min may imply that the preformed radioligand: antibody complex started to dissociate upon addition to the tissue preparation but that this dissociation was slow and took longer than 5 minutes.

The experiments using the 5 min incubation, showed slight differences in the abilities of the Mabs to inhibit [^{125}I]-α-human CGRP binding in the three membrane preparations. The amino acid residues 32-37 appear to be important for receptor

binding in all three membrane preparations. Amino acids 1-13 also appear to be important in the liver with 1-13 and 19-27 important in the cerebellum.

The experiments using the 30 min incubation period showed that the entire CGRP molecule is required for successful receptor binding in the rat liver, spleen and cerebellum. CD1 and Cn1 in particular inhibited binding in all three preparations. CD1 was raised against epitope 2 of CGRP, amino acids 13-19 (diagram 3.1), the area of the CGRP molecule incorporating the α-helix (Lynch & Kaiser, 1988), implying this region was important in CGRP receptor binding in all tissues. These findings agree with previous reports that the entire CGRP molecule is required for successful receptor-ligand interactions, such as that by O'Connell *et al.* (1993). This group reported that a CGRP analogue lacking the carboxy-phenylalanineamide was unable to bind to the CGRP receptor present on L6 skeletal myocytes.

The conflicting data, regarding the amino acid residues important for CGRP receptor binding, obtained using the two incubation periods needs to be examined in greater detail in future experiments. Further studies regarding the affinity of the Mabs for [^{125}I]-α-human CGRP also need to be performed.

3.5.3 Conclusions

The orders of potency based upon fitted IC_{50} values, obtained using the CGRP analogues and fragments showed that different CGRP receptors may exist in rat liver, splenic and cerebellar membranes. Due to large inter-experimental variations conclusive interpretation of these results is not possible. Further experimentation may however be able to clarify this problem. The results obtained using the panel of monoclonal antibodies and a 30 min experimental incubation, showed that the rat liver, spleen and cerebellar membrane preparations possessed similar binding characteristics. The entire CGRP molecule was necessary for receptor binding in all of the rat membrane preparations.

The results presented in this thesis indicate that small differences may exist between the CGRP receptors present in rat liver, spleen and cerebellar but no positive conclusions can be drawn based solely upon these results.

It was the intention to use information obtained from these studies for use in subsequent functional assays. The competition binding studies should have provided information regarding the affinities of CGRP and CGRP analogues at the CGRP receptor whilst the Mab study should have provided information regarding the amino acid residues necessary for successful receptor: ligand binding. This information could have been of assistance in helping to choose receptor agonists and antagonists for use in future experiments examining CGRP receptor classification.

As the experimental problems associated with these studies prevented their continuation (section 3.6) a second, less documented species, the guinea-pig, was chosen for the subsequent studies examining CGRP and amylin receptor classification and characterisation.

3.6 Problems with radioligand binding

Interpretation of the results obtained in this study for CGRP binding characteristics and structure-activity relationships must be treated with caution, due to the variability in the levels of specific binding produced with [^{125}I]-α-human CGRP in consecutive experiments (table 3.4). This phenomenon occurred irregularly but with sufficient frequency as to make progress with the radioligand binding experiments almost impossible.

Experiment number	Specific binding / cpm
1	4450
2	1470
3	1363
4	7387
5	4441
6	4036
7	5288
8	1301
9	7551

Table 3.4 *Specific binding of [^{125}I]-α-human CGRP on rat cerebellar membranes. in nine consecutive experiments. Specific binding expressed in counts per minute.*

The amounts of tissue and radioligand used in each incubation were consistent and yet the variability of specific binding between experiments remained. All physical parameters were investigated, such as the storage of radioligand and tissue at -20°C, effects of Sigmacoat or dimethylsilane (used to coat plasticware) upon buffer pH, experimental temperature variation and buffer composition. No physical reasons for this variability in binding were apparent and thus these experiments were eventually abandoned as their ability to provide valid consistent data was highly questionable.

Many binding studies have reported little differences in the affinity constants obtained using CGRP receptor antagonists such as the fragment $CGRP_{(8-37)}$ yet functional studies using the same tissues have shown great variation in pA_2 values obtained implying that differences in the tissue receptors exist (Dennis *et al.*, 1989; Mimeault *et al.*, 1991). This may suggest that functional studies are a more reliable and valid way to characterise and classify CGRP receptors.

Chapter 4

CGRP and amylin receptor classification in the guinea-pig ileum.

4.1 Introduction

4.1.1 Effects of CGRP and amylin in the ileum

CGRP-like immunoreactivity is distributed extensively in the myenteric and submucosal plexuses of the intestine, especially in the cell bodies of enteric neurones and in fibres which have been traced directly to the mucosa of the guinea-pig small intestine, particularly in the connective tissue among the crypts of Lieberkuhn (Feher *et al.*, 1986; Furness *et al.*, 1985). CGRP has been shown to act on distinct neural pathways supplying the longitudinal and circular muscles of the guinea-pig ileum as well as on the muscle cells themselves, suggesting an involvement in the regulation of intestinal motility (Holzer *et al.*, 1989).

The mammalian small intestine contains nerve fibres which originate from extrinsic (primary afferent) and intrinsic neurones of the myenteric and submucosal plexuses (Ekblad *et al.*, 1987; Feher *et al.*, 1986). In the rat, myenteric CGRP neurones issue projections within the myenteric plexus and circular muscle (Ekblad *et al.*, 1987), whereas in the guinea-pig the myenteric neurones supply the mucosa alone (Costa *et al.*, 1986) suggesting species differences occur. CGRP receptors on the myenteric neurones in the guinea-pig have been shown to possess a high degree of specific CGRP binding (Palmer *et al.*, 1986).

CGRP has been shown to have multiple effects on the guinea-pig ileum, with rat-α-CGRP producing both relaxation and contraction of the longitudinal smooth muscle, and contraction of the circular smooth muscle (Bartho *et al.*, 1987; Holzer *et al.*, 1989). The CGRP-induced ileal contraction has been shown to occur via a concentration-dependent release of acetylcholine from the myenteric plexus, an effect mediated by cholinergic interneurones (Mulholland & Jaffer, 1990). Relaxation of the longitudinal muscle however occurs by direct action on the smooth muscle itself (Schworer *et al.*, 1991; Bartho 1991). The direct relaxant effect of rat-α-CGRP was sensitive to antagonism by 1μM $CGRP_{(8\text{-}37)}$ (Dennis *et al.*, 1990) and the contractile effect on the circular muscle was sensitive to antagonism by 3μM $CGRP_{(8\text{-}37)}$ (Bartho *et al.*, 1991/1993) implying the involvement of $CGRP_1$ receptors (Dennis *et al.*,

1989). Previously $CGRP_{(8-37)}$ has been shown to be ineffective against rat-α-CGRP-induced cholinergic contraction of the longitudinal muscle but was capable of antagonising the direct relaxant effect. This suggests the possible existence of 2 receptor subtypes, post-synaptic $CGRP_1$ receptors mediating relaxation and presynaptic $CGRP_2$ receptors mediating contraction (Schworer *et al.*, 1991; Bartho *et al.*, 1992,1993)

4.2 Aims and objectives

The previous study utilising CGRP, its analogues; Tyr°-CGRP and [Cys $(ACM)_{2,7}$]-human CGRP and a panel of monoclonal antibodies raised against CGRP (chapter 3) did not produce any conclusive information regarding the classification and characterisation of CGRP receptors in the rat liver, spleen and cerebellum. For this reason it was decided to proceed with any further examination of CGRP receptors using a second animal species, the guinea-pig, in order to try and obtain information regarding CGRP receptor classification and characterisation in one animal species.

The aim of this study was to further classify the previously reported CGRP receptors in the isolated guinea-pig ileum by utilising CGRP agonists and a number of CGRP fragments previously shown to possess antagonistic properties in this or other isolated tissue preparations. At present, the only reported CGRP receptor antagonists are fragments of the CGRP molecule, use of these anatgonists could also therefore provide information regarding the amino acid residues important for binding at the CGRP receptors studied. The putative amylin receptor antagonist AC187 (Young *et al.*, 1994) and the amylin fragment human $amylin_{(8-37)}$ were used, alongside human and rat amylin, to identify possible amylin receptors in the guinea-pig ileum and examine the possible cross-reactivity between CGRP and amylin in this preparation.

4.3 Methods and Materials

The experimental protocol is as outlined in chapter 2, section 2.2. The nature of the response obtained using human-α-CGRP and rat-α-CGRP on the ileum preparations (figure 4.1) was found to be mediated by a direct action on the smooth muscle. Addition of atropine (1μM) to the preparations was not found to affect the CGRP-induced responses thereby indicating that cholinergic interneurones were not involved in mediating the response. Atropine was not used routinely in these experiments. The CGRP receptors responsible for direct relaxation of ileal smooth muscle have been reported to be sensitive to antagonism by $CGRP_{(8-37)}$ and therefore type 1. It was decided to use $CGRP_{(8-37)}$ alongside other reported CGRP receptor antagonists; Tyr^{0}-$CGRP_{(28-37)}$ and $CGRP_{(19-37)}$, to try and obtain a clearer picture of CGRP receptor classification in the guinea-pig ileum.

Thiorphan was dissolved in ethanol to give a stock of 10mM and used at 10μM. Vehicle controls were performed using 0.1% v/v ethanol (see figure 4.3). The ileal preparations were pre-incubated with thiorphan for 30 mins prior to the addition of human-α-CGRP.

4.3.1 Data evaluation

EC_{50} values were obtained by EBDA-LIGAND and analysed statistically as described in section 2.4. n = number of animals utilised, rather than individual tissue responses. When duplicate concentration-response curves were constructed on tissues from the same animal, values were normalised and means produced prior to statistical analysis. All results were normalised as percentage of control response, where control responses are defined as stimulated twitch amplitude prior to addition of CGRP or other agonists.

4.4 Results

Initially the effects of human-α-CGRP, human-β-CGRP and rat-α-CGRP on the electrically-evoked twitch contractions of the guinea-pig ileum were examined. All of these peptides produced concentration-dependent inhibition of twitch amplitude (figure 4.2), a finding in agreement with previous reports (Dennis *et al.*, 1990; Schworer *et al.*, 1991). Rat amylin was also found to cause a similar inhibition of twitch height, but with a lower potency than the 3 forms of CGRP.

The potency order, as determined by EC_{50} values ± s.e.m (figure 4.2) was: Rat-α-CGRP (2.7 ± 4.9nM) = human-α-CGRP (7.7 ± 8.7nM) > human-β-CGRP (41.0 ± 13.0nM) > rat amylin (200 ± 49.0nM).

The concentration-response curves produced by human-α-CGRP and rat-α-CGRP appear to be bi-phasic. The initial inhibition of twitch height was followed by a slight reversal of the effect at 0.3μM CGRP, a result that was routinely observed. CGRP has been previously shown to have a bi-phasic effect on the guinea-pig ileum, producing direct relaxation of longitudinal muscle and cholinergically mediated contraction of circular and longitudinal muscle.

Thiorphan, an inhibitor of endopeptidase 24:11, the enzyme responsible for breakdown of endogenous CGRP (Davies *et al.*, 1992), has been reported to potentiate the effects of human-α-CGRP in the rat vas deferens (EC_{50} values of 4.4nM for CGRP alone and 14.1nM for CGRP in the presence of 10μM thiorphan) (Longmore *et al.*, 1994). In this study, thiorphan (10μM) and its vehicle control, ethanol, were tested in conjunction with human-α-CGRP on the guinea-pig ileum

Neither 10μM thiorphan nor its vehicle control (0.1% ethanol), potentiated the effect produced by human-α-CGRP on the stimulated twitch height (figure 4.3). One possible explanation for the discrepancy between results produced here and previous reports of the actions of thiorphan, may be explained by possible low levels of endopeptidase 24:11 in the guinea-pig ileal preparations used in the experiments

conducted here. Any inhibition of endopeptidase 24:11 by thiorphan would not thereby produce any increase in the effect caused by exogenous application of CGRP. Thiorphan was not used routinely in subsequent experiments.

4.4 .1 Human-α-CGRP

As can be seen in figures 4.1 and 4.2, human-α-CGRP potently inhibited the electrically-evoked twitch contractions of the guinea-pig ileum. All of the CGRP fragments and amylin receptor antagonists tested against the human-α-CGRP-induced response, produced statistically significant shifts in the concentration-response curve produced by the agonist alone.

The CGRP fragment, $CGRP_{(8-37)}$ has previously been reported to act as an antagonist at the $CGRP_1$ receptor and is the current basis of CGRP receptor subtype classification.

At 1μM and 3μM, $CGRP_{(8-37)}$ significantly shifted the human-α-CGRP concentration-response curve such that the calculated EC_{50} values were increased from 7.7 ± 8.7nM for agonist alone to 110 ± 9.6nM and 260 ± 130nM respectively for human-α-CGRP plus 1μM and 3μM $CGRP_{(8-37)}$ (figure 4.4) The estimated pA_2 value for this data, obtained from averaging dose-ratios (data not shown) was 7.2.

The ability of $CGRP_{(8-37)}$ to produce a shift to the right of the concentration-response curve, without affecting the maximal human-α-CGRP effect, suggests that $CGRP_{(8-37)}$ is a potent competitive antagonist in the guinea-pig ileum. This result also indicates that the amino acid residues 8-37 are important for CGRP receptor binding.

Two further CGRP fragments; $CGRP_{(19-37)}$ (3μM) and Tyr^0-$CGRP_{(28-37)}$ (10μM) were subsequently screened against the human-α-CGRP response. These fragments have previously been reported to effectively antagonise CGRP in guinea-pig atria (Rovero *et al.*, 1991) and opossum internal anal sphincter (Chakder & Rattan, 1992)

respectively. These fragments did not alter the stimulated twitch amplitude when added alone (data not shown) but did antagonise the human-α-CGRP-induced response.

The calculated EC_{50} values for human-α-CGRP and human-α-CGRP plus 3μM $CGRP_{(19-37)}$ were 7.7 ± 8.7nM and 210 ± 89.0nM respectively producing an estimated pA_2 value of 6.7 (see figure 4.5)

The calculated EC_{50} values for human-α-CGRP and human-α-CGRP plus 10μM Tyr°-$CGRP_{(28-37)}$ were 7.7 ± 8.7nM and 88.0 ± 1.9nM respectively producing an estimated pA_2 value of 6.0 (see figure 4.6).

$CGRP_{(19-37)}$ and Tyr°-$CGRP_{(28-37)}$ were found not to be as potent antagonists against human-α-CGRP, as $CGRP_{(8-37)}$ (see figures 4.4-4.6 and table 4.1). These results suggest that no single amino acid residue in the section of the CGRP molecule between residues 8-27 can be highlighted as essential for receptor binding, but the potency of antagonism appears to decrease as the fragments become shorter: $CGRP_{(8-37)} > CGRP_{(19-37)} > Tyr^{\circ}$-$CGRP_{(28-37)}$

The amylin receptor antagonists; human $amylin_{(8-37)}$ (1μM and 3μM) and AC187 (10μM) were also tested against the human-α-CGRP-induced inhibition of electrically-evoked twitch amplitude.

Figure 4.7a shows the effects of 1μM human $amylin_{(8-37)}$ on the human-α-CGRP response on the guinea-pig ileum. It appears from the graph that at 1nM and 10nM human-α-CGRP, $amylin_{(8-37)}$ caused antagonism, an effect absent at the higher concentrations, implying that the antagonism observed was not really competitive. 3μM, $amylin_{(8-37)}$ did not produce any rightward shift of the control response curve (figure 4.7b). The heterogeneity of these results indicate that this antagonist may not be a reliable tool for use in this preparation.

AC187 (10μM) also caused significant antagonism of the control response curve produced by human-α-CGRP (see figure 4.8).

	Human-α-CGRP (EC_{50} ± s.e.m / nM)
Control	7.7 ± 8.7 (n = 52)
1μM CGRP (8-37)	110 ± 9.6* (n = 9)
3μM CGRP (8-37)	260 ± 130* (n = 5)
3μM CGRP (19-37)	210 ± 89.0* (n = 4)
10μM Tyr°-CGRP (28-37)	88.0 ± 1.9* (n = 6)
1μM Human amylin (8-37)	74.0 ± 23.0 (n = 7)
10μM AC187	340 ± 89.0* (n = 3)

Table 4.1 *EC_{50} values ± s.e.m for human-α-CGRP alone and in the presence of a variety of antagonists on the isolated guinea-pig ileum. All values were taken from figures 4.2 & 4.4-4.8 All values shown are nM. * indicates significant difference from control, tested by one-way ANOVA and post-hoc Dunnett's test ($P \leq 0.05$).*

The overall potency order for the CGRP and amylin fragments compared to human-α-CGRP alone, based upon calculated EC_{50} values (figures 4.2-4.8 and table 4.1) was: $CGRP_{(8-37)}$ > ($amylin_{(8-37)}$) > $CGRP_{(19-37)}$ > AC187 > Tyr°-$CGRP_{(28-37)}$. ($Amylin_{(8-37)}$ is tentatively included in this order of potency, but the results obtained using this antagonist need to be investigated further).

4.4.2 Rat-α-CGRP

Rat-α-CGRP inhibited electrically-evoked twitch contractions of the guinea-pig isolated ileum in a manner similar to that observed with human-α-CGRP. The inhibition curve produced by rat-α-CGRP (figure 4.2) gave a calculated EC_{50} value ± s.e.m of 2.7 ± 4.9nM, a value not statistically different to that obtained for human-α-CGRP (7.7 ± 8.7nM).

The five CGRP and amylin receptor antagonists screened against the human-α-CGRP-induced inhibition of stimulated twitch height were also tested against the response elicited by rat-α-CGRP.

As can be seen in figure 4.9, 1μM $CGRP_{(8-37)}$ significantly antagonised the rat-α-CGRP-induced control response. The calculated EC_{50} values ± s.e.m were; rat-α-CGRP, 2.7 ± 4.9nM and rat-α-CGRP plus 1μM $CGRP_{(8-37)}$, 34.6 ± 12.0nM. $CGRP_{(8-37)}$ appears to be a competitive antagonist of the rat-α-CGRP-induced control response in the guinea-pig ileum, as $CGRP_{(8-37)}$ produced a rightward shift of concentration-response curve without affecting the maximal rat-α-CGRP effect. This result indicates that amino acid residues 8-37 are important in rat-α-CGRP receptor binding but the EC_{50} value (± s.e.m) produced was not as great as that seen for human-α-CGRP plus 1μM $CGRP_{(8-37)}$, 110nM (± 9.6) indicating a possible difference between the ileal receptor recognising human-α-CGRP and that recognising rat-α-CGRP (see section 4.7).

1μM $CGRP_{(19-37)}$ produced significant competitive antagonism of the rat-α-CGRP control response (figure 4.10) (EC_{50} values ± s.e.m: rat-α-CGRP = 2.7 ± 4.9nM, rat-α-CGRP plus 1μM $CGRP_{(19-37)}$ = 170 ± 14.0nM) . Based upon EC_{50} values obtained for rat-α-CGRP in the presence of this antagonist and $CGRP_{(8-37)}$, $CGRP_{(19-37)}$ appeared to more potent at antagonising the control response induced by rat-α-CGRP than $CGRP_{(8-37)}$.

10μM Tyr°-$CGRP_{(28-37)}$ also antagonised the rat-α-CGRP-induced control response (figure 4.11) (EC_{50} values ± s.e.m: rat-α-CGRP = 2.7 ± 4.9nM, rat-α-CGRP plus 10μM Tyr°-$CGRP_{(28-37)}$ = 19.4 ± 4.6nM) but appeared to be less potent than $CGRP_{(19-37)}$, which seems from these results (figures 4.9-4.11 & table 4.2) to produce the greatest degree of antagonism of the rat-α-CGRP-induced response in the guinea-pig ileum.

The CGRP receptor antagonists produced the following order of potency (see table 4.2):

$CGRP_{(19-37)} > CGRP_{(8-37)} > Tyr^{\circ}\text{-}CGRP_{(28-37)}$.

The amylin receptor antagonists, human $amylin_{(8-37)}$ and AC187 were tested against the rat-α-CGRP-induced inhibition of stimulated twitch amplitude in the guinea-pig ileum.

It is difficult to conclude whether human $amylin_{(8-37)}$ is an antagonist of the rat-α-CGRP-induced response in this preparation based upon the results obtained here. The large errors seen in figure 4.12 preclude conclusive interpretation of the results and further experiments need to be conducted to increase the n numbers in an attempt to decrease these errors. The approximate EC_{50} value (± s.e.m) for rat-α-CGRP plus 1μM human $amylin_{(8-37)}$ = 170nM (± 74.0).

AC187 (10μM) caused a significant rightward shift of the rat-α-CGRP-induced control response curve with a calculated EC_{50} value ± s.e.m of 84.0 ± 12.0nM (figure 4.13).

	Rat-α-CGRP (EC_{50} values ± s.e.m / nM)
Control	2.7 ± 4.9 (n = 39)
1μM CGRP (8-37)	34.6 ± 12.0* (n = 10)
1μM CGRP (19-37)	170 ± 14.0* (n = 9)
10μM Tyr°-CGRP (28-37)	19.4 ± 4.6* (n = 6)
1μM Human amylin (8-37)	170 ± 74.0 (n = 4)
10μM AC187	84.0 ± 12.0* (n = 3)

Table 4.2 *EC_{50} values ± s.e.m for rat-α-CGRP alone and in the presence of a variety of antagonists on the isolated guinea-pig ileum. All values were taken from figures 4.2 & 4.9-4.13. All values shown are nM. * indicates significant difference from control, tested by one-way ANOVA and post-hoc Dunnett's test ($P \leq 0.05$).*

Overall the order of relative potencies for the CGRP and amylin receptor antagonists against rat-α-CGRP, based upon the calculated EC_{50} values obtained from figures 4.9-4.13 and table 4.2 was:

$Amylin_{(8-37)} = CGRP_{(19-37)} > CGRP_{(8-37)} > AC187 > Tyr^{0}\text{-}CGRP_{(28-37)}$.

The pattern of antagonism of rat-α-CGRP compared with human-α-CGRP by this panel of antagonists was broadly similar but with some interesting differences, especially between $CGRP_{(19-37)}$ and $CGRP_{(8-37)}$.

4.4.3 Human-β-CGRP

Human-β-CGRP caused a similar effect on the electrically field stimulated guinea-pig ileum as was seen with human-α-CGRP and rat-α-CGRP (figure 4.2) but with a lower potency, EC_{50} values of 41.0 ± 13.0nM for human-β-CGRP compared to 7.7 ± 8.7nM for human-α-CGRP and 2.7 ± 4.9nM for rat-α-CGRP. These values were not found to differ statistically from one another.

The only CGRP receptor antagonist to be tested against the human-β-CGRP-induced inhibition of electrically-evoked twitch contraction, was $CGRP_{(8-37)}$ (1μM) (figure 4.14).

This CGRP receptor antagonist did not appear to produce significant antagonism of the human-β-CGRP-induced response. The estimated EC_{50} value ± s.e.m for human-β-CGRP plus 1μM $CGRP_{(8-37)}$ was 103 ± 17.0nM. Higher concentrations of human-β-CGRP need to be employed to gain an accurate value for this measurement as the agonist response in the presence of the antagonist did not reach a plateau at the higher concentrations utilised.

Further concentrations of $CGRP_{(8-37)}$ and the use of other CGRP and amylin antagonists would be required before any firm conclusions concerning the receptor at which human-β-CGRP acts in the guinea-pig ileum.

4.4.4 Rat amylin

Rat amylin caused a concentration-dependent inhibition of electrically field stimulated twitch contractions in the guinea-pig ileum (figure 4.2). The calculated EC_{50} value ± s.e.m was 200 ± 49.0nM, a value significantly different to those found for the other agonists tested in this preparation (human-α-CGRP, 7.7 ± 8.9nM; rat-α-CGRP, 2.7 ± 4.9nM; human-β-CGRP, 41.0 ± 13.0nM). Rat amylin had a significantly lower potency than the other peptide agonists tested and only caused inhibition of twitch amplitude in 15% (18/120) of the preparations in which it was tested. $CGRP_{(8-37)}$ (1μM), $CGRP_{(19-37)}$ (1μM),Tyr^{0}-$CGRP_{(28-37)}$ (1μM) and human $amylin_{(8-37)}$ (1μM) were tested against the rat amylin-induced control response.

Figure 4.15 shows the effects of some of the CGRP and amylin antagonists utilised against rat amylin in this preparation. No significant antagonism of the rat amylin-induced response was achieved using these antagonists at the concentrations tested (table 4.3). Higher concentrations of these antagonists need to be utilised to ascertain whether or not they can antagonise the control response to rat amylin in this preparation.

	Rat amylin (EC_{50} values ± s.e.m /nM)
Control	200 ± 49.0 (n = 18)
1μM CGRP$_{(8-37)}$	515 ± 112 (n = 3)
1μM CGRP$_{(19-37)}$	373 ± 55.0 (n = 4)
1μM Tyr°-CGRP$_{(28-37)}$	192 ± 21.1 (n = 4)
1μM Human amylin$_{(8-37)}$	480 ± 22.0 (n = 6)

Table 4.3 *EC_{50} values ± s.e.m for rat amylin alone and in the presence of a variety of antagonists on the isolated guinea-pig ileum. All values shown are nM. Values for rat amylin, CGRP$_{(19-37)}$ and Tyr°-CGRP$_{(28-37)}$ were taken from figures 4.2 and 4.15, the others from data not shown.*

Human amylin was tested on the electrically-evoked twitch contractions of the guinea-pig ileum but was not found to cause any inhibition of twitch amplitude at concentrations up to 1μM.

	Human-α-CGRP	Rat-α-CGRP
Control	**1.0**	**1.0**
1μM CGRP (8-37)	14.4	12.7
3μM CGRP (19-37)	27.5	(1μM) 62.3
10μM Tyr°- CGRP (28-37)	11.5	7.1
1μM Human amylin (8-37)	9.7	62.3
10μM AC187	44.4	30.8

Table 4.4 *Comparison of fold shifts produced by CGRP and amylin receptor antagonists on the control responses to human-α-CGRP and rat-α-CGRP in the guinea-pig ileum. Fold-shifts were calculated by division of EC_{50} values taking agonist values as 1.0. Where comparable concentrations of antagonist were not utilised the concentration is given ( ) above.*

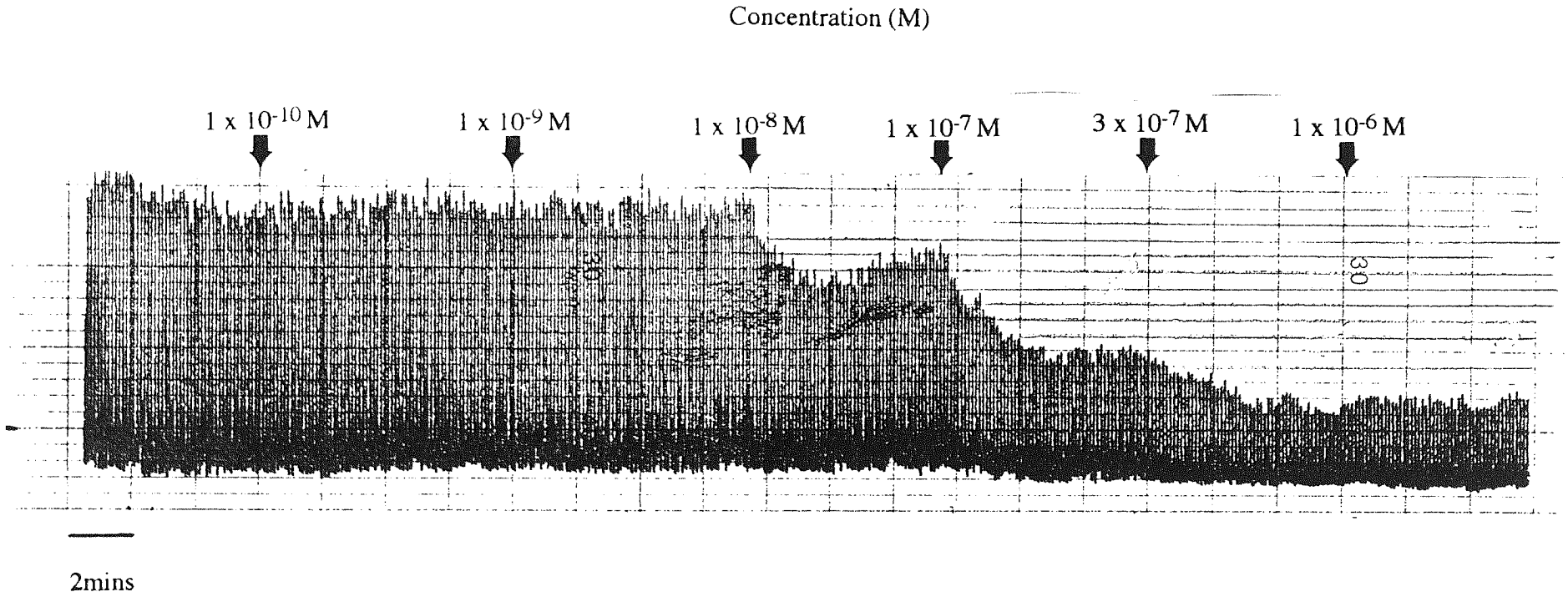


Figure 4.1 *Representative trace illustrating the concentration-related relaxant effect of human-α-CGRP in the electrically evoked twitch contractions of guinea-pig isolated ileum. The chart speed was set at 30 cm / hour.*

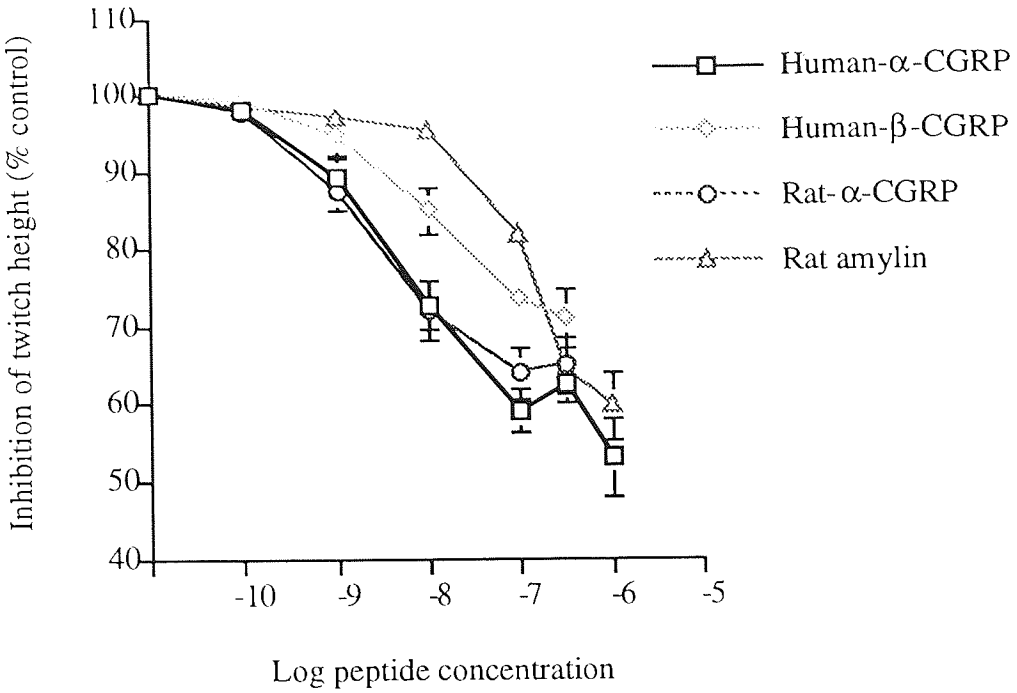


Figure 4.2 *Inhibition of electrically-evoked twitch contraction of the isolated guinea-pig ileum by increasing concentrations of human-α-CGRP (□), human-β-CGRP (◇), rat-α-CGRP (○) and rat amylin (Δ). Values of EC_{50} from fits ± s.e.m: human-α-CGRP = 7.7 ± 8.7nM, human-β-CGRP = 41.0 ± 13.0nM, rat-α-CGRP = 2.7 ± 4.9nM and rat amylin = 200 ± 49.0nM. s.e.mean shown by the vertical lines. The points represent means of 52, 8, 39 and 18 separate experiments respectively. Controls were taken as twitch amplitude prior to addition of agonist.*

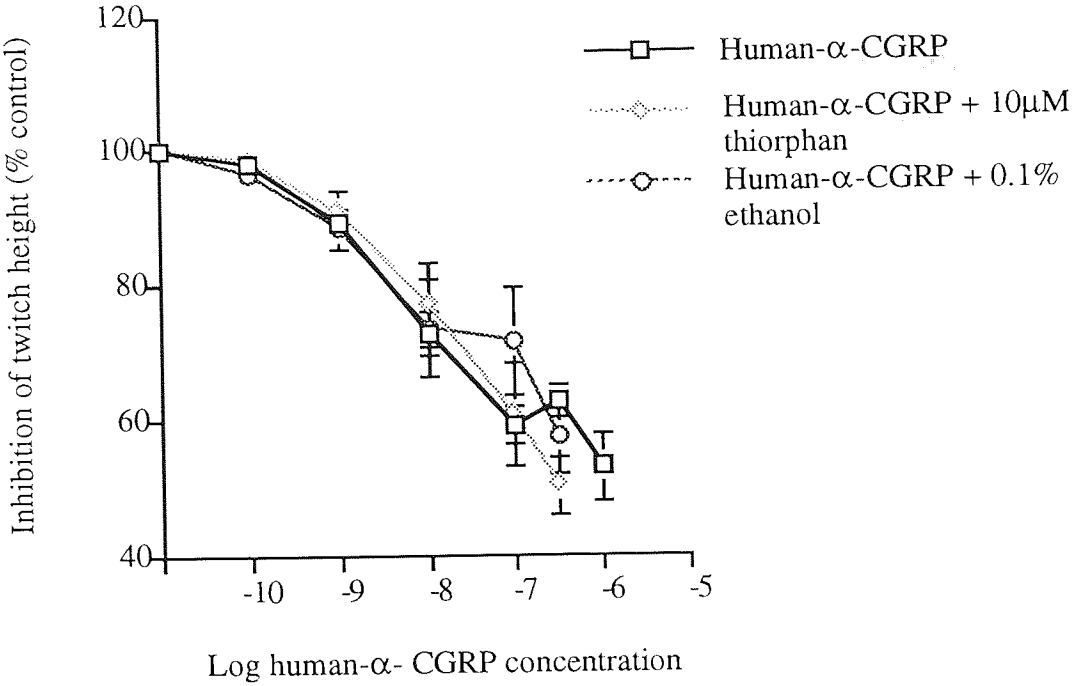


Figure 4.3 *Inhibition of electrically-evoked twitch contraction of the isolated guinea-pig ileum by increasing concentrations of human-α-CGRP (□), human-α-CGRP + thiorphan (10μM) (◇), and human-α-CGRP + ethanol (0.1%) (○). Values of EC_{50} from fits ± s.e.m: human-α-CGRP = 7.7 ± 8.7nM, human-α-CGRP + thiorphan = 33.0 ± 7.3nM, human-α-CGRP + ethanol = 7.5 ± 2.5nM. s.e.mean shown by the vertical lines. The points represent means of 52, 9 and 8 separate experiments respectively. Controls were taken as twitch amplitude prior to addition of agonist.*

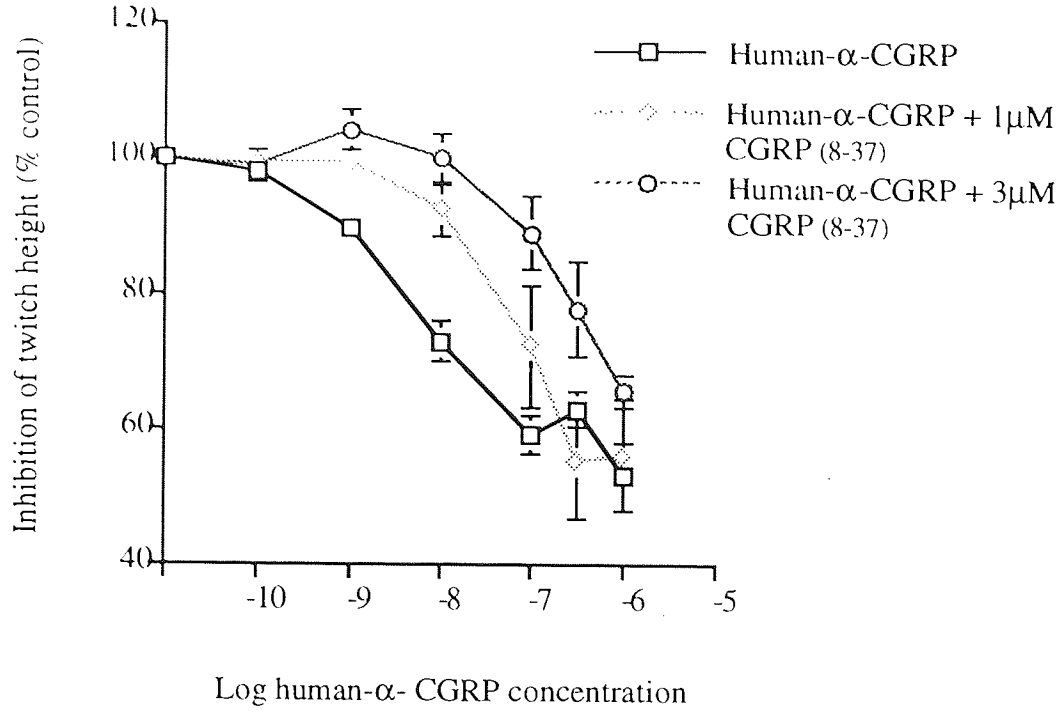


Figure 4.4 *Inhibition of electrically-evoked twitch contraction of the isolated guinea-pig ileum by increasing concentrations of human-α-CGRP (□), human-α-CGRP + 1μM CGRP$_{(8-37)}$ (◇), and human-α-CGRP + 3μM CGRP$_{(8-37)}$ (○). Values of EC$_{50}$ from fits ± s.e.m: human-α-CGRP = 7.7 ± 8.7nM, human-α-CGRP + 1μM CGRP$_{(8-37)}$ = 110 ± 9.6nM, human-α-CGRP + 3μM CGRP$_{(8-37)}$ = 260 ± 130nM. s.e.mean shown by the vertical lines. The points represent means of 52, 9 and 5 separate experiments respectively. Controls were taken as twitch amplitude prior to addition of agonist.* The estimated pA_2 value for this data = 7.2

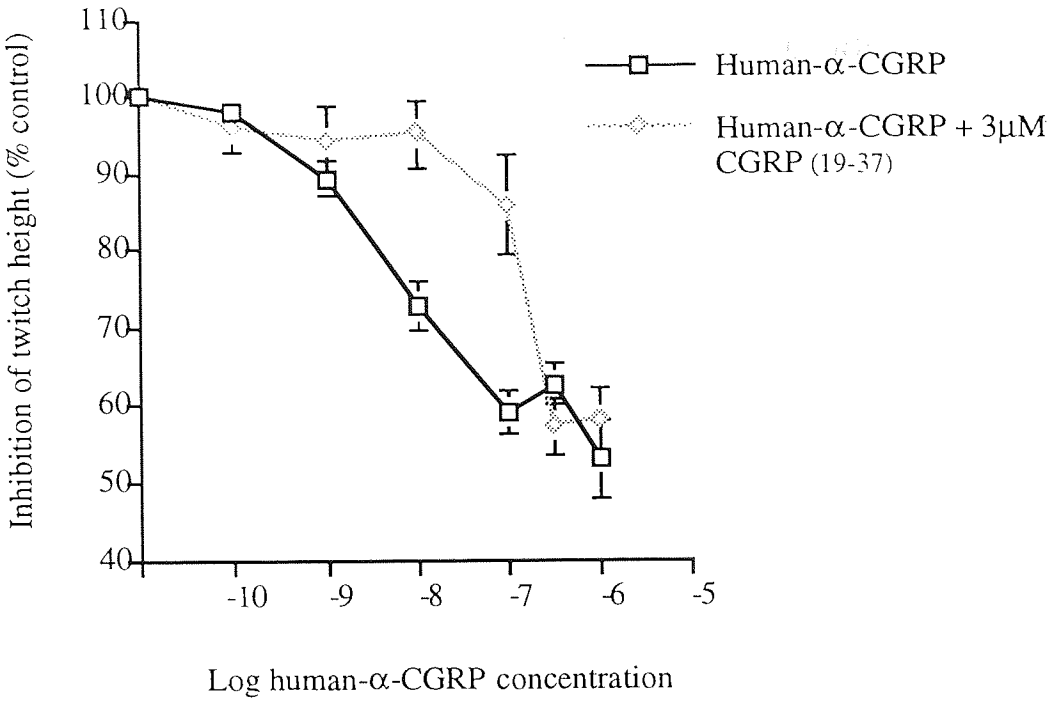


Figure 4.5 *Inhibition of electrically-evoked twitch contraction of the isolated guinea-pig ileum by increasing concentrations of human-α-CGRP (□), human-α-CGRP + 3μM CGRP$_{(19-37)}$ (◇). Values of EC_{50} from fits ± s.e.m: human-α-CGRP = 7.7 ± 8.7nM, human-α-CGRP + 3μM CGRP$_{(19-37)}$ = 210 ± 89.0nM. s.e.mean shown by the vertical lines. The points represent means of 52 and 4 separate experiments respectively. Controls were taken as twitch amplitude prior to addition of agonist.*

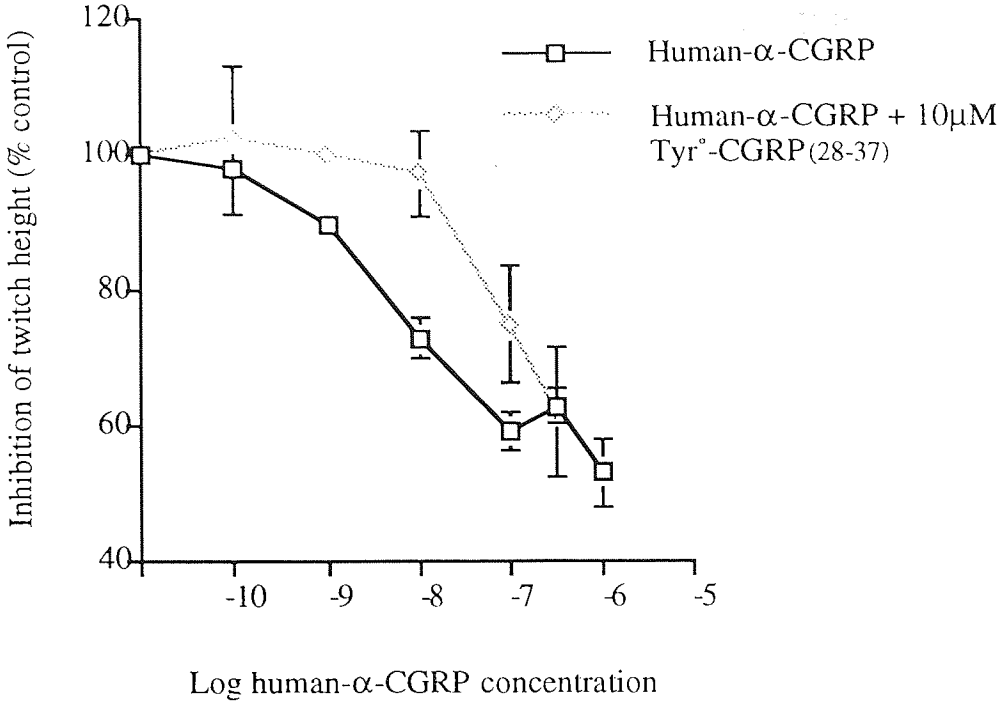


Figure 4.6 *Inhibition of electrically-evoked twitch contraction of the isolated guinea-pig ileum by increasing concentrations of human-α-CGRP (□), human-α-CGRP + 10μM Tyr°-CGRP(28-37) (◇). Values of EC_{50} from fits ± s.e.m: human-α-CGRP = 7.7 ± 8.7nM, human-α-CGRP + 10μM Tyr°-CGRP(28-37) = 88.0 ± 1.9nM. s.e.mean shown by the vertical lines. The points represent means of 52 and 6 separate experiments respectively. Controls were taken as twitch amplitude prior to addition of agonist.*

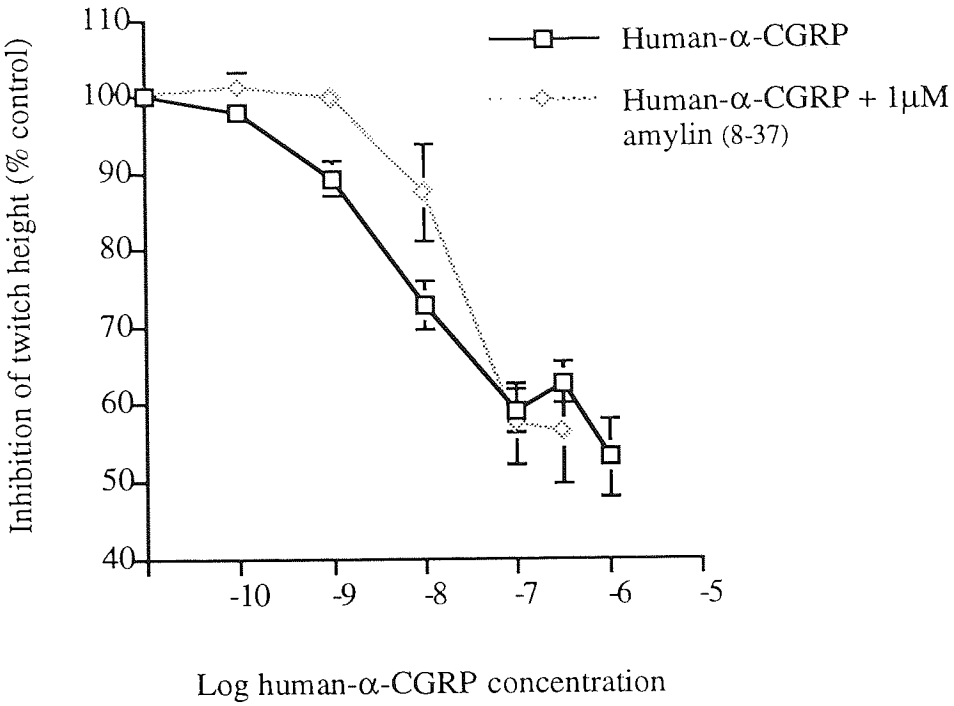


Figure 4.7a *Inhibition of electrically-evoked twitch contraction of the isolated guinea-pig ileum by increasing concentrations of human-α-CGRP (□), human-α-CGRP + 1μM amylin$_{(8-37)}$ (◇). Values of EC_{50} from fits ± s.e.m: human-α-CGRP = 7.7 ± 8.7nM, human-α-CGRP + 1μM amylin$_{(8-37)}$ = 74.0 ± 23.0nM. s.e.mean shown by the vertical lines. The points represent means of 52 and 7 separate experiments respectively. Controls were taken as twitch amplitude prior to addition of agonist.*

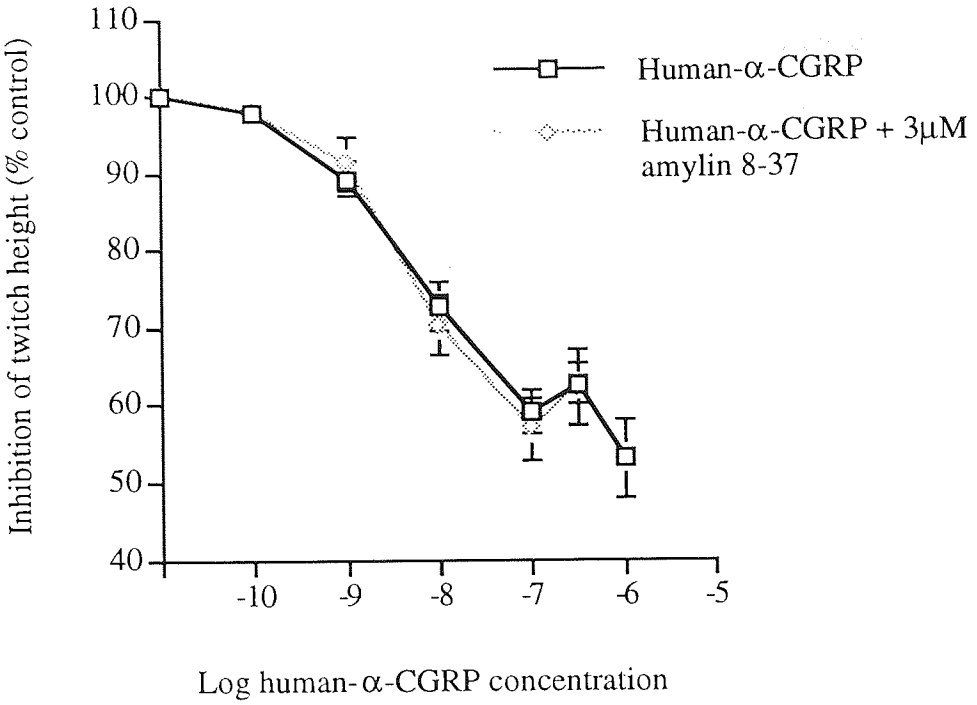


Figure 4.7b *Inhibition of electrically-evoked twitch contraction of the isolated guinea-pig ileum by increasing concentrations of human-α-CGRP (□), human-α-CGRP + 3μM CGRP$_{(8-37)}$ (◇). Values of EC$_{50}$ from fits ± s.e.m: human-α-CGRP = 7.7 ± 8.7nM, human-α-CGRP + 3μM amylin$_{(8-37)}$ = 19.3 ± 12.0nM. s.e.mean shown by the vertical lines. The points represent means of 52 and 6 separate experiments respectively. Controls were taken as twitch amplitude prior to addition of agonist.*

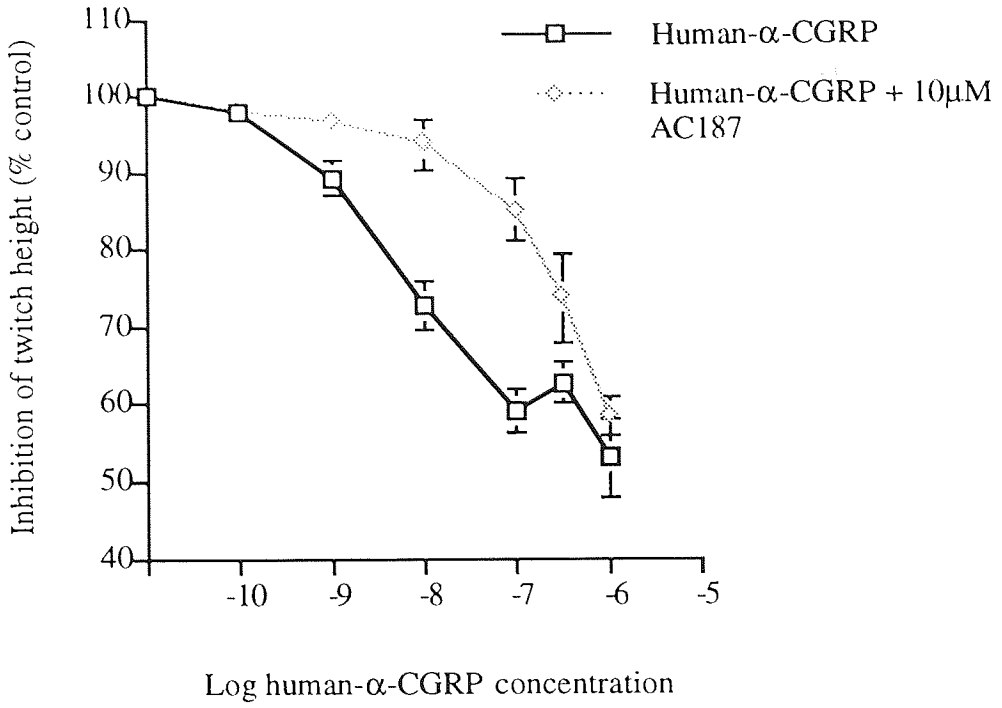


Figure 4.8 *Inhibition of electrically-evoked twitch contraction of the isolated guinea-pig ileum by increasing concentrations of human-α-CGRP (□), human-α-CGRP + 10μM AC187 (◇). Values of EC_{50} from fits ± s.e.m: human-α-CGRP = 7.7 ± 8.7nM, human-α-CGRP + 10μM AC187 = 340 ± 29.6nM. s.e.mean shown by the vertical lines. The points represent means of 52 and 3 separate experiments respectively. Controls were taken as twitch amplitude prior to addition of agonist.*

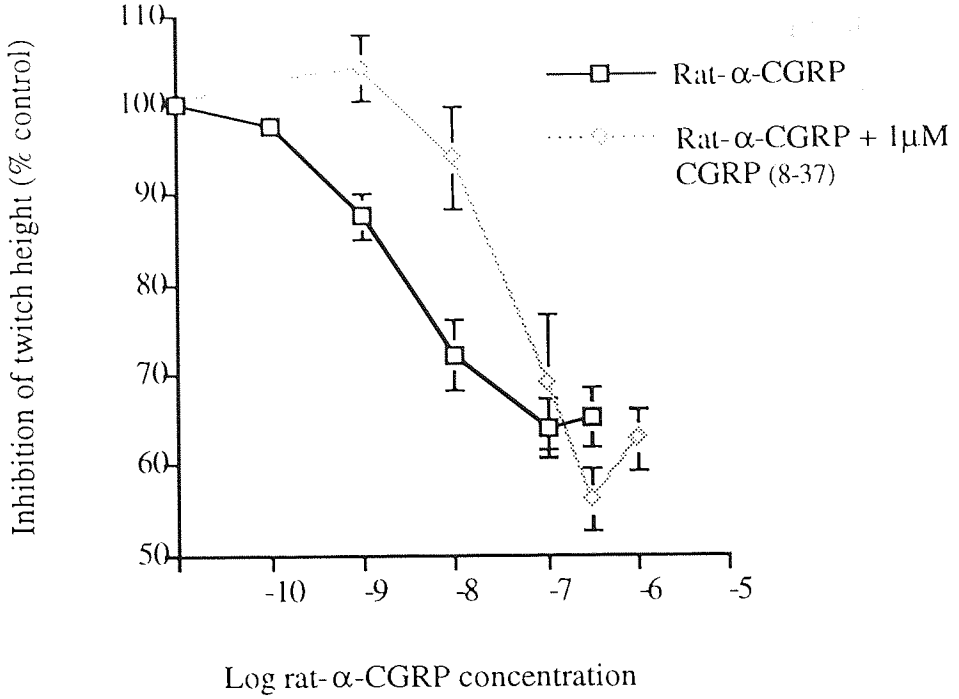


Figure 4.9 *Inhibition of electrically-evoked twitch contraction of the isolated guinea-pig ileum by increasing concentrations of rat-α-CGRP (□), rat-α-CGRP + 1μM CGRP$_{(8-37)}$ (◇). Values of EC$_{50}$ from fits ± s.e.m: rat-α-CGRP = 2.7 ± 4.9nM, rat-α-CGRP + 1μM CGRP$_{(8-37)}$ = 34.6 ± 12.0nM. s.e.mean shown by the vertical lines. The points represent means of 39 and 10 separate experiments respectively. Controls were taken as twitch amplitude prior to addition of agonist.*

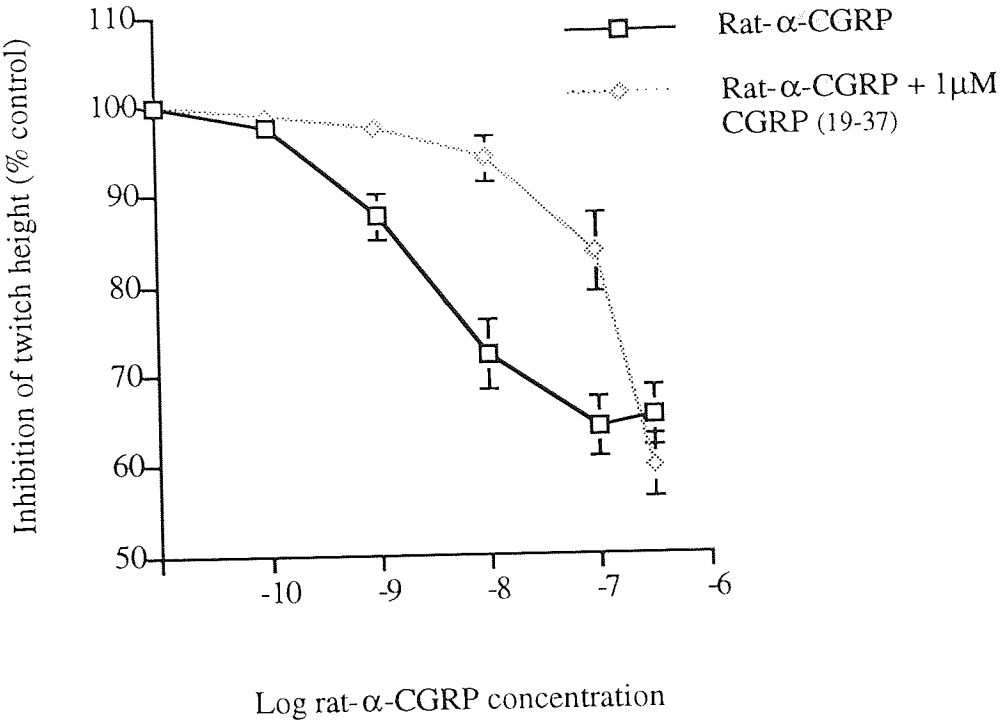


Figure 4.10 *Inhibition of electrically-evoked twitch contraction of the isolated guinea-pig ileum by increasing concentrations of rat-α-CGRP (□), rat-α-CGRP + 1μM CGRP$_{(19-37)}$ (◇). Values of EC$_{50}$ from fits ± s.e.m: rat-α-CGRP = 2.7 ± 4.9nM, rat-α-CGRP + 1μM CGRP$_{(19-37)}$ = 170 ± 14.0nM. s.e.mean shown by the vertical lines. The points represent means of 39 and 9 separate experiments respectively. Controls were taken as twitch amplitude prior to addition of agonist.*

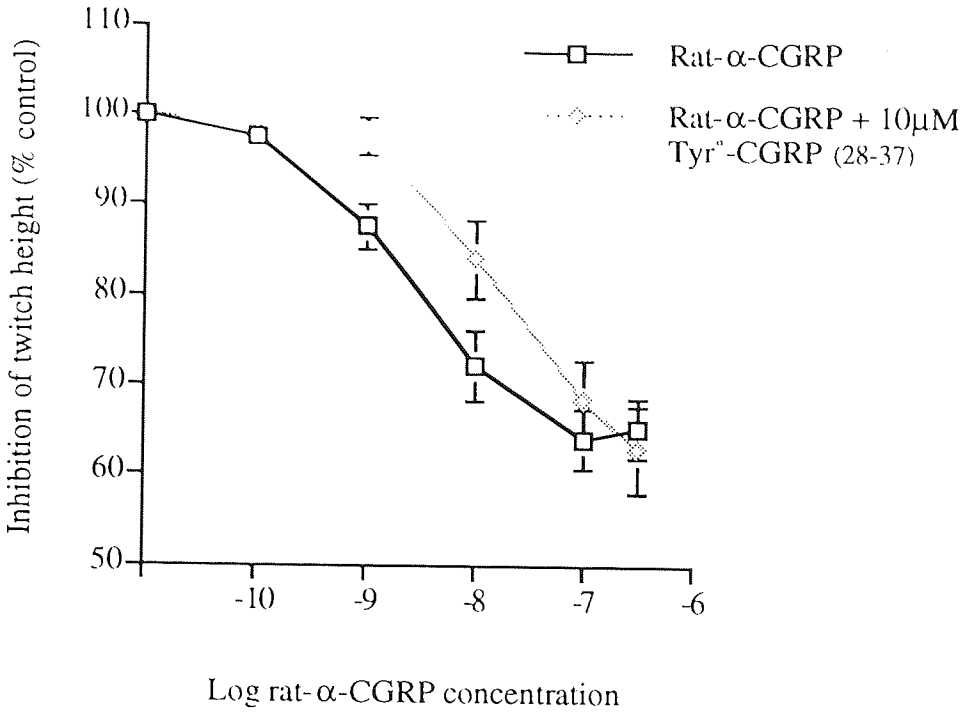


Figure 4.11 *Inhibition of electrically-evoked twitch contraction of the isolated guinea-pig ileum by increasing concentrations of rat-α-CGRP (□), rat-α-CGRP + 10μM Tyr°-CGRP$_{(28-37)}$ (◇). Values of EC_{50} from fits ± s.e.m: rat-α-CGRP = 2.7 ± 4.9nM, rat-α-CGRP + 10μM Tyr°-CGRP$_{(28-37)}$ = 19.4 ± 4.6nM. s.e.mean shown by the vertical lines. The points represent means of 39 and 6 separate experiments respectively. Controls were taken as twitch amplitude prior to addition of agonist.*

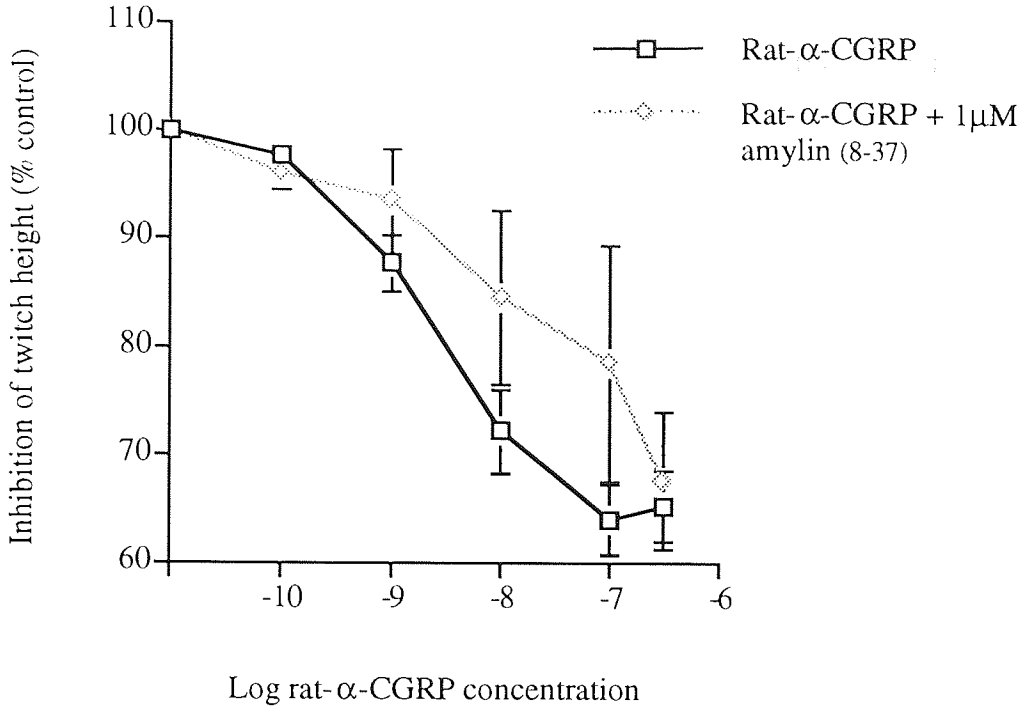


Figure 4.12 *Inhibition of electrically-evoked twitch contraction of the isolated guinea-pig ileum by increasing concentrations of rat-α-CGRP (□), rat-α-CGRP + 1μM amylin$_{(8-37)}$ (◇). Values of EC_{50} from fits ± s.e.m: rat-α-CGRP = 2.7 ± 4.9nM, rat-α-CGRP + 1μM amylin$_{(8-37)}$ = approximately 170 ± 74.0nM. s.e.mean shown by the vertical lines. The points represent means of 39 and 4 separate experiments respectively. Controls were taken as twitch amplitude prior to addition of agonist.*

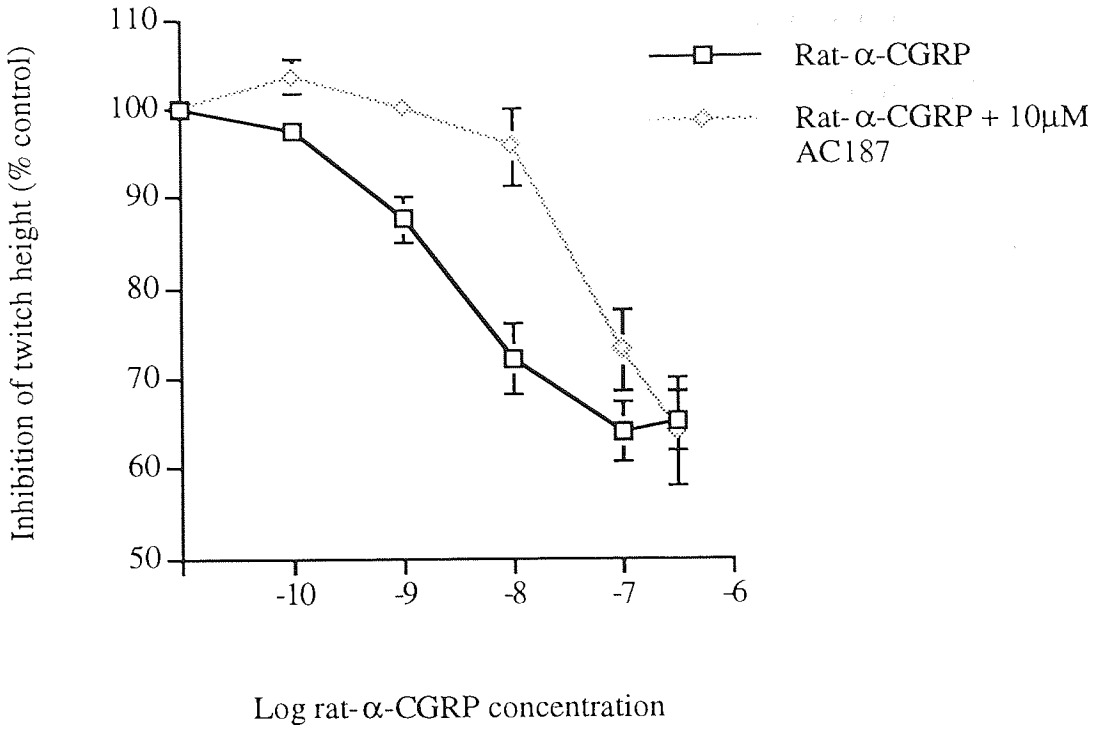


Figure 4.13 *Inhibition of electrically-evoked twitch contraction of the isolated guinea-pig ileum by increasing concentrations of rat-α-CGRP (□), rat-α-CGRP + 10μM AC187 (◇). Values of EC_{50} from fits ± s.e.m: rat-α-CGRP = 2.7 ± 4.9nM, rat-α-CGRP + 10μM AC187 = 84.0 ± 12.0nM. s.e.mean shown by the vertical lines. The points represent means of 39 and 3 separate experiments respectively. Controls were taken as twitch amplitude prior to addition of agonist.*

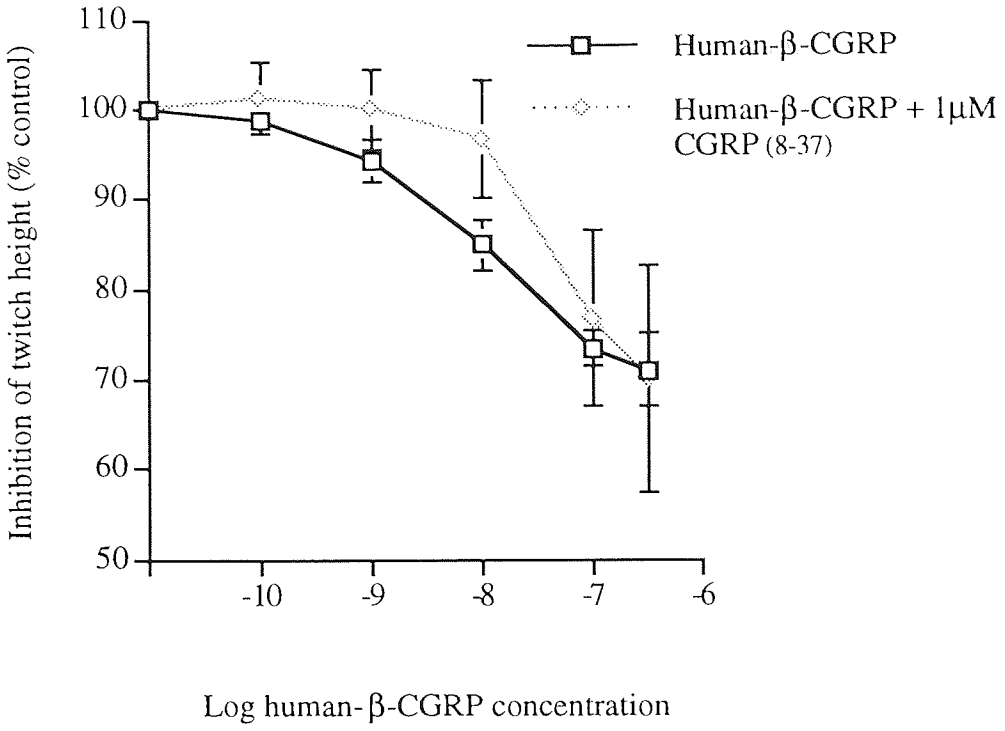


Figure 4.14 *Inhibition of electrically-evoked twitch contraction of the isolated guinea-pig ileum by increasing concentrations of human-β-CGRP (□), human-β-CGRP + 1μM CGRP$_{(8-37)}$ (◇). Values of EC_{50} from fits ± s.e.m: human-β-CGRP = 41.0 ± 13.0nM, human-β-CGRP + 1μM CGRP$_{(8-37)}$ = 103 ± 17.0nM. s.e.mean shown by the vertical lines. The points represent means of 8 and 3 separate experiments respectively. Controls were taken as twitch amplitude prior to addition of agonist.*

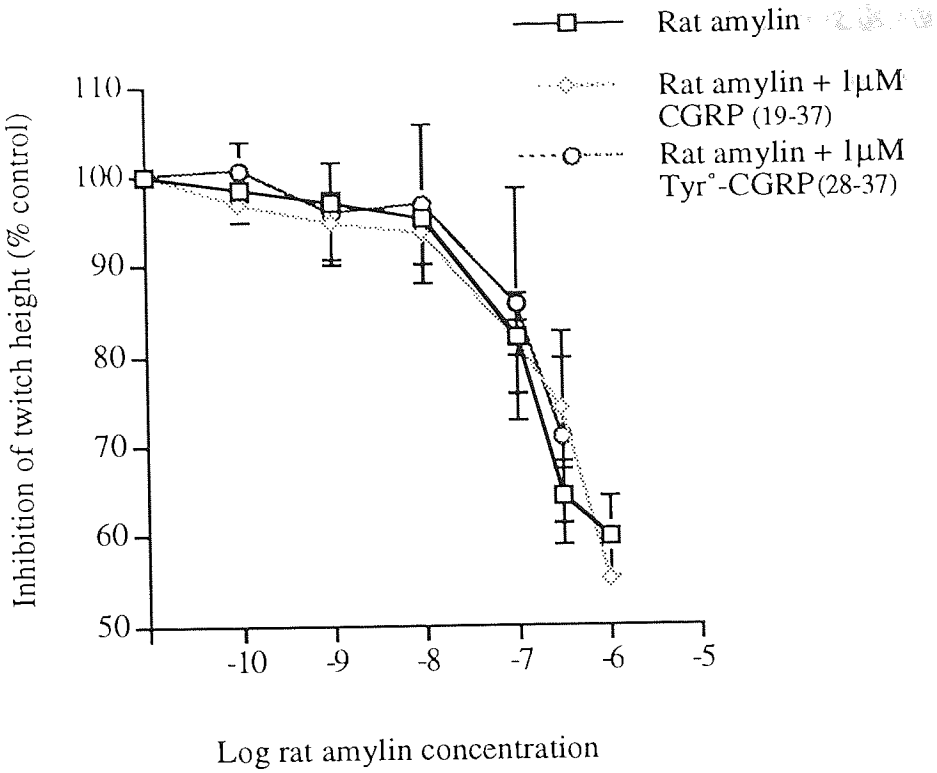


Figure 4.15 *Inhibition of electrically-evoked twitch contraction of the isolated guinea-pig ileum by increasing concentrations of rat amylin (□), rat amylin + 1μM $CGRP_{(19-37)}$ (◇), and rat amylin + 1μM Tyr°-$CGRP_{(28-37)}$ (○). Values of EC_{50} from fits ± s.e.m: rat amylin = 200 ± 49.0nM, rat amylin + 1μM $CGRP_{(19-37)}$ = 373 ± 55.0nM, rat amylin + 1μM Tyr°-$CGRP_{(28-37)}$ = 192 ± 21.1nM. s.e.mean shown by the vertical lines. The points represent means of 18, 4 and 4 separate experiments respectively. Controls were taken as twitch amplitude prior to addition of agonist.*

4.5 Discussion and Conclusions

4.5.1 CGRP and amylin receptor classification in the guinea-pig ileum

The results obtained from work on the guinea-pig ileum, support and extend the current pharmacology of CGRP and amylin. They provide evidence to support the theory that separate and distinct receptors for CGRP and amylin exist in this preparation, an idea proposed by previous studies on rat vas deferens, CHO-K1 cells and rat soleus muscle (Guiliani *et al.*, 1992; D'Santos *et al.*, 1992, Beaumont *et al.*, 1995).

Previous reports utilising the same methodology used here and the CGRP receptor antagonist $CGRP_{(8\text{-}37)}$, have proposed the existence of CGRP receptor subtypes in the guinea-pig ileum. CGRP produces relaxation of the electrically stimulated ileum by acting directly on the smooth muscle via receptors sensitive to $CGRP_{(8\text{-}37)}$. $CGRP_{(8\text{-}37)}$ has been shown to be ineffective against rat-α-CGRP-induced cholinergic contraction of longitudinal muscle (Dennis *et al.*, 1990; Bartho *et al.*, 1993). Dennis *et al* (1990) obtained a pA_2 value for the effects of $CGRP_{(8\text{-}37)}$ against human-α-CGRP in the guinea-pig ileum, of 7.2, a value in agreement with the estimated pA_2 found here of 7.2 (figure 4.4). Present CGRP receptor classification is based upon the antagonistic effects of $CGRP_{(8\text{-}37)}$ where $CGRP_1$ receptors are antagonised by this fragment but $CGRP_2$ receptors are not. These results show that mainly $CGRP_1$ receptors are present on the guinea-pig ileum and that activation of such receptors by CGRP produces smooth muscle relaxation directly.

In this study 1μM $CGRP_{(8\text{-}37)}$ was slightly more potent at antagonising the human-α-CGRP response than the rat-α-CGRP-induced response. The human-β-CGRP-induced response was however resistant to antagonism by 1μM $CGRP_{(8\text{-}37)}$. These results are consistent with the existence of 2 receptor subtypes in the guinea-pig ileum, one sensitive to human-α-CGRP and the other sensitive to human-β-CGRP and rat-α-CGRP. In the guinea-pig basilar artery $CGRP_{(8\text{-}37)}$ antagonised the human-α-CGRP response but not the human-β-CGRP-induced response (Jansen *et al.*, 1992)

supporting this hypothesis that the 2 forms of human CGRP are able to differentiate between CGRP receptor subtypes.

Two other CGRP fragments; $CGRP_{(19\text{-}37)}$ and $Tyr^{\circ}\text{-}CGRP_{(28\text{-}37)}$ were tested on this preparation to examine their antagonistic abilities, specificity of action and potencies. Differences between the actions of $CGRP_{(8\text{-}37)}$, $CGRP_{(19\text{-}37)}$ and $Tyr^{\circ}\text{-}CGRP_{(28\text{-}37)}$ may highlight differences in the areas of the CGRP molecule necessary for receptor binding.

$CGRP_{(19\text{-}37)}$ (3μM), like $CGRP_{(8\text{-}37)}$, highlighted differences between responses induced by human-α-CGRP and rat-α-CGRP. This antagonist was more potent at antagonising the rat-α-CGRP-induced inhibition of stimulated twitch height of the guinea-pig ileum than human-α-CGRP. This result taken in conjunction with the fact that $CGRP_{(8\text{-}37)}$ was slightly more potent at antagonising the human-α-CGRP-induced response compared to the rat-α-CGRP-induced response leads to the suggestion that human-α- and rat-α-CGRP may be working via different receptor subtypes in the guinea-pig ileum.

The human-α-CGRP induced response was most potently antagonised by $CGRP_{(8\text{-}37)}$, implying that amino acid residues 8-37 are important in human-α-CGRP receptor recognition. However, the rat-α-CGRP-induced response was most potently antagonised by $CGRP_{(19\text{-}37)}$ implying that the amino acid residues 19-37 are important for rat-α-CGRP receptor recognition. As $CGRP_{(8\text{-}37)}$ was not as potent an anatgonist as $CGRP_{(19\text{-}37)}$ against the rat-α-CGRP-induced effects this result may imply residues 8-18 reduce affinity at this receptor. Amino acids 8-18 have been reported to adopt the α-helical structure reported to be essential for the interaction of human-α-CGRP with its receptors (Lynch & Kaiser, 1988). This implies that the α-helical section is required for potent antagonism at the human-α-CGRP receptor but not at the rat-α-CGRP receptor.

Rat amylin was tested on this preparation to investigate the presence of amylin receptors and to examine any cross-reactivity between the CGRP and amylin receptors present.

The potency of the rat amylin-induced response was lower than that seen for human-α-CGRP, human-β-CGRP and rat-α-CGRP (figure 4.2) (26, 4.8 and 73 times, respectively). None of the CGRP amylin receptor antagonists caused antagonism of the rat amylin-induced response which implies the existence of a separate receptor at which rat amylin is an agonist. Given the low potency at which rat amylin was seen to work, it may be that the receptor present on the guinea-pig ileum is not an amylin receptor but another peptide receptor at which rat amylin could cross-react or simply that the amylin receptor is present at low density.

AC187 was 1.4 times more potent at antagonising the human-α-CGRP-induced response compared to the rat-α-CGRP-induced response in the guinea-pig ileum. Previous reports found AC187 had high affinity for amylin receptors on rat skeletal muscle and a lower affinity for the rat L6 skeletal muscle myocyte $CGRP_1$ receptors (Beaumont *et al.*, 1995). The results presented in this thesis suggest that human-α-CGRP is acting through its own distinct CGRP receptor in the guinea-pig ileum. As AC187 causes significant antagonism of the human-α-CGRP-induced response at this receptor, it does not appear to be acting as a specific amylin receptor antagonist in the guinea-pig ileum. Due to erratic responses with rat amylin it was not possible to investigate amylin sensitivity to AC187 in this preparation.

4.5.2 Conclusions

The results presented here suggest the existence of at least two receptors for CGRP on the guinea-pig isolated ileum; one recognising human-α-CGRP and a second recognising rat-α-CGRP and human-β-CGRP. In general, the antagonism produced by the CGRP fragments used appears to show that each of the putative receptor types has a different pattern of selectivity. The human-α-CGRP receptor being sensitive to $CGRP_{(8\text{-}37)}$ and the rat-α-CGRP receptor being sensitive to $CGRP_{(19\text{-}37)}$. Human-β-CGRP was insensitive to antagonism by $CGRP_{(8\text{-}37)}$ implying that it may be acting

via a $CGRP_2$ receptor but as activation of this receptor type in the ileum is reported to produce contraction this is unlikely. This result may imply that human-β-CGRP is working through a receptor distinct to the CGRP receptors present on the ileum or that the concentration of $CGRP_{(8\text{-}37)}$ was not high enough to produce competitive antagonism.

The structural requirements for high affinity binding to the aforementioned receptor subtypes may differ with amino acid residues 8-37 important for type 1 and 19-37 important for type 2. The mechanism of action of rat amylin is not clear and it may be working either through a specific amylin receptor, or in light of its low potency in this preparation it may be working via another separate peptide receptor. It is not however, working via a CGRP receptor.

Chapter 5

CGRP and amylin receptor classification in the guinea-pig vas deferens.

5.1 Introduction

5.1.1 CGRP and amylin receptor classification

As previously explained (chapter 1, section 1.2.1.1), the current CGRP receptor classification is based upon studies using rat vas deferens and guinea-pig atria and their sensitivity to antagonism by [Cys $(ACM)_{2,7}$]-human CGRP and $CGRP_{(8-37)}$. The rat vas deferens have been shown to possess $CGRP_2$ receptors which are reported to be insensitive to $CGRP_{(8-37)}$ (Quirion *et al.*, 1992; Mimeault *et al.*, 1991; Dennis *et al.*, 1990; Maggi *et al.*, 1991).

In the rat vas deferens rat-α-CGRP, rat-β-CGRP and the related peptide, amylin have been shown to cause concentration-dependent inhibition of electrically-evoked twitch contraction (Guiliani *et al.*, 1992). The authors have proposed the existence of two receptors; one activated by CGRP and another selective for amylin. This conclusion is based upon the resistance of the amylin-induced effects to antagonism by $CGRP_{(8-37)}$ (3μM), whilst human-α-CGRP is sensitive to antagonism by this CGRP fragment (pA_2 of 6.55; Maggi *et al.*, 1991) and that amylin is 100 times less potent than human-α-CGRP. Interestingly, $CGRP_{(8-37)}$ was able to distinguish between the two CGRP isoforms as the effect of human-β-CGRP was not inhibited by $CGRP_{(8-37)}$ (1μM) (Longmore *et al.*, 1994).

Little work using CGRP has been performed on guinea-pig vas deferens yet this preparation has been reported to possess $CGRP_2$ receptors (Quirion *et al.*, 1992; Mimeault *et al.*, 1991). The guinea-pig vas deferens has been shown to have a population of temperature-sensitive, high affinity binding sites for CGRP that are coupled to a G-protein (Van Rossum *et al.*, 1993). Electrically-evoked twitch contractions of the guinea-pig vas deferens have been shown to be composed of 2 phases both due to neurotransmitter release, an initial twitch response mediated by adenosine 5'-triphosphate (ATP) followed by a longer sustained relaxant phase mediated by noradrenaline. CGRP has been reported to inhibit the initial twitch response induced by ATP, by pre-junctional modulation of purinergic transmission (Ellis & Burnstock, 1989).

5.2 Aims and objectives

This study was designed to compliment previous work investigating CGRP and amylin receptor classification on the guinea-pig ileum (chapter 4). These experiments were designed to investigate the classification of CGRP and amylin receptors on guinea-pig isolated vas deferens in order to draw comparisons between CGRP receptors within the same animal species. The same CGRP receptor and amylin receptor agonists and antagonists were utilised as for the study on the ileum; human-α-CGRP, rat-α-CGRP, $CGRP_{(8-37)}$, $CGRP_{(19-37)}$ and Tyr^{0}-$CGRP_{(28-37)}$. This should allow subsequent comparisons between results obtained in the vas deferens and those obtained in the ileum. The rat vas deferens are reported to possess $CGRP_2$ receptors and this was also investigated using $CGRP_{(8-37)}$.

Human and rat amylin and the amylin receptor antagonists AC187 and $amylin_{(8-37)}$ were also used in order to examine cross-reactivity between amylin and CGRP receptors in this preparation.

The novel hypotensive peptide, adrenomedullin, which has substantial sequence homology with CGRP, has recently been reported to be capable of cross-reacting with CGRP receptors. Preliminary studies using this peptide were also carried out.

5.3 Methods and materials

The protocol is as outlined in chapter 2, section 2.2. CGRP is reported to produce relaxation of the rat vas deferens by sustaining the relaxant effects produced by noradrenaline and by pre-junctional inhibition of the purinergic contractions produced by ATP during electrical stimulation.

Thiorphan (10μM) was dissolved in ethanol and vehicle control experiments using 0.1% ethanol were conducted. The vehicle was not found to affect the tissue preparations on which it was tested (see figure 5.5). The vas deferens were incubated with thiorphan for 30 mins prior to addition of human-α-CGRP ± 3μM $CGRP_{(8-37)}$, human-α-CGRP ± 1μM human $amylin_{(8-37)}$ or rat amylin ± 1μM $CGRP_{(8-37)}$.

5.3.1 Data evaluation

EC_{50} values were obtained by EBDA-LIGAND and analysed statistically as described in section 2.4. n was taken as the number of animals, not individual tissue responses. When duplicate concentration-response curves were constructed on tissues from the same animal, values were normalised and means produced prior to statistical analysis. All results were normalised as % control response, where control values were determined as the stimulated twitch height prior to addition of agonist. Fold-shifts were calculated by division of EC_{50} values, taking the values calculated from the agonist control curves as 1.0. Maximal effects were not always achieved due to limited availability of agonist concentrations.

5.4 Results

Human-α-CGRP (figure 5.1), rat-α-CGRP, human amylin and rat amylin all produced concentration-dependent inhibition of electrically-evoked twitch contractions of the guinea-pig vas deferens (figure 5.2). The calculated EC_{50} values (± s.e.m) for the peptide agonists were, human-α-CGRP, 10.5 ± 3.3nM; rat-α-CGRP, 23.7 ± 3.6nM; human amylin, 166 ± 48.6nM and rat amylin, 99.0 ± 11.0nM.

The novel hypotensive agent adrenomedullin$_{(13-52)}$ has been shown to possess specific receptors on vascular smooth muscle cells with which CGRP has been shown to interact (Eguchi *et al.*, 1994). Adrenomedullin$_{(13-52)}$ produced concentration-dependent inhibition of stimulated twitch contraction in the guinea-pig vas deferens (see figure 5.3). The calculated EC_{50} value ± s.e.m was estimated to be 44.2 ± 2.1nM.

5.4.1 Responses to CGRP

The working hypothesis that CGRP has its own receptors on the guinea-pig vas deferens was investigated using human-α-CGRP and rat-α-CGRP and a number of CGRP receptor antagonists: Tyr°-$CGRP_{(28-37)}$, $CGRP_{(8-37)}$, $CGRP_{(19-37)}$, human and rat $amylin_{(8-37)}$, AC187 (acetyl-Asn^{30}, Tyr^{32}]-salmon $calcitonin_{(8-32)}$ (Young *et al.*, 1994) and $adrenomedullin_{(22-52)}$ ($AM_{(22-52)}$).

Tyr°-$CGRP_{(28-37)}$ (10μM) antagonised the human-α-CGRP-induced inhibition of twitch contraction (figure 5.4 & table 5.1), with calculated EC_{50} values ± s.e.m of human-α-CGRP, 10.5 ± 3.3nM and human-α-CGRP plus 10μM Tyr°-$CGRP_{(28-37)}$, 76.4 ± 10.8nM. Rat-α-CGRP was not antagonised by this CGRP receptor antagonist at the concentration tested (1.5μM). The calculated EC_{50} values ± s.e.m were rat-α-CGRP, 23.7 ± 3.6nM and rat-α-CGRP plus 1.5μM Tyr°-$CGRP_{(28-37)}$, 20.0 ± 5.0nM. This antagonist exhibited a low potency of action so further CGRP receptor antagonists were examined against the human-α-CGRP and rat-α-CGRP-induced control responses.

Human-α-CGRP was antagonised by 10μM $CGRP_{(19-37)}$ (figure 5.5 & table 5.1) with calculated EC_{50} values ± s.e.m of human-α-CGRP, 10.5 ± 3.3nM and human-α-CGRP plus 10μM $CGRP_{(19-37)}$, 119 ± 12.0nM. The rat-α-CGRP-induced response was not antagonised by $CGRP_{(19-37)}$ (10μM). The calculated EC_{50} values ± s.e.m were rat-α-CGRP, 23.7 ± 3.6nM and rat-α-CGRP plus 10μM $CGRP_{(19-37)}$, 19.8 ± 3.1nM.

$CGRP_{(8-37)}$ was examined against the human-α-CGRP and rat-α-CGRP-induced inhibition of stimulated twitch contraction. At concentrations up to 10μM, $CGRP_{(8-37)}$ did not cause antagonism of the human-α-CGRP or the rat-α-CGRP-induced responses (data not shown). The calculated EC_{50} values were human-α-CGRP, 10.5 ± 3.3nM and human-α-CGRP plus 3μM $CGRP_{(8-37)}$, 14.3 ± 2.8nM; rat-α-CGRP, 23.7 ± 3.6nM and rat-α-CGRP plus 10μM $CGRP_{(8-37)}$, 41.0 ± 10.5nM (values not significantly different from control $P \leq 0.05$).

Thiorphan (10μM), an inhibitor of endopeptidase 24:11, the enzyme responsible for breakdown of endogenous CGRP (Davies *et al.*, 1992), has been reported to potentiate the effects of CGRP in the rat vas deferens (Longmore *et al.*, 1994). The effects of 10μM thiorphan, upon the effects induced by CGRP, were examined in the guinea-pig vas deferens alongside its vehicle control, 0.1% ethanol (see figure 5.6) Thiorphan was subsequently used to examine the effects of 3μM $CGRP_{(8-37)}$ on the human-α-CGRP-induced response (data not shown). Thiorphan was not found to produce any significant shift of the concentration-response curve produced by human-α-CGRP (figure 5.6) nor the concentration-response curve produced by human-α-CGRP in the presence of 3μM $CGRP_{(8-37)}$.

The calculated EC_{50} values ± s.e.m were human-α-CGRP, 10.5 ± 3.3nM; human-α-CGRP plus 10μM thiorphan, 22.1 ± 10.8nM and human-α-CGRP plus 10μM thiorphan plus 3μM $CGRP_{(8-37)}$, 36.0 ± 13.8nM.

The CGRP receptor antagonists examined against the human-α-CGRP-induced inhibition of stimulated twitch contraction showed that $CGRP_{(19-37)}$ and Tyr^{0}-$CGRP_{(28-37)}$ caused significant antagonism (see table 5.1). $CGRP_{(8-37)}$ did not antagonise the CGRP-induced control responses, a finding which contrasts with those obtained using the rat vas deferens where human-α-CGRP-induced effects were weakly antagonised by this CGRP fragment at 1μM (Maggi *et al.*, 1991).

Three amylin receptor antagonists were tested against the human-α-CGRP and rat-α-CGRP-induced control responses: human $amylin_{(8-37)}$, rat $amylin_{(8-37)}$ and AC187 (figures 5.7 and 5.8). The novel hypotensive agent $adrenomedullin_{(13-52)}$ inhibited electrically-evoked twitch contractions in the guinea-pig vas deferens (figure 5.3), so the adrenomedullin fragment $AM_{(22-52)}$ was also tested against the CGRP-induced responses (see table 5.1).

The human-α-CGRP-induced response was not antagonised by 3μM human amylin$_{(8-37)}$ nor 3μM rat amylin$_{(8-37)}$ (data not shown).The calculated EC_{50} values ± s.e.m were human-α-CGRP, 10.5 ± 3.3nM, human-α-CGRP plus 3μM human amylin$_{(8-37)}$, 19.3 ± 12.0nM and human-α-CGRP plus 3μM rat amylin$_{(8-37)}$, 3.4 ± 5.8nM. Thiorphan did not potentiate the human-α-CGRP-induced response and did not alter the effects of 1μM human amylin$_{(8-37)}$ on the human-α-CGRP-induced response, calculated EC_{50} values ± s.e.m of human-α-CGRP, 10.5 ± 3.3nM, human-α-CGRP plus 10μM thiorphan, 21.1 ± 10.8nM and human-α-CGRP plus 10μM thiorphan plus 1μM human amylin$_{(8-37)}$, 13.0 ± 3.4nM.

The rat-α-CGRP-induced response was significantly antagonised by 1μM human amylin$_{(8-37)}$ (figure 5.7), calculated EC_{50} values ± s.e.m were rat-α-CGRP, 23.7 ± 3.6nM and rat-α-CGRP plus 1μM human amylin$_{(8-37)}$, 303 ± 28.7nM. Rat amylin$_{(8-37)}$ was not tested against rat-α-CGRP.

AC187 (10μM) was tested against the CGRP-induced responses in the guinea-pig vas deferens. No significant antagonism of the agonist responses was observed, calculated EC_{50} values ± s.e.m were human-α-CGRP, 10.5 ± 3.3nM, human-α-CGRP plus 10μM AC187, 13.6 ± 2.0nM; rat-α-CGRP, 23.7 ± 3.6nM and rat-α-CGRP plus 10μM AC187, 22.5 ± 19.5nM (figure 5.8).

The adrenomedullin fragment, AM$_{(22-52)}$ was examined against the CGRP-induced control responses for any possible antagonistic effects. At the concentration tested (0.3μM) no antagonism of the CGRP-induced responses was seen (data not shown). Calculated EC_{50} values ± s.e.m were human-α-CGRP, 10.5 ± 3.3nM, human-α-CGRP plus 0.3μM AM$_{(22-52)}$, 45.8 ± 25.1nM; rat-α-CGRP, 23.7 ± 3.6nM and rat-α-CGRP plus 0.3μM AM$_{(22-52)}$, 41.3 ± 18.9nM. Further experiments using higher concentrations of this peptide antagonist need to be performed.

Inhibitory fragment	Human-α-CGRP (EC_{50} ± s.e.m / nM)	Rat-α-CGRP (EC_{50} ± s.e.m / nM)
Control	10.5 ± 3.3 (n = 58)	23.7 ± 3.6 (n= 38)
Tyr°-CGRP(28-37)	(10μM) 76.4 ± 10.8 * (n = 5)	(1.5μM) 20.0 ± 5.1 (n = 4)
CGRP(19-37)	(10μM) 119 ± 12.0 * (n = 5)	(10μM) 19.8 ± 3.1 (n = 3)
CGRP(8-37)	(3μM) 14.3 ± 2.8 (n = 5)	(10μM) 41.0 ± 10.5 (n = 4)
Human-amylin(8-37)	(3μM) 19.3 ± 12.0 (n = 8)	(1μM) 303 ± 28.7 * (n = 4)
Rat-amylin(8-37)	(3μM) 3.4 ± 5.8 (n = 4)	ND
AC187	(10μM) 13.6 ± 2.0 (n = 3)	(10μM) 22.5 ± 19.5 (n = 3)
Adrenomedullin (22-52)	(0.3μM) 45.8 ± 25.1 (n = 4)	(0.3μM) 41.3 ± 18.9 (n = 4)

Table 5.1 *EC_{50} values ± s.e.m for human-α-CGRP and rat-α-CGRP alone and in the presence of a variety of antagonists on the isolated guinea-pig vas deferens. All values were taken from figures 5.2 & 5.4-5.8. All values shown are nM. * indicates significant inhibition of response for agonist alone found using a one-way ANOVA and post-hoc Dunnett's test ($P \leq 0.05$). ND = not determined.*

The response to human-α-CGRP was significantly antagonised by 10μM Tyr°-$CGRP_{(28-37)}$ and 10μM $CGRP_{(19-37)}$. 7 and 11 fold shifts were produced for the agonist response curves in the presence of the antagonist (see table 5.3). Rat-α-CGRP was significantly antagonised by 1μM human $amylin_{(8-37)}$ and a 12 fold shift of the

control curve was shown to be produced in the presence of the antagonist (see table 5.3).

5.4.2 Responses to amylin

As shown in section 5.4 and figure 5.2, human and rat amylin caused concentration-dependent inhibition of stimulated twitch contractions in the guinea-pig isolated vas deferens. The responses induced by these agonists were not significantly different to each other.

The same panel of CGRP receptor antagonists were utilised as for the study using human-α-CGRP and rat-α-CGRP (section 5.4.1)

Tyr°-CGRP$_{(28-37)}$ (10μM) did not significantly antagonise rat amylin or human amylin-induced inhibition of stimulated twitch contraction (see figure 5.9 & table 5.2). The calculated EC_{50} values ± s.e.m were human amylin, 166 ± 48.6nM, human amylin plus 10μM Tyr°-CGRP$_{(28-37)}$, 145 ± 9.7nM; rat amylin, 99.0 ± 11.0nM and rat amylin plus 10μM Tyr°-CGRP$_{(28-37)}$, 94.6 ± 28.0nM.

CGRP$_{(19-37)}$ (10μM) significantly antagonised the rat amylin-induced inhibition of stimulated twitch amplitude, producing a 2-fold shift of the response curve to rat amylin in the presence of the antagonist (see figure 5.10 and tables 5.2 & 5.3). The approximate fitted EC_{50} values ± s.e.m were rat amylin, 99.0 ± 11.0nM and rat amylin plus 10μM CGRP$_{(19-37)}$, 163 ± 19.4nM. The EC_{50} value for rat amylin plus 10μM CGRP$_{(19-37)}$ was only approximate as the response curve does not plateau at the higher concentrations used. The human amylin-induced control response was not antagonised by 10μM CGRP$_{(19-37)}$ the calculated EC_{50} values ± s.e.m were human amylin, 166 ± 48.6nM and human amylin plus 10μM CGRP$_{(19-37)}$, 75.5 ± 21.6nM.

$CGRP_{(8-37)}$ significantly antagonised both the rat and human amylin-induced responses (figure 5.11). $CGRP_{(8-37)}$ had a higher potency against rat amylin-induced responses than human amylin-induced responses (table 5.2). The calculated EC_{50} values ± s.e.m were rat amylin, 99.0 ± 11.0nM, rat amylin plus 1μM $CGRP_{(8-37)}$, 202 ± 14.3nM; human amylin 166 ± 48.6 and human amylin plus 1μM $CGRP_{(8-37)}$, 455 ± 54.3nM.

Thiorphan (10μM) was added to tissues prior to construction of the rat amylin control curves but made no significant difference to the EC_{50} values (± s.e.m): rat amylin, 99.0 ± 11.0nM and rat amylin plus 10μM thiorphan, 35.0 ± 25.0nM. Thiorphan (10μM) did not significantly affect the 1μM $CGRP_{(8-37)}$-induced antagonism of the rat amylin-induced control response. Calculated EC_{50} values ± s.e.m were rat amylin, 99.0 ± 11.0nM, rat amylin plus 1μM $CGRP_{(8-37)}$, 202 ± 14.3nM and rat amylin plus 1μM $CGRP_{(8-37)}$ plus 10μM thiorphan, 117 ± 27.6nM.

The results obtained using $CGRP_{(19-37)}$ and $CGRP_{(8-37)}$ may imply that amino acid residues 8-18 are important for receptor interactions at the amylin recognising receptor on the guinea-pig vas deferens. When these amino acid residues are combined with residues 19-37, binding affinity was seen to increase by approximately 34% against human amylin i.e. binding seems to be proportional to length of fragment. In the guinea-pig vas deferens amylin has been shown to be antagonised by CGRP receptor antagonists which were unable to recognise CGRP (section 5.4.1). This implies that amylin may have its own receptors in the guinea-pig vas deferens and that $CGRP_{(8-37)}$ is not selective for CGRP receptors in this preparation.

Human $amylin_{(8-37)}$ and rat $amylin_{(8-37)}$ (3μM) were tested against the amylin-induced control responses in the guinea-pig vas deferens. Human $amylin_{(8-37)}$ significantly antagonised the rat amylin-induced response (figure 5.12) but not that induced by human amylin. The calculated EC_{50} values ± s.e.m were: rat amylin, 99.0 ± 11.0nM, rat amylin plus 1μM human $amylin_{(8-37)}$, 234 ± 22.8nM; human amylin, 166 ± 48.6nM and human amylin plus 1μM human $amylin_{(8-37)}$, 279 ± 20.9nM. Rat

amylin$_{(8-37)}$ (3μM) was an ineffective antagonist of the rat amylin-induced responses (data not shown). The EC_{50} values ± s.e.m were rat amylin, 99.0 ± 11.0nM and rat amylin plus 3μM rat amylin$_{(8-37)}$, 154 ± 15.2nM. Rat amylin$_{(8-37)}$ was not examined against the human amylin-induced control response. The results suggest that the two types of amylin have distinct receptor subtypes in this preparation. They also may imply that human amylin$_{(8-37)}$ is receptor specific in this preparation for the 'rat amylin' receptor. Human and rat amylin differ in 6 amino acids between residues 18-29 therefore receptor differences may exist capable of distinguishing between the peptides.

AC187 (10μM) antagonised the rat amylin-induced response (figure 5.13) but not the response induced by human amylin (see table 5.2). The calculated EC_{50} values ± s.e.m were, rat amylin, 99.0 ± 11.0nM, rat amylin plus 10μM AC187, 277 ± 57.7nM; human amylin, 166 ± 48.6nM and human amylin plus 10μM AC187, 149 ± 46.0nM.
In common with AC187, AM$_{(22-52)}$ (0.3μM) was able to distinguish between the different types of amylin used, as rat amylin was subject to antagonism (figure 5.14) (EC_{50} values ± s.e.m of, rat amylin, 99.0 ± 11.0nM and rat amylin plus 0.3μM AM$_{(22-52)}$, 273 ± 28.0nM) whilst the effects of human amylin were not (EC_{50} values ± s.e.m were: human amylin, 166 ± 48.6nM and human amylin plus 0.3μM AM$_{(22-52)}$, 139 ± 32.2nM).
These results obtained using AC187 and AM$_{(22-52)}$ support the idea that distinct amylin receptors exist in the guinea-pig vas deferens which are able to distinguish between rat and human amylin.

Inhibitory fragment	Human amylin (EC_{50} ± s.e.m / nM)	Rat amylin (EC_{50} ± s.e.m / nM)
Control	166 ± 48.6 (n = 37)	99.0 ± 11.0 (n = 47)
Tyr°-CGRP(28-37) (10μM)	94.6 ± 28.0 (n = 3)	145 ± 9.7 (n = 4)
CGRP(19-37) (10μM)	75.5 ± 21.6 (n = 4)	163 ± 19.4* (n = 4)
CGRP(8-37)	(3μM) 455 ± 54.3 * (n = 5)	(1μM) 202 ± 14.3 * (n = 4)
Human-amylin(8-37) (3μM)	279 ± 20.9 (n = 4)	234 ± 22.8 * (n = 7)
Rat-amylin(8-37) (3μM)	ND	154 ± 15.2 (n = 3)
AC187 (10μM)	149 ± 46.0 (n = 9)	277 ± 57.7 * (n = 4)
Adrenomedullin (22-52) (0.3μM)	139 ± 32.2 (n = 4)	273 ± 28.0 * (n = 5)

Table 5.2 *EC_{50} values ± s.e.m for human amylin and rat amylin alone and in the presence of a variety of antagonists on the isolated guinea-pig vas deferens. All values were taken from figures 5.2 & 5.9-5.14. All values shown are nM. * indicates significant inhibition of response to agonist alone found using a one-way ANOVA and post-hoc Dunnett's test ($P \leq 0.05$). ND = not determined.*

Overall the pattern of antagonism of human and rat amylin-induced inhibition of electrically-evoked twitch contractions of the guinea-pig vas deferens was distinct for the 2 agonists used. Human amylin-induced responses were significantly antagonised by 3μM $CGRP_{(8-37)}$, producing a 3-fold shift from the control response curve (table 5.3). The rat amylin-induced responses were significantly antagonised by $CGRP_{(19-}$

$_{37)}$ (10μM), $CGRP_{(8-37)}$ (1μM), human $amylin_{(8-37)}$ (3μM), AC187 (10μM) and $AM_{(22-52)}$ (0.3μm).

5.4.3 Responses to adrenomedullin$_{(13-52)}$

As shown in section 5.4, figure 5.3, $adrenomedullin_{(13-52)}$ produced concentration-dependent inhibition of electrically-evoked twitch contractions of the guinea-pig vas deferens. The calculated EC_{50} value (± s.e.m), 44.2 (± 2.1nM), a value not significantly different to the values obtained for CGRP.

$Adrenomedullin_{(13-52)}$-induced control responses were not significantly shifted by the addition of 1μM $CGRP_{(8-37)}$ or 0.3μM $AM_{(22-52)}$ (figure 5.15). The calculated EC_{50} values ± s.e.m were, $adrenomedullin_{(13-52)}$, 44.2 ± 2.1nM, $adrenomedullin_{(13-52)}$ plus 1μM $CGRP_{(8-37)}$, 72.2 ± 17.0nM and $adrenomedullin_{(13-52)}$ plus 0.3μM $AM_{(22-52)}$, 44.2 ± 2.1nM.

Further work using this agonist and an extensive range of antagonists needs to be performed particularly as the guinea-pig vas deferens appears to be a tissue in which reproducible effects using adrenomedullin can be achieved

	Human-α-CGRP	Rat-α-CGRP	Human amylin	Rat amylin
Control	**1.0**	**1.0**	**1.0**	**1.0**
Tyr°-CGRP$_{(28-37)}$ (10μM)	7.3	(1.5μM) 0.8	0.6	1.5
CGRP$_{(19-37)}$ (10μM)	11.3	0.8	0.5	1.6
CGRP$_{(8-37)}$ (3μM)	1.4	(10μM) 1.7	2.7	(1μM) 2.0
Human amylin$_{(8-37)}$ (3μM)	1.8	12.8	1.7	2.4
Rat amylin (8-37) (3μM)	0.3	ND	ND	1.6
AC187 (10μM)	1.3	0.9	0.9	2.8
AM$_{(22-52)}$ (0.3μM)	4.4	1.7	0.8	2.8

Table 5.3 *Comparison of fold-shifts produced by CGRP and amylin receptor antagonists on the control responses to human-α-CGRP, rat-α-CGRP, human amylin and rat amylin in the guinea-pig vas deferens. Fold-shifts were calculated by division of EC_{50} values taking agonist values as 1.0. ND = value not determined. Where comparable concentrations of antagonist were not utilised the concentration is given ( ) above.*

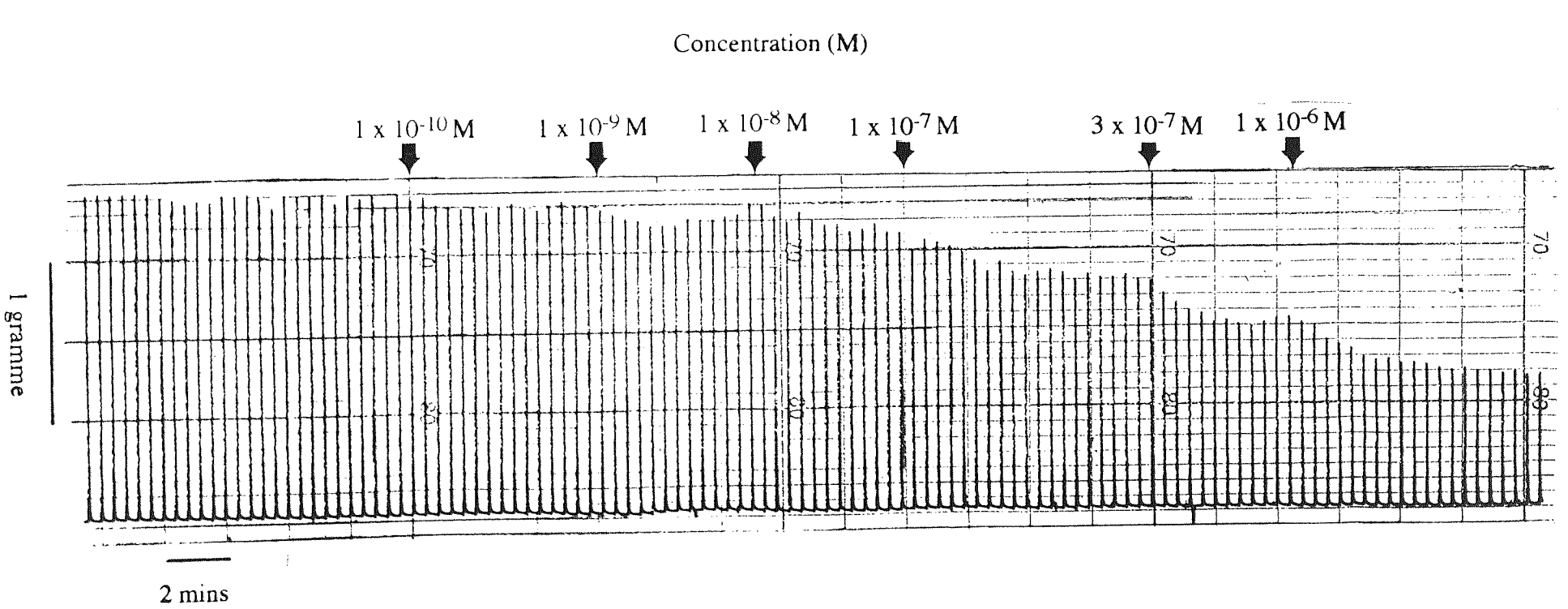


Figure 5.1 *Representative trace illustrating the concentration-related relaxant effect of human-α-CGRP in the electrically evoked twitch contractions of guinea-pig isolated vas deferens. The chart speed was set at 30 cm / hour.*

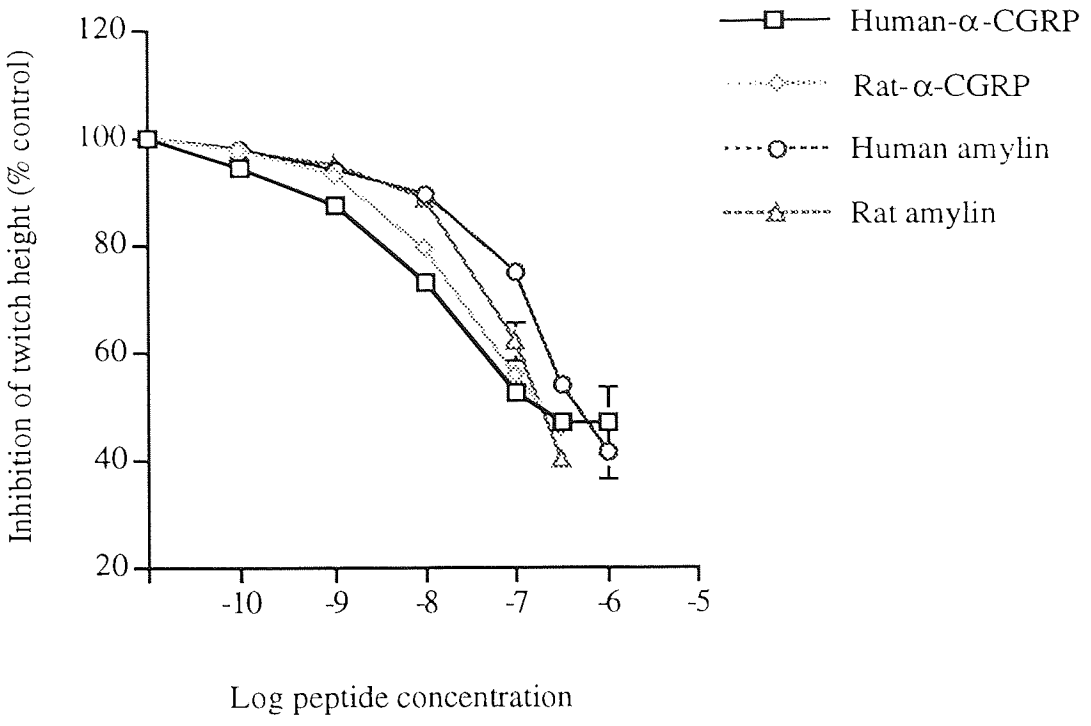


Figure 5.2 *Inhibition of electrically-evoked twitch contraction of the isolated guinea-pig vas deferens by increasing concentrations of human-α-CGRP (□), rat-α-CGRP (◇), human amylin (○) and rat amylin (△). Values of EC_{50} from fits ± s.e.m were human-α-CGRP = 10.5 ± 3.3nM, rat-α-CGRP = 23.7 ± 3.6nM, human amylin = 166 ± 48.6nM and rat amylin = 99.0 ± 11.0nM. s.e.mean shown by the vertical lines. The points represent means of 58, 38, 47 and 37 separate experiments respectively. Controls were taken as twitch amplitude prior to addition of agonist.*

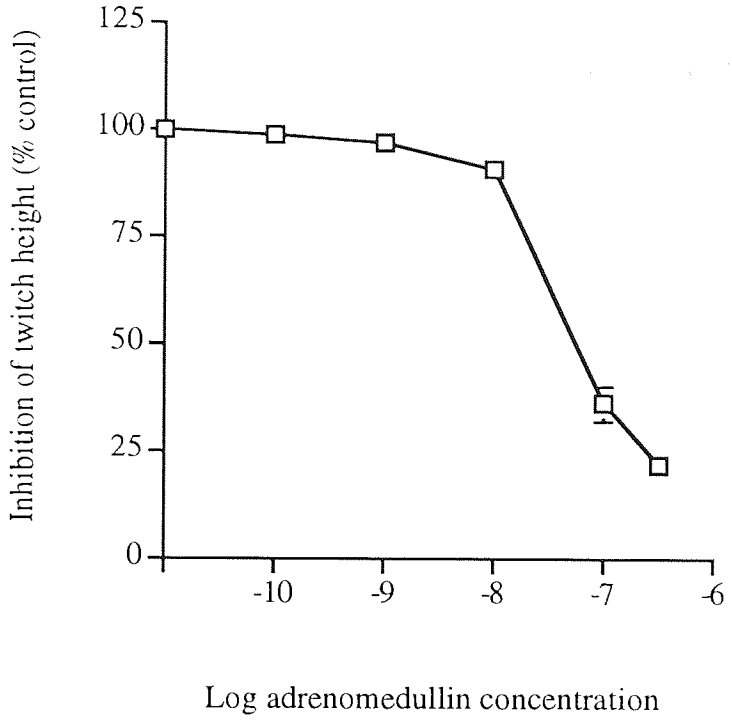


Figure 5.3 *Inhibition of electrically-evoked twitch contraction of the isolated guinea-pig vas deferens by increasing concentrations of adrenomedullin$_{(13-52)}$ (□). Value of EC_{50} from fit ± s.e.m was Adrenomedullin$_{(13-52)}$ = 44.2 ± 2.1nM. s.e.mean shown by the vertical lines. The points represent a mean of 6 separate experiments.*

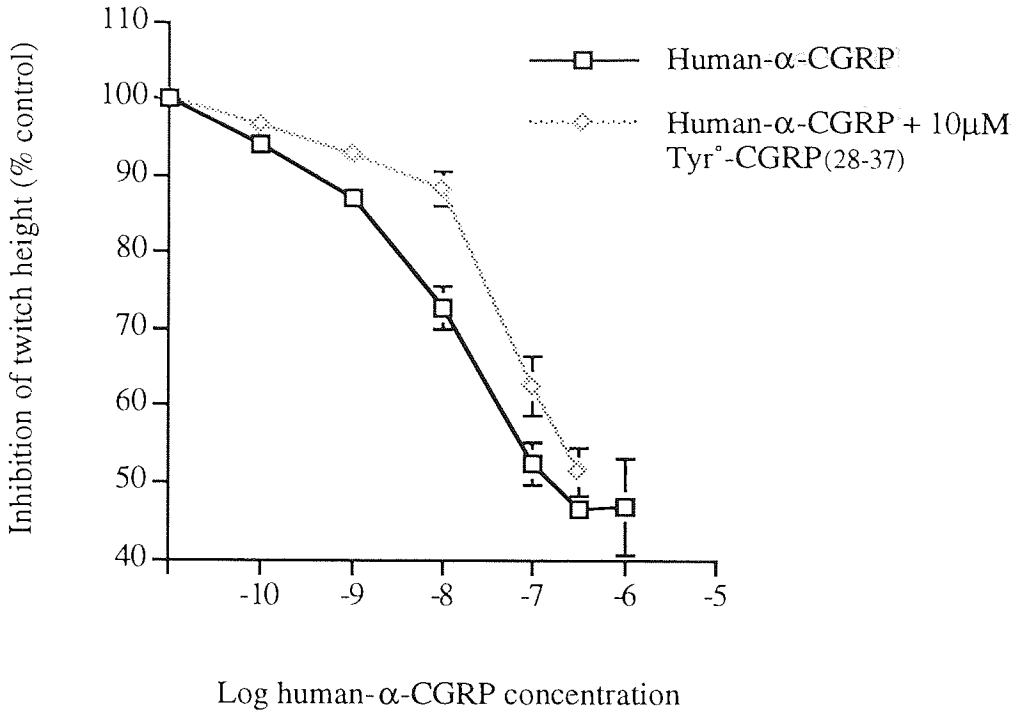


Figure 5.4 *Inhibition of electrically-evoked twitch contraction of the isolated guinea-pig vas deferens by increasing concentrations of human-α-CGRP (□), human-α-CGRP + 10μM Tyr°-CGRP$_{(28-37)}$ (◇). Values of EC_{50} from fits ± s.e.m were, human-α-CGRP = 10.5 ± 3.3nM, human-α-CGRP + 10μM Tyr°-CGRP$_{(28-37)}$ = 76.4 ± 10.8nM. s.e.mean shown by the vertical lines. The points represent means of 58 and 5 separate experiments respectively. Controls were taken as twitch amplitude prior to addition of agonist.*

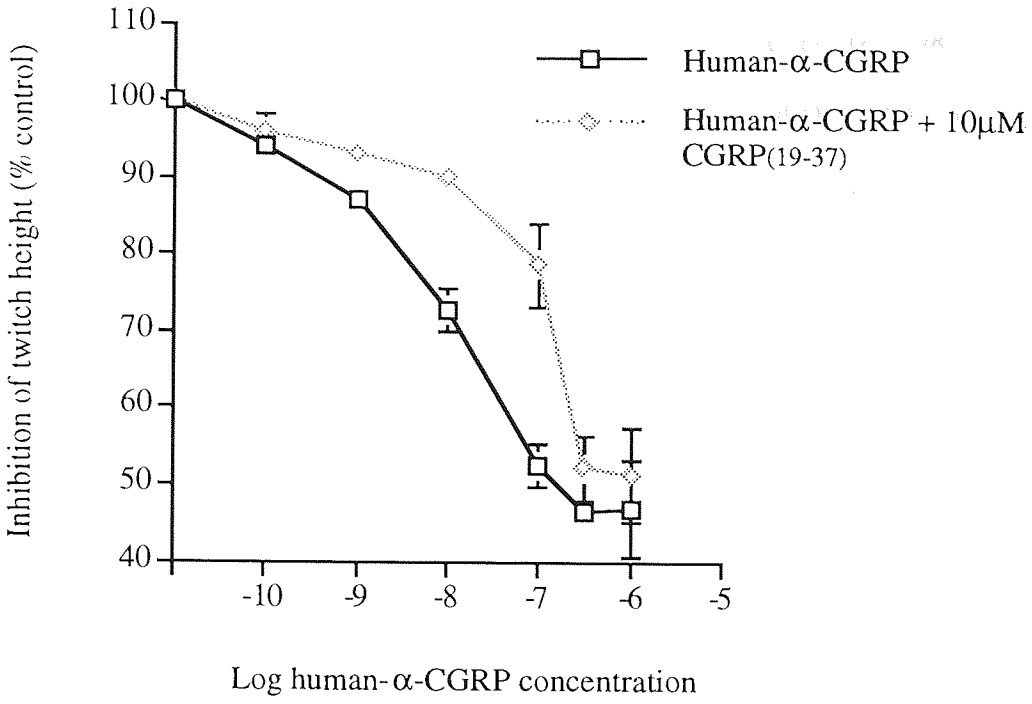


Figure 5.5 *Inhibition of electrically-evoked twitch contraction of the isolated guinea-pig vas deferens by increasing concentrations of human-α-CGRP (□), human-α-CGRP + 10μM CGRP$_{(19-37)}$ (◇). Values of EC_{50} from fits ± s.e.m were, human-α-CGRP = 10.5 ± 3.3nM, human-α-CGRP + 10μM CGRP$_{(19-37)}$ = 119 ± 12.0nM. s.e.mean shown by the vertical lines. The points represent means of 58 and 5 separate experiments respectively. Controls were taken as twitch amplitude prior to addition of agonist.*

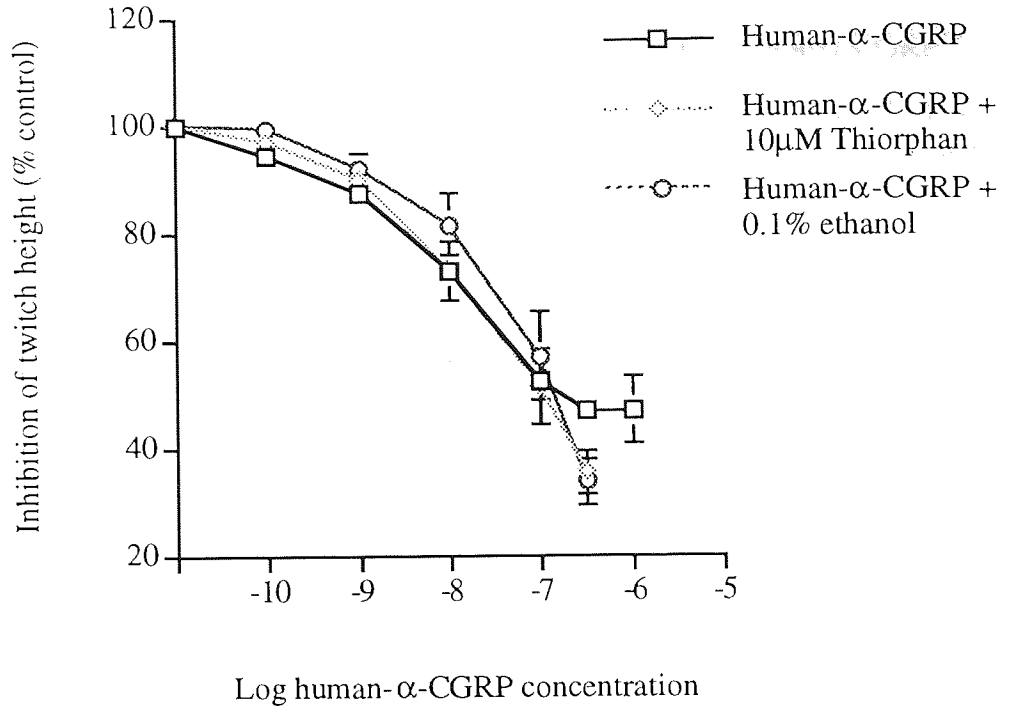


Figure 5.6 *Inhibition of electrically-evoked twitch contraction of the isolated guinea-pig vas deferens by increasing concentrations of human-α-CGRP (□), human-α-CGRP + thiorphan (10μM) (◇), and human-α-CGRP + ethanol (0.1%) (○). Values of EC_{50} from fits ± s.e.m were, human-α-CGRP = 10.5 ± 3.3nM, human-α-CGRP + thiorphan = 22.1 ± 10.8nM, human-α-CGRP + ethanol = 81.3 ± 20.0nM. s.e.mean shown by the vertical lines. The points represent means of 58, 13 and 9 separate experiments respectively. Controls were taken as twitch amplitude prior to addition of agonist.*

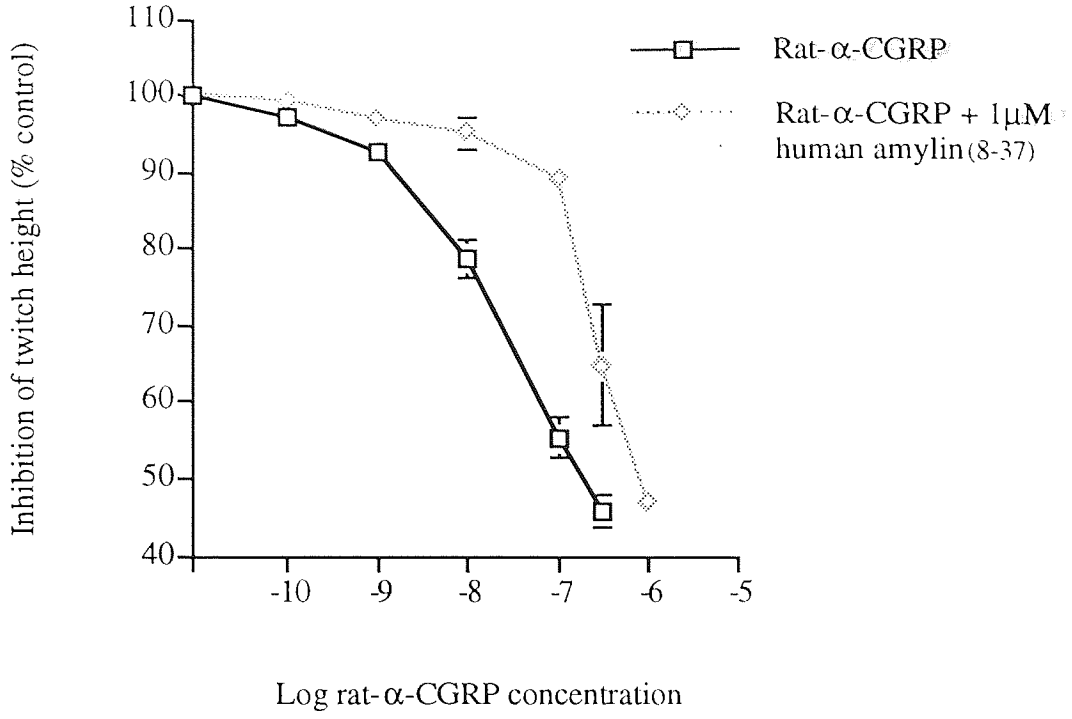


Figure 5.7 *Inhibition of electrically-evoked twitch contraction of the isolated guinea-pig vas deferens by increasing concentrations of rat-α-CGRP (□), rat-α-CGRP + 1μM amylin(8-37) (◇). Values of EC_{50} from fits ± s.e.m were, rat-α-CGRP = 23.7 ± 3.6nM, rat-α-CGRP + 1μM human amylin(8-37) = 303 ± 28.7nM. s.e.mean shown by the vertical lines. The points represent means of 38 and 4 separate experiments respectively. Controls were taken as twitch amplitude prior to addition of agonist.*

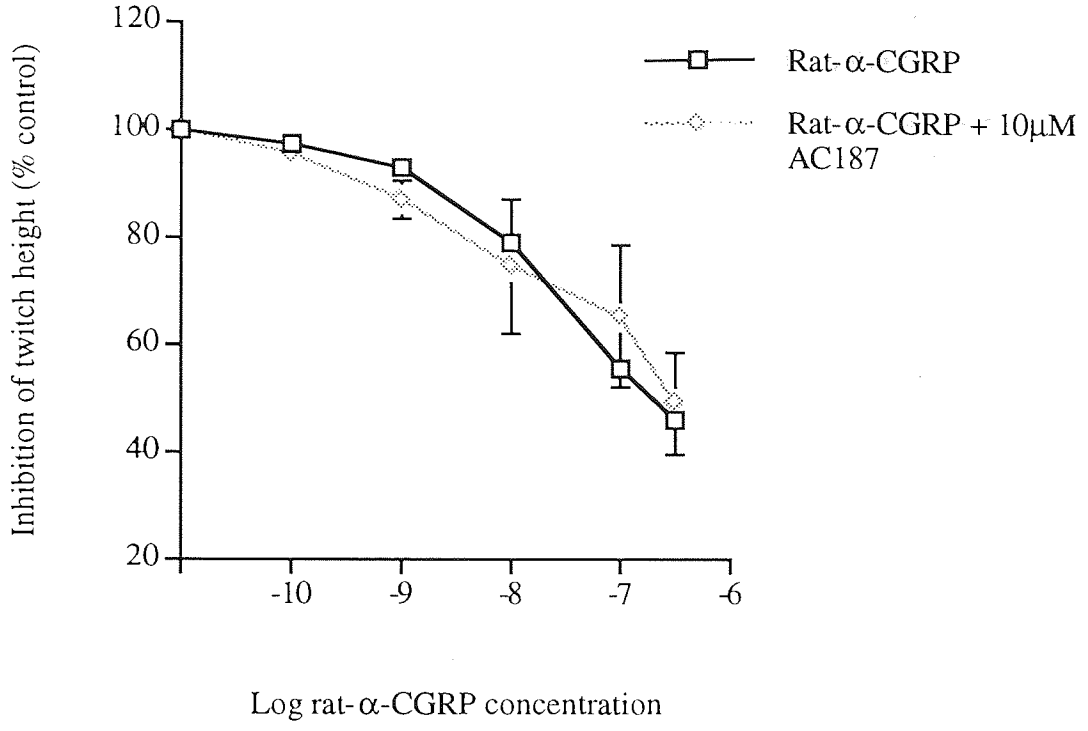


Figure 5.8 *Inhibition of electrically-evoked twitch contraction of the isolated guinea-pig vas deferens by increasing concentrations of rat-α-CGRP (□), rat-α-CGRP + 10μM AC187 (◇). Values of EC_{50} from fits ± s.e.m were, rat-α-CGRP = 23.7 ± 3.6nM, rat-α-CGRP + 10μM AC187 = 22.5 ± 19.5nM. s.e.mean shown by the vertical lines. The points represent means of 38 and 4 separate experiments respectively. Controls were taken as twitch amplitude prior to addition of agonist.*

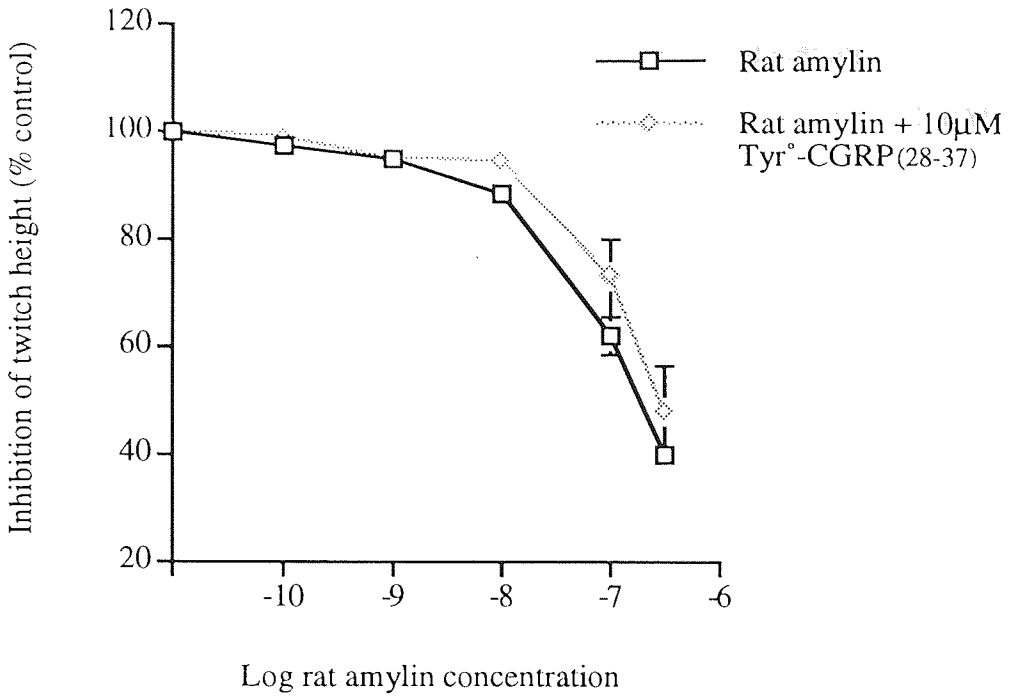


Figure 5.9 *Inhibition of electrically-evoked twitch contraction of the isolated guinea-pig vas deferens by increasing concentrations of rat amylin (□), rat amylin + 10μM Tyr°-CGRP(28-37) (◇). Values of EC_{50} from fits ± s.e.m were, rat amylin = 99.0 ± 11.0nM, rat amylin + 10μM Tyr°-CGRP(28-37) = 145 ± 9.7nM. s.e.mean shown by the vertical lines. The points represent means of 47 and 3 separate experiments respectively. Controls were taken as twitch amplitude prior to addition of agonist.*

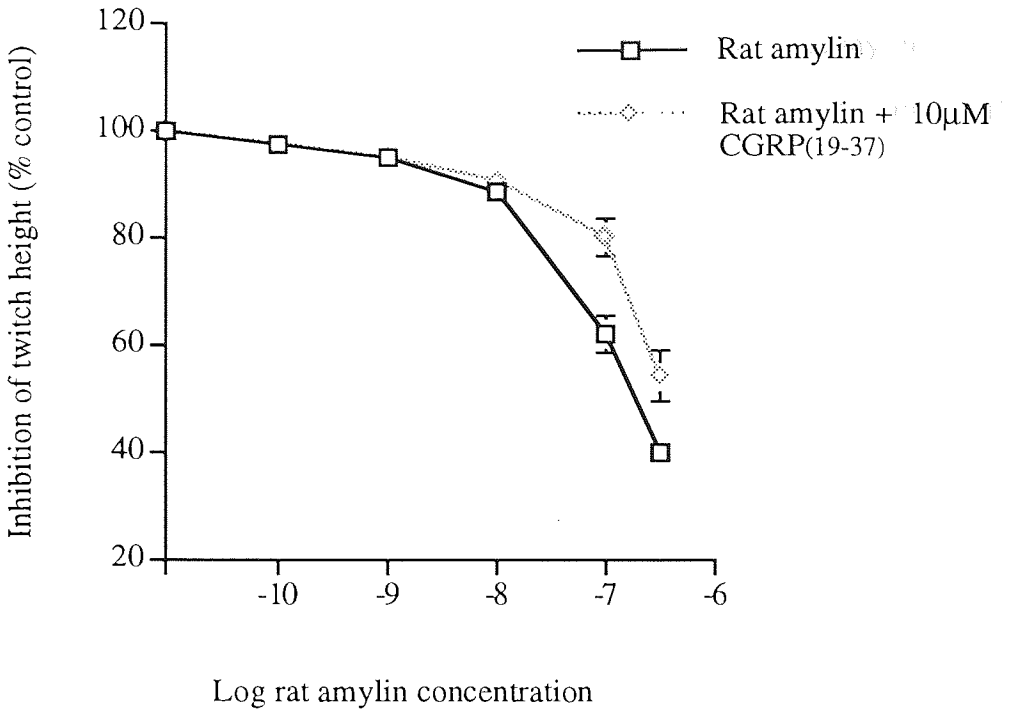


Figure 5.10 *Inhibition of electrically-evoked twitch contraction of the isolated guinea-pig vas deferens by increasing concentrations of rat amylin (□), rat amylin + 10μM CGRP$_{(19-37)}$ (◇). Values of EC_{50} from fits ± s.e.m were, rat amylin = 99.0 ± 11.0nM, rat amylin + 10μM CGRP$_{(19-37)}$ = 163 ± 19.4nM. s.e.mean shown by the vertical lines. The points represent means of 47 and 4 separate experiments respectively. Controls were taken as twitch amplitude prior to addition of agonist.*

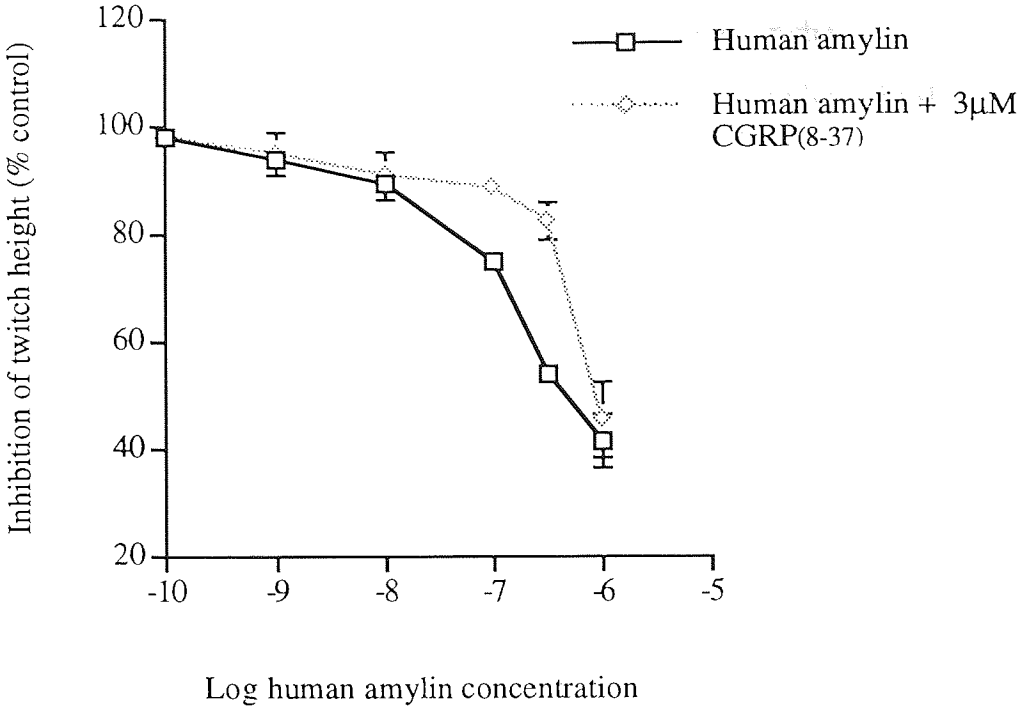


Figure 5.11 *Inhibition of electrically-evoked twitch contraction of the isolated guinea-pig vas deferens by increasing concentrations of human amylin (□), human amylin + 3μM CGRP$_{(8-37)}$ (◇). Values of EC$_{50}$ from fits ± s.e.m were, human amylin = 166 ± 48.6nM, human amylin + 3μM CGRP$_{(8-37)}$ = 455 ± 54.3nM. s.e.mean shown by the vertical lines. The points represent means of 37 and 5 separate experiments respectively. Controls were taken as twitch amplitude prior to addition of agonist.*

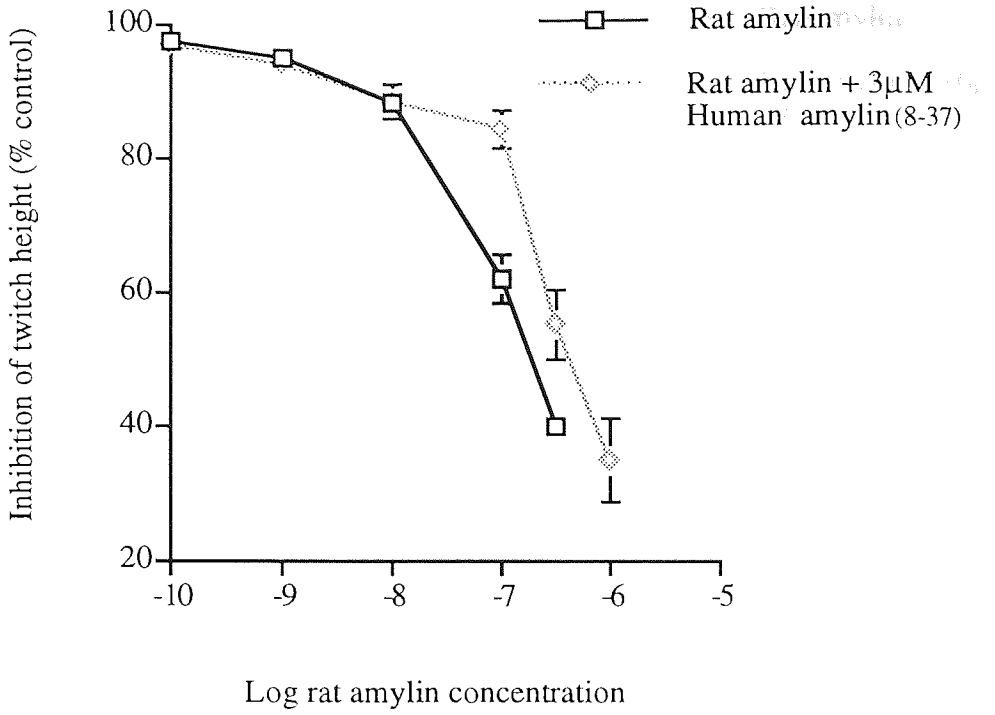


Figure 5.12 *Inhibition of electrically-evoked twitch contraction of the isolated guinea-pig vas deferens by increasing concentrations of rat amylin (□), rat amylin + 3μM human amylin$_{(8-37)}$ (◇). Values of EC_{50} from fits ± s.e.m were, rat amylin = 99.0 ± 11.0nM, rat amylin + 3μM human amylin$_{(8-37)}$ = 234 ± 22.8nM. s.e.mean shown by the vertical lines. The points represent means of 47 and 3 separate experiments respectively. Controls were taken as twitch amplitude prior to addition of agonist.*

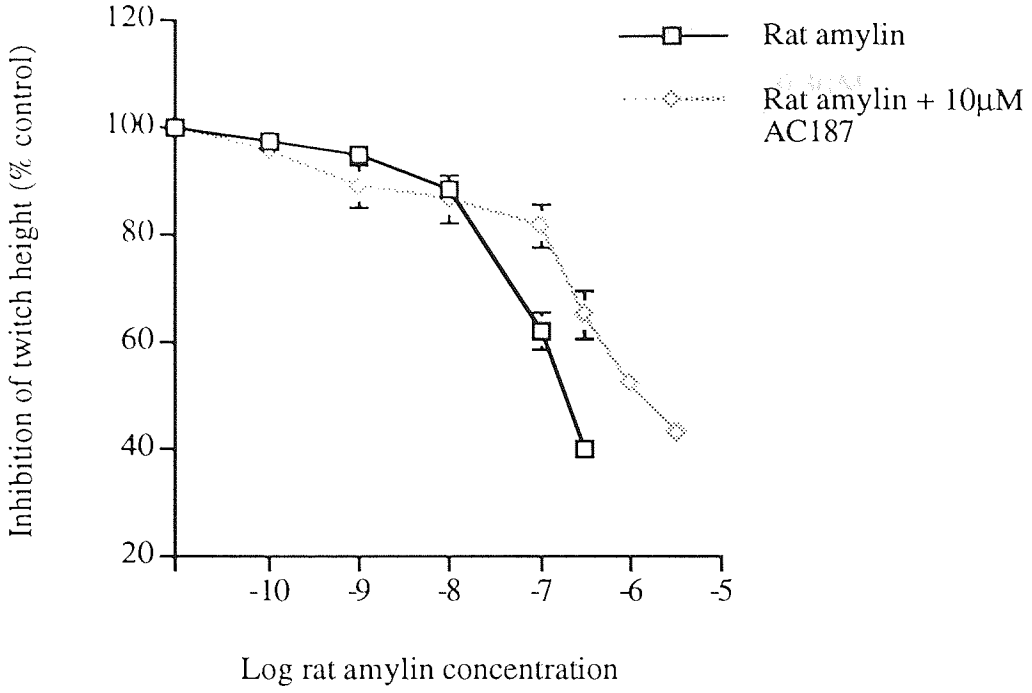


Figure 5.13 *Inhibition of electrically-evoked twitch contraction of the isolated guinea-pig vas deferens by increasing concentrations of rat amylin (□), rat amylin + 10μM AC187 (◇). Values of EC_{50} from fits ± s.e.m were, rat amylin = 99.0 ± 11.0nM, rat amylin + 10μM AC187 = 277 ± 57.7nM. s.e.mean shown by the vertical lines. The points represent means of 47 and 4 separate experiments respectively. Controls were taken as twitch amplitude prior to addition of agonist.*

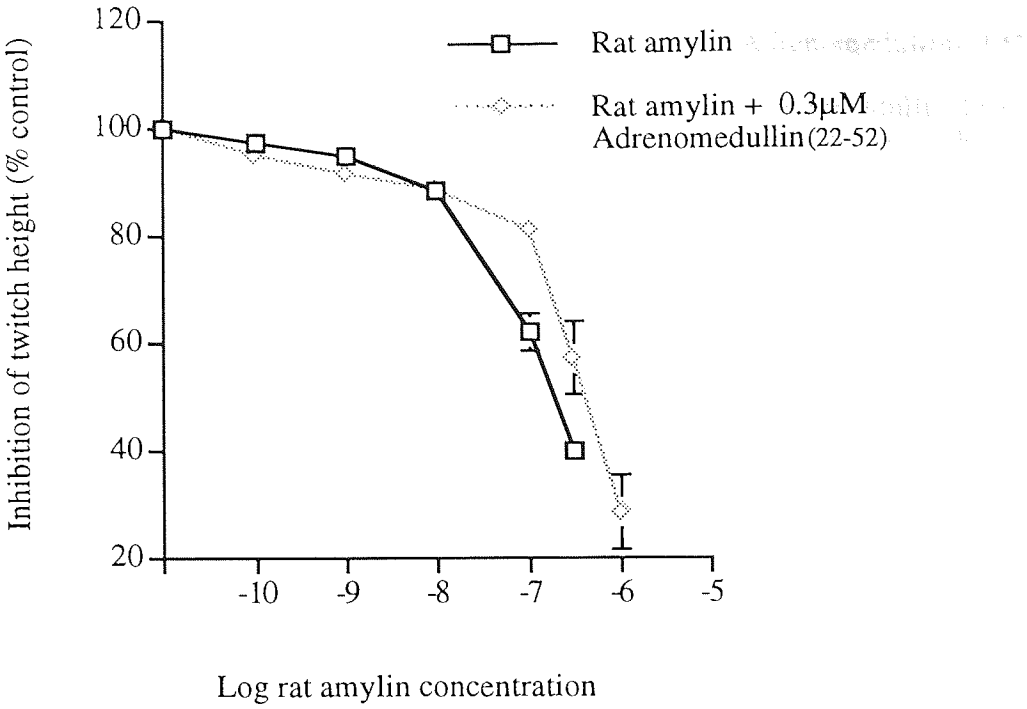


Figure 5.14 *Inhibition of electrically-evoked twitch contraction of the isolated guinea-pig vas deferens by increasing concentrations of rat amylin (□), rat amylin + 0.3μM adrenomedullin(22-52) (◇). Values of EC_{50} from fits ± s.e.m were, rat amylin = 99.0 ± 11.0nM, rat amylin + 0.3μM adrenomedullin(22-52) = 273nM ± 28.0nM. s.e.mean shown by the vertical lines. The points represent means of 47 and 5 separate experiments respectively. Controls were taken as twitch amplitude prior to addition of agonist.*

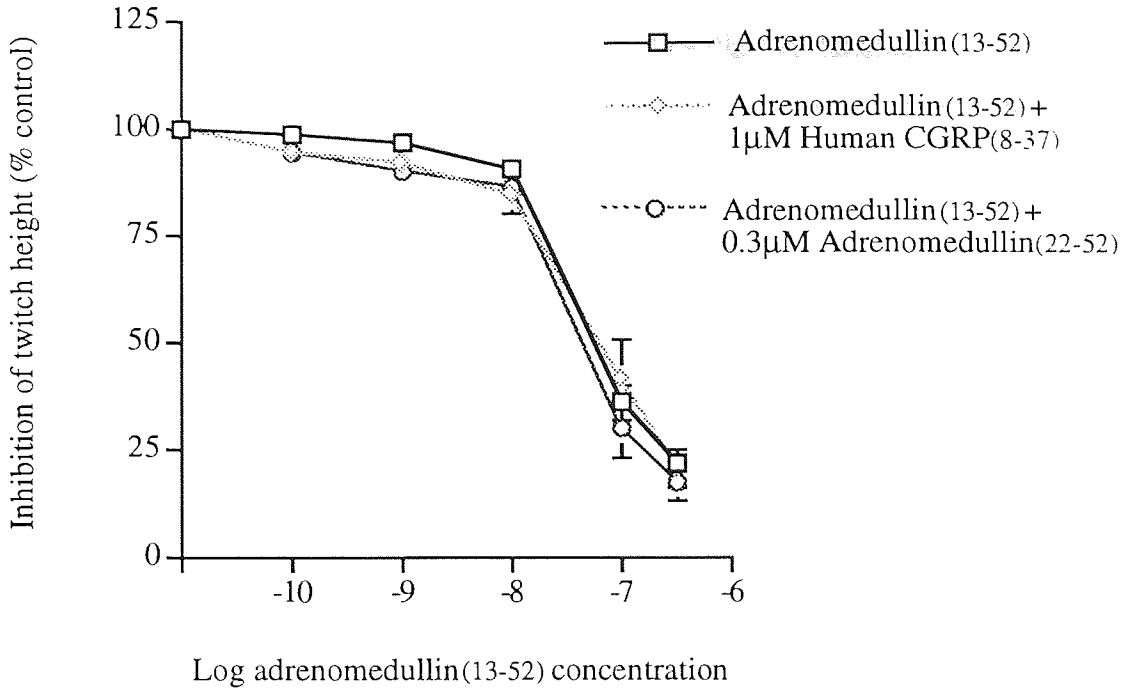


Figure 5.15 *Inhibition of electrically-evoked twitch contraction of the isolated guinea-pig vas deferens by increasing concentrations of adrenomedullin$_{(13-52)}$ (□), adrenomedullin$_{(13-52)}$ + 1μM CGRP$_{(8-37)}$ (◇), and adrenomedullin$_{(13-52)}$ + adrenomedullin$_{(22-52)}$ (○). Values of EC_{50} from fits ± s.e.m were, adrenomedullin$_{(13-52)}$ = 44.2 ± 2.1nM, adrenomedullin$_{(13-52)}$ + 1μM CGRP$_{(8-37)}$ = 72.2 ± 17.0nM and adrenomedullin$_{(13-52)}$ + 0.3μM adrenomedullin$_{(22-52)}$ = 40.0 ± 6.4nM. s.e.mean shown by the vertical lines. The points represent means of 6, 3 and 3 separate experiments respectively. Controls were taken as twitch amplitude prior to addition of agonist.*

5.5 Discussion and Conclusions

5.5.1 Classification of CGRP receptors on the guinea-pig vas deferens

It has been previously suggested that distinct receptors exist which are able to distinguish between CGRP and amylin (Dennis *et al.*, 1990), yet cross-reactivity of amylin at a distinct CGRP receptor has also been proposed (Chantry *et al.*, 1991). In the rat vas deferens $CGRP_{(8-37)}$ has been shown to antagonise rat-α- and rat-β-CGRP responses, albeit with low potency, but it was ineffective against amylin-induced responses. This has led to suggestions that the rat vas deferens express distinct CGRP and amylin receptors (Giuliani *et al.*, 1992). The rat vas deferens is reported to possess $CGRP_2$ receptors which are insensitive or have a low sensitivity to $CGRP_{(8-37)}$ (Dennis *et al.*, 1990, Mimeault *et al.*, 1991; Maggi *et al.*, 1991; Giuliani *et al.*, 1992). In the guinea-pig vas deferens, the results reported in this thesis showed $CGRP_{(8-37)}$ was ineffective against the CGRP-induced responses (see table 5.1), a finding which, based upon the current receptor classification, indicated that $CGRP_2$ receptors are also present on the guinea-pig vas deferens. $CGRP_{(8-37)}$ significantly antagonised the responses induced by rat and human amylin (see figure 5.11 & table 5.2) implying that this CGRP receptor antagonist may not be highly selective in its actions.

The extent of species differences is not well documented yet the current CGRP receptor classification is based upon results from two different species; rat and guinea-pig. The present classification also relies heavily on results obtained using $CGRP_{(8-37)}$ even though other CGRP fragments have been reported as having antagonistic properties in other preparations.

5.5.2 Classification of amylin receptors on the guinea-pig vas deferens

Results presented in this thesis (section 5.4.2) support the theory that distinct CGRP and amylin receptors exist in the guinea-pig vas deferens.

AC187 has previously been reported as possessing antagonistic properties at distinct amylin receptors in rats *in vivo* (Young *et al.*, 1994). In the experiments conducted here (section 5.4.1), AC187 was shown to be unable to antagonise human-α-CGRP and rat-α-CGRP-induced responses (see figure 5.8 & table 5.1) but did antagonise responses induced by rat amylin showing it is selective in its activity (figure 5.13). Results obtained using Tyr^{0}-$CGRP_{(28-37)}$ and $AM_{(22-52)}$ also support the theory that amylin is acting at receptors on the guinea-pig vas deferens distinct to those at which CGRP produces it actions. The CGRP fragment significantly antagonised the human-α-CGRP-induced responses (figure 5.4) but not the amylin-induced responses (figure 5.9), whilst $AM_{(22-52)}$ antagonised rat amylin-induced responses (figure 5.14) but not those of human-α-CGRP.

The amylin receptors shown here appear to differ from others reported on rat soleus muscle, where AC187 was 300 times more potent at antagonising amylin responses than was seen here (Beaumont *et al.*, 1995). Amylin receptors found on guinea-pig urinary bladder and trachea may resemble those shown here, where $CGRP_{(8-37)}$ did not antagonise CGRP-induced responses but significantly antagonised amylin-induced responses (Giuliani *et al.*, 1992; Bhogal *et al.*, 1993).

Overall these results suggest that amylin has its own receptors on the guinea-pig vas deferens which are distinct from the CGRP receptors found on this preparation. These amylin receptors show similarities to those found on other guinea-pig preparations but differ from those found on rat soleus muscle. These results, like those found for CGRP, section 5.5.1, indicate that species differences exist between amylin receptors.

5.5.3 Classification of selective receptor subtypes on the guinea-pig vas deferens

The results presented in sections 5.4.1 and 5.4.2, raise the possibility that the guinea-pig vas deferens possess CGRP and amylin receptors. The idea that these receptors

can distinguish between the human and rat forms of their endogenous ligands is also a possibility.

	Human-α-CGRP	Rat-α-CGRP	Human amylin	Rat amylin	AM 13-52
Tyr°-CGRP-(28-37)	√	x	x	x	-
CGRP(19-37)	√	x	x	√	-
CGRP(8-37)	x	x	√	√	x
Amylin(8-37)	x	√	x	√	-
AC187	x	x	x	√	-
AM (22-52)	x	x	x	√	x

Table 5.4 *Summary of the pattern of antagonism of CGRP, amylin and adrenomedullin $_{13-52}$ (AM_{13-52}) by peptide fragments in the guinea-pig vas deferens. √ indicates statistically significant antagonism of the agonist control response, x indicates no antagonism occurred,- indicates not determined.*

Each peptide agonist appeared to have an individual pharmacological profile and the antagonists used revealed receptor heterogeneity. For example, amylin$_{(8-37)}$ antagonised rat-α-CGRP and rat amylin-induced responses but CGRP$_{(19-37)}$ antagonised only the human-α-CGRP and rat amylin-induced responses.

The results obtained using human-α-CGRP and rat-α-CGRP and the CGRP receptor antagonists show that the two forms of CGRP do not appear to be working via the same receptor subtype. The results using the CGRP receptor antagonists may imply that rat-α-CGRP is either not working via a CGRP receptor in the guinea-pig vas deferens or that the CGRP receptor at which this agonist is working is not sensitive to the CGRP receptor antagonists used here. The rat-α-CGRP-induced responses were

antagonised by amylin$_{(8-37)}$ which may imply that this receptor antagonist is not selective in its actions.

Human and rat amylin do not appear to be working at the same amylin receptor in the guinea-pig vas deferens. Based upon results obtained using the various CGRP and amylin receptor antagonists, it appears that the receptor through which human amylin is working is insensitive to the amylin receptor antagonists used here. The rat amylin-induced response was antagonised by both the CGRP and amylin receptor antagonists implying that these fragments do not show high receptor selectivity in this preparation.

The antagonists were not, as expected, 100% selective and therefore Schild analysis would be required to investigate their actions more fully. Although over-interpretation of these results would not be advisable, it is interesting to speculate that each of the peptide agonists has its own receptor in the guinea-pig vas deferens.

Antagonism of the human-α-CGRP-induced response by Tyr°-CGRP$_{(28-37)}$ and CGRP$_{(19-37)}$ indicates that the C-terminus may be important in binding to the putative human-α-CGRP receptor. This tentative conclusion was only based on the studies carried out here and as no N-terminal fragments were tested it is difficult to conclude which areas of the CGRP molecule are essential for human-α-CGRP receptor interactions.

The effects of AM$_{(13-52)}$ and CGRP have been previously compared on rat and cat pulmonary vascular flow. CGRP was 3 times more potent than AM$_{(13-52)}$ in the rat preparation yet AM$_{(13-52)}$ was more potent than CGRP in the cat preparation (Nossamen *et al.*, 1995). This indicates a species difference in the effects of these 2 peptides although it is impossible to say if this was at the level of the receptor. In the guinea-pig vas deferens, CGRP and AM$_{(13-52)}$ had effects which were not significantly different to one another. AM$_{(13-52)}$ may possibly be working through one of the putative CGRP receptors in this preparation, as like human-α-CGRP and rat-α-CGRP, the control response induced by AM$_{(13-52)}$ was not antagonised by CGRP$_{(8-37)}$ or AM$_{(22-52)}$.

5.5.4 Conclusions

These experiments suggest that distinct CGRP and amylin receptors exist on the guinea-pig vas deferens. The amylin receptors, purported to exist on the guinea-pig vas deferens are distinct from $CGRP_1$ or $CGRP_2$ receptors. The CGRP receptor present on the guinea-pig vas deferens, based upon results with $CGRP_{(8\text{-}37)}$, is type 2. The selectivity shown by the CGRP and amylin receptor antagonists (see table 5.4) show that human-α-CGRP, rat-α-CGRP, rat amylin and human amylin appear to act via different receptor subtypes in the guinea-pig vas deferens. Further work needs to be conducted to establish the precise receptor types at which they act. $AM_{(13\text{-}52)}$ may work through a CGRP receptor subtype on the guinea-pig vas deferens as it shows the same pattern of antagonism as CGRP but further work would be required to confirm this.

Chapter 6

CGRP receptor classification in the guinea-pig atria.

6.1 Introduction

6.1.1 Effects of CGRP on the heart

CGRP has been shown to possess species-dependent, potent cardiovascular effects including vasodilatation, and positive chronotropic and inotropic effects upon the heart. The positive inotropic and chronotropic effects of CGRP on guinea-pig, human and rat atria are reported to be mediated via CGRP release from the peripheral terminals of capsaicin-sensitive sensory neurones (Franco-Cereceda & Lundberg, 1985; Marshall *et al.*, 1986; Saito *et al.*, 1987; Goto *et al.*, 1992). Application of capsaicin to these sensory nerves caused depletion of endogenous stores of CGRP resulting in the rapid development of tachyphylaxis (Miyauchi *et al.*, 1988). CGRP has been shown to be a neurotransmitter of these non-adrenergic, non-cholinergic (NANC) nerves in guinea-pig, human and rat atria (Saito *et al.*, 1987; Goto *et al.*, 1992). The positive inotropic effect induced by CGRP on the heart is not affected by adrenergic, histaminergic or serotonergic antagonists suggesting CGRP acts directly on cardiac myocytes, probably via its own receptors (Ishikawa *et al.*, 1988).

In the ventricles there are several bundles of CGRP-like immunoreactive nerves in the pericardium, but not in the myocardium, and it has been suggested that circulating CGRP activates specific receptors on the ventricles to produce subsequent inotropic effects on the whole heart (Miyauchi *et al.*, 1988; Mulderry *et al.*, 1985). Numerous CGRP-like immunoreactive nerves are reported to exist in the myocardium of rat atria and radioligand binding experiments have shown higher densities of CGRP receptors to exist in the right atrium compared to the left (Sigrist *et al.*, 1986; Mulderry *et al.*, 1985). In the guinea-pig, CGRP binding has been shown to mainly occur in the atria and the CGRP receptors found here are reported to exist in multiple affinity states (Van Rossum *et al.*, 1993; Chatterjee *et al.*, 1991; Gertheimer *et al.*, 1986).

The actions of human-α-CGRP on the guinea-pig atria are antagonised by $CGRP_{(8-37)}$ and $CGRP_{(12-37)}$; pA_2 values of 7.0-7.5 and 6.0-6.1 being obtained for these receptor antagonists respectively (Quirion *et al.*, 1992; Dennis *et al.*, 1989, 1990;

Guiliani *et al.*, 1992). Based upon these results and the present receptor classification the guinea-pig atria appears to possess $CGRP_1$ receptors (Dennis *et al.*, 1990).

6.2 Aims and objectives

Following the investigations into receptor classification and characterisation in the guinea-pig ileum and vas deferens (chapters 4 and 5) the guinea-pig atria were chosen as a third tissue type in which to study CGRP receptors. The guinea-pig ileum was shown to possess $CGRP_1$ receptors and the vas deferens $CGRP_2$ receptors. The aim of these experiments was to see if the CGRP receptors present on the atria showed similarities in their antagonism by CGRP and amylin fragments to the CGRP receptors found in the other guinea-pig tissues examined. The guinea-pig atria have previously been shown to possess $CGRP_1$ receptors and that activation of these receptors by rat-α-CGRP produces positive inotropic and chronotropic effects.

This study was also designed in order to verify previous reports of CGRP-induced effects in the guinea-pig atria and to further classify the CGRP receptors present by use of the CGRP and amylin receptor antagonists currently available. The receptor antagonists used were the same as utilised in the previous studies on ileum and vas deferens; $CGRP_{(8\text{-}37)}$, $CGRP_{(19\text{-}37)}$, Tyr°-$CGRP_{(28\text{-}37)}$, AC187 and $amylin_{(8\text{-}37)}$.

6.3 Methods and Materials

The protocol is as described in chapter 2, section 2.2. The atria were separated in order to electrically stimulate the left atrium and examine the effects of human-α-CGRP on chronotropy and inotropy in the guinea-pig heart. Electrical stimulation, using platinum electrodes placed parallel to the tissue, was found to yield reproducible responses and produce a stable inotropic baseline. Human-α-CGRP produced concentration-dependent responses suitable for measurement of antagonistic effects of the CGRP fragments.

The atrial preparations were found to become desensitised to the effects of CGRP following the construction of a single concentration-response curve. To overcome this, control concentration-response curves using human-α-CGRP, were constructed on the left and right atria of the guinea-pig (n = 14 and 20 respectively) whilst the effects of CGRP and amylin receptor antagonists on these responses were subsequently examined on tissues from a second animal. This methodology prevented paired analysis of data.

6.3.1 Data evaluation

Statistical analysis of calculated EC_{50} values was by one-way ANOVA and post-hoc Dunnett's test ($P \leq 0.05$) taken to be significant. n values correspond to the number of animals used not individual tissue responses. Results were normalised as % control value. Inotropic responses were measured taking the initial twitch height as 0%. Chronotropic responses were measured by counting individual beats over a 4 sec period and then converting the results to beats per minute. Chronotropic results were subsequently normalised taking the initial response as 0%.

6.4 Results

Human-α-CGRP caused a concentration-dependent positive inotropic effect on the guinea-pig isolated spontaneously beating right atria and on the electrically driven left atria (see figures 6.1a, 6.1b & 6.2). Human-α-CGRP produced a positive chronotropic effect on the spontaneously beating right atrium also in a concentration-dependent manner.

from one another implying that human-α-CGRP acts via a similar mechanism to produce positive inotropic effects on the right and left atria.

Human-α-CGRP caused a concentration-dependent positive chronotropic effect on the spontaneously beating right atrium with an EC_{50} (± s.e.m) of 78.9 (± 42.9nM) (see figure 6.3).

A panel of CGRP and amylin receptor antagonists were used to investigate the human-α-CGRP-induced control responses in the left and right atria: Tyr°-$CGRP_{(28-37)}$, $CGRP_{(8-37)}$, $CGRP_{(19-37)}$, $amylin_{(8-37)}$ and AC187 (acetyl-Asn^{30}, Tyr^{32}]-salmon $calcitonin_{(8-32)}$ (Young *et al.*, 1994) (see table 6.1).

10μM Tyr°-$CGRP_{(28-37)}$ was found to weakly antagonise the human-α-CGRP-induced inotropic response on the right atrium (figure 6.4). The calculated EC_{50} value ± s.e.m for human-α-CGRP plus 10μM Tyr°-$CGRP_{(28-37)}$ was 46.6 ± 4.2nM, a value found to be significantly different from that obtained for human-α-CGRP alone (10.4 ± 18.2nM).

The human-α-CGRP-induced positive inotropic effect seen on the left atrium was antagonised more potently by 10μM Tyr°-$CGRP_{(28-37)}$, to the extent that it was almost completely abolished at concentrations upto 300nM (see figure 6.5). No EC_{50} value could be obtained for the concentration-response curve of human-α-CGRP in the presence of 10μM Tyr°-$CGRP_{(28-37)}$ due to the flat nature of the plot.

The positive chronotropic effect induced by human-α-CGRP was not significantly antagonised by 10μM Tyr°-$CGRP_{(28-37)}$ (figure 6.6) (EC_{50} values ± s.e.m were, human-α-CGRP = 78.9 ± 42.9nM and human-α-CGRP plus 10μM Tyr°-$CGRP_{(28-37)}$, 71.7 ± 17.9nM).

The human-α-CGRP-induced positive inotropic effect on the guinea-pig isolated right and left atria was not significantly antagonised by the CGRP receptor antagonist $CGRP_{(8-37)}$ at concentrations up to 3μM (figure 6.7) (EC_{50} values ± s.e.m were, on the right atrium, human-α-CGRP, 10.4 ± 18.2nM, human-α-CGRP plus 1μM $CGRP_{(8-37)}$, 9.9 ± 2.1nM and on the left atrium, human-α-CGRP, 41.4 ± 8.9nM, human-α-CGRP plus 1μM $CGRP_{(8-37)}$, 112 ± 49.8nM). These results contrast to those obtained in previous studies by other workers.

The human-α-CGRP-induced chronotropic effect on the right atrium was significantly antagonised by $CGRP_{(8-37)}$ (1μM) (figure 6.8) (EC_{50} value ± s.e.m for human-α-CGRP, 78.9 ± 42.9nM and human-α-CGRP plus 1μM $CGRP_{(8-37)}$, 558 ± 37.0nM. This latter value corresponds to an apparent pA_2 value of 6.8, similar to that found by other workers in previous studies.

$CGRP_{(19-37)}$ (3μM) did not significantly antagonise the human-α-CGRP-induced inotropic response (figure 6.9) (calculated EC_{50} value ± s.e.m for human-α-CGRP plus 3μM $CGRP_{(19-37)}$, 27.7 ± 21.0nM). Similarly the human-α-CGRP-induced chronotropic response was not antagonised by 3μM $CGRP_{(19-37)}$ (EC_{50} value ± s.e.m for human-α-CGRP plus 3μM $CGRP_{(19-37)}$, 11.6 ± 4.1nM).

Two amylin receptor antagonists were tested against the human-α-CGRP-induced responses on the left and right atria. 10μM AC187 was tested against the human-α-CGRP-induced inotropic responses on the left and right atria, and on the chronotropic responses induced by human-α-CGRP on the right atrium (figure 6.10 & table 6.1). No significant antagonism of the human-α-CGRP-induced responses was seen using this antagonist at the concentration utilised (see table 6.1).

The second amylin receptor antagonist, $amylin_{(8-37)}$ (1μM) was tested against the human-α-CGRP-induced inotropic response in the guinea-pig left atrium. 1μM $amylin_{(8-37)}$ did not significantly antagonise the human-α-CGRP-induced positive inotropic response in the left atrium (EC_{50} values ± s.e.m of human-α-CGRP, 41.4 ± 8.9nM and human-α-CGRP plus 1μM $amylin_{(8-37)}$, 17.2 ± 4.7nM) (figure 6.11).

	Tissues		
	EC_{50} ± s.e.m (nM)		
	Right atrium / inotropy	**Right atrium / chronotropy**	**Left atrium / inotropy**
Human-α-CGRP	10.4 ± 18.2 (n = 20)	78.9± 42.9 (n = 20)	41.4 ± 8.9 (n =14)
10μM Tyr°-CGRP(28-37)	46.6 ± 4.2* (n = 6)	71.1 ± 17.9 (n = 6)	ND (n =6)
1μM CGRP(8-37)	9.9 ± 2.1 (n = 5)	558 ± 37.0*(n = 4)	112 ± 49.8 (n = 6)
3μM CGRP(19-37)	27.7 ± 21.0 (n = 5)	116 ± 4.1 (n = 3)	ND
10μM AC187	115 ± 80 (n = 6)	196 ± 31.5 (n = 4)	177 ± 867 (n = 6)
1μM Amylin(8-37)	ND	ND	17.2 ± 47.0 (n = 5)

Table 6.1 *EC_{50} values ± s.e.m for human-α-CGRP alone and in the presence of a variety of antagonists on the guinea-pig isolated right and left atria. All values were taken from figures 6.2-6.11. All values shown are nM. * indicates significant difference from the agonist alone found using a one-way ANOVA followed by a post-hoc Dunnett's test ($P \leq 0.05$). ND = value not determined.*

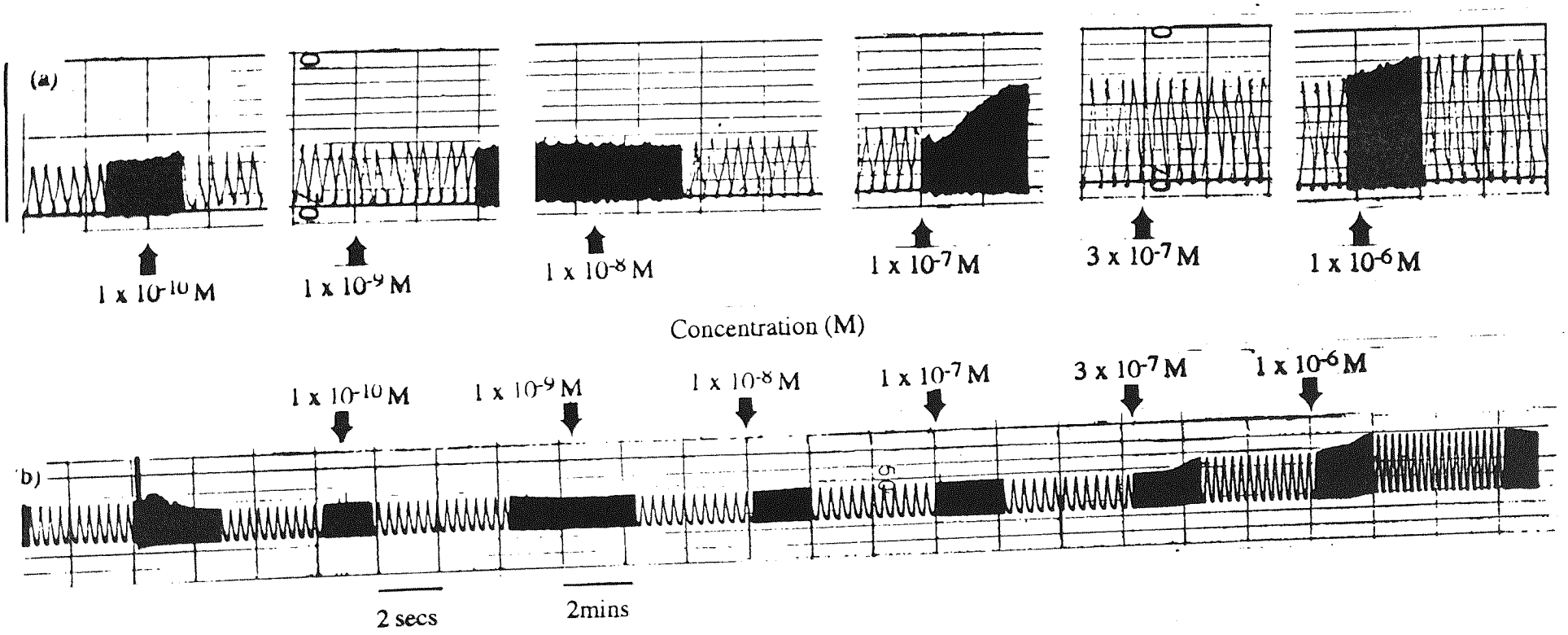


Figure 6.1 *Representative trace illustrating the concentration-related inotropic effects of human-α-CGRP in the electrically driven guinea-pig isolated left atrium (a) and the inotropic and chronotropic effects of human-α-CGRP in the spontaneously beating guinea-pig isolated right atrium (b). Measurement of inotropic effects were recorded as increase in twitch height following the addition of human-α-CGRP. Chronotropic responses were measured as individual beats/min over a 4 sec period, following the increase of the chart speed to 30 cm / min in order to observe individual beats.*

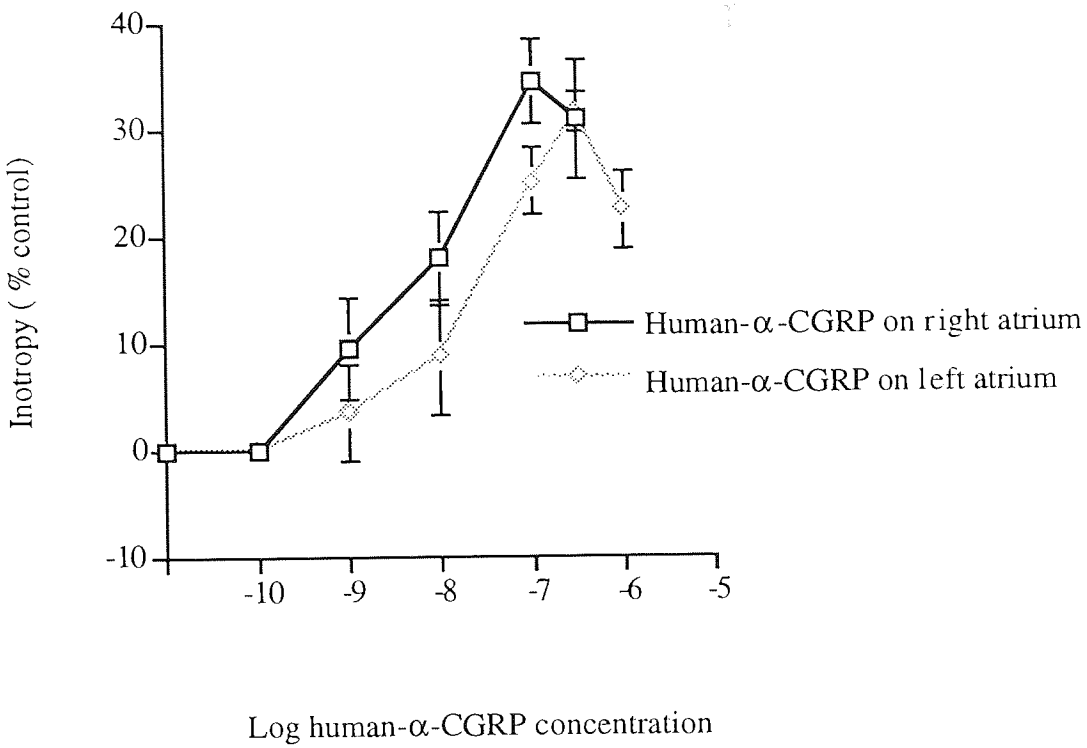


Figure 6.2 *Positive inotropic effect induced by human-α-CGRP on the guinea-pig isolated spontaneous right atrium (□) and electrically driven left atrium(◇). Values of EC_{50} from fits ± s.e.m were human-α-CGRP on the right atrium = 10.4 ± 18.2nM, human-α-CGRP on the left atrium = 41.0 ± 8.9nM. s.e.mean shown by the vertical lines. The points represent means of 20 and 14 separate experiments respectively. Controls were taken as twitch amplitude prior to addition of agonist.*

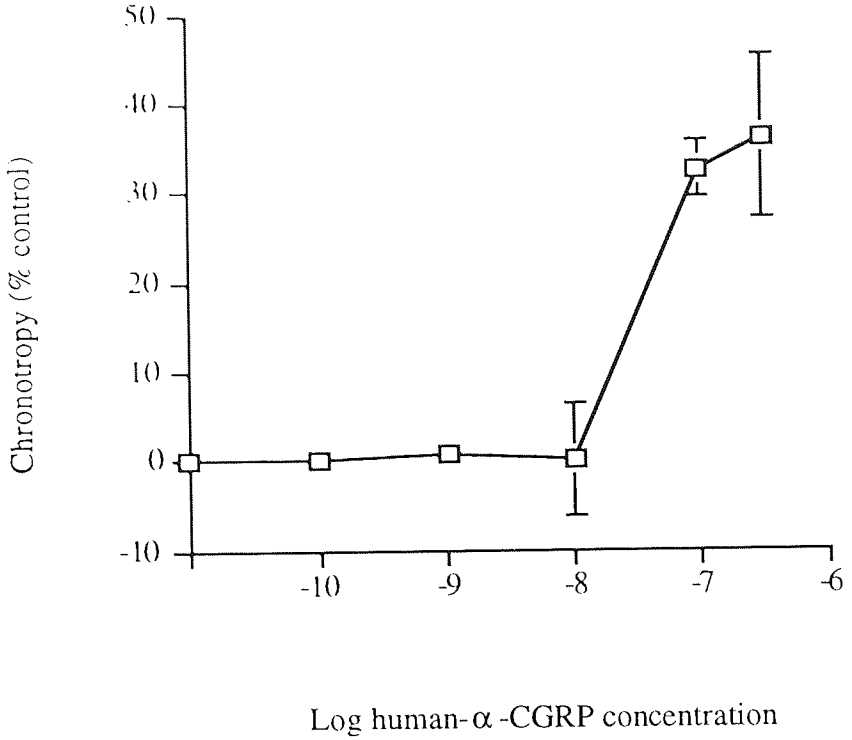


Figure 6.3 *Positive chronotropic effect induced by human-α-CGRP on the guinea-pig isolated spontaneous right atrium (□). Values of EC_{50} from fits ± s.e.m were, human-α-CGRP = 78.9 ± 42.9nM. s.e.mean shown by the vertical lines. The points represent a mean of 20 separate experiments. Controls were taken as heart rate prior to addition of test substance.*

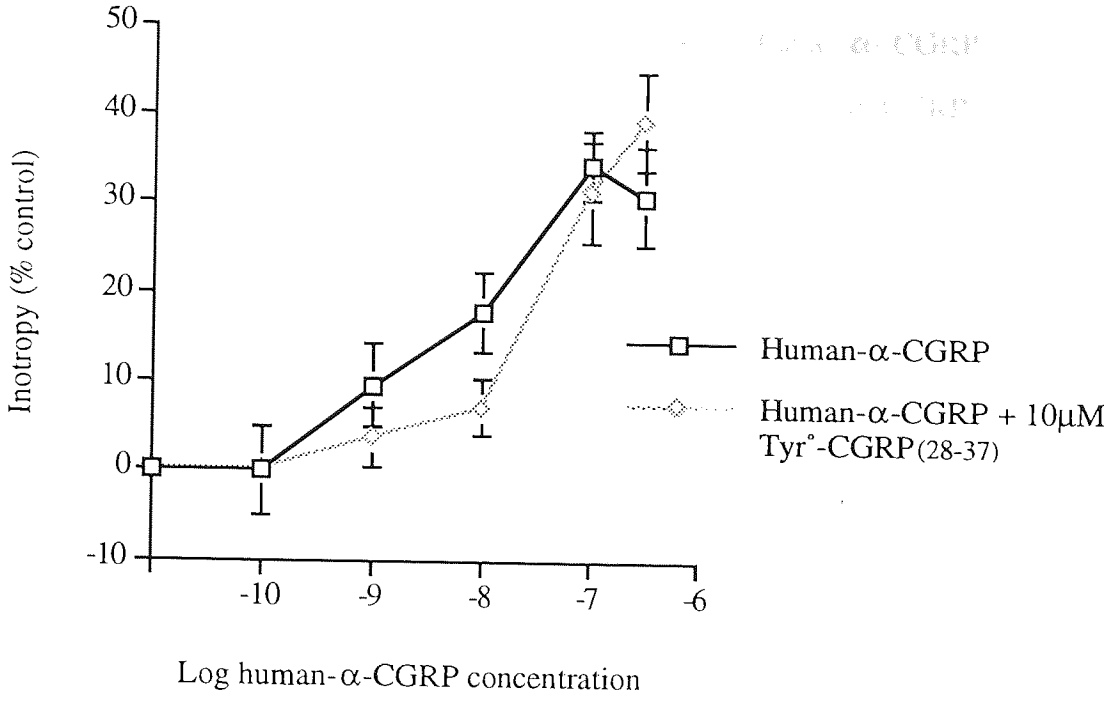


Figure 6.4 *Positive inotropic effect induced by human-α-CGRP on the guinea-pig isolated spontaneous right atrium (□) and in the presence of 10μM Tyr°-CGRP(28-37) (◇). Values of EC_{50} from fits ± s.e.m were, human-α-CGRP = 10.4 ± 18.2nM, human-α-CGRP + 10μM Tyr°-CGRP(28-37) = 46.6 ± 4.2nM. s.e.mean shown by the vertical lines. The points represent means of 20 and 6 separate experiments respectively. Controls were taken as twitch amplitude prior to addition of test substance.*

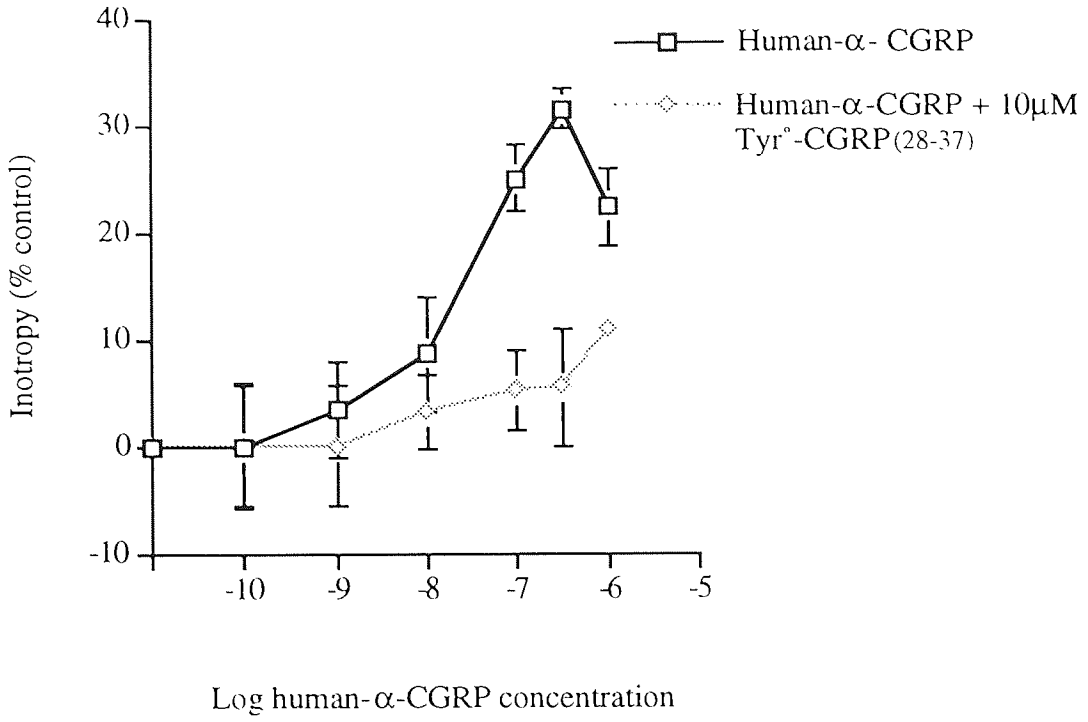


Figure 6.5 *Positive inotropic effect induced by human-α-CGRP on the guinea-pig isolated electrically driven left atrium (□) and in the presence of 10μM Tyr°-CGRP(28-37) (◇). Values of EC_{50} from fits ± s.e.m were, human-α-CGRP = 41.4 ± 8.9nM, human-α-CGRP + 10μM Tyr°-CGRP(28-37) = not determined. s.e.mean shown by the vertical lines. The points represent means of 14 and 6 separate experiments respectively. Controls were taken as twitch amplitude prior to addition of test substance.*

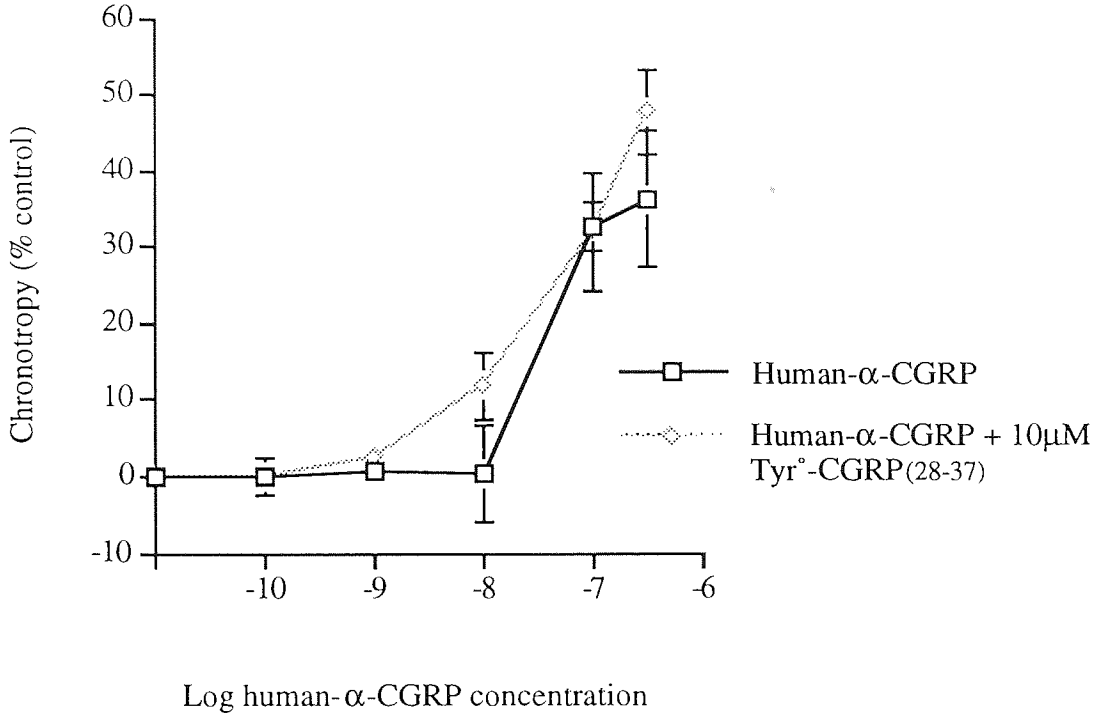


Figure 6.6 *Positive chronotropic effect induced by human-α-CGRP on the guinea-pig isolated spontaneous right atrium (□) and in the presence of 10μM Tyr°-CGRP(28-37) (◇). Values of EC_{50} from fits ± s.e.m were, human-α-CGRP = 10.4 ± 18.2nM, human-α-CGRP + 10μM Tyr°-CGRP(28-37) = 71.1 ± 17.9nM. s.e.mean shown by the vertical lines. The points represent means of 20 and 6 separate experiments respectively. Controls were taken as heart rate prior to addition of test substance.*

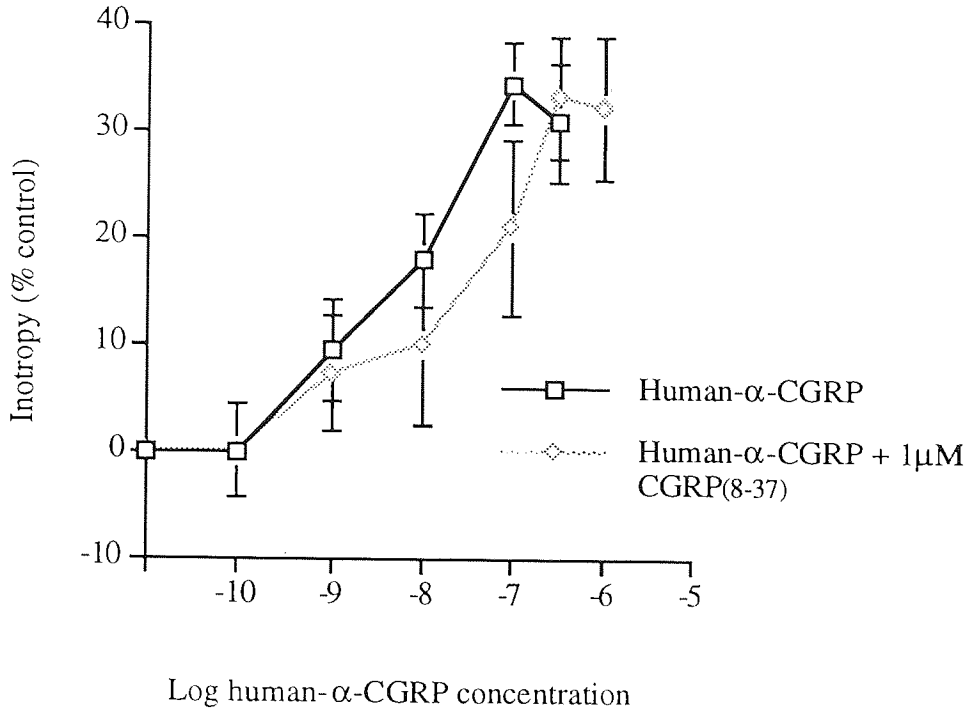


Figure 6.7 *Positive inotropic effect induced by human-α-CGRP on the guinea-pig isolated spontaneous right atrium (□) and in the presence of 1μM CGRP$_{(8-37)}$ (◇). Values of EC$_{50}$ from fits ± s.e.m were, human-α-CGRP = 10.4 ± 18.2nM, human-α-CGRP + 1μM CGRP$_{(8-37)}$ = 9.9 ± 2.1nM. s.e.mean shown by the vertical lines. The points represent means of 20 and 5 separate experiments respectively. Controls were taken as twitch amplitude prior to addition of test substance.*

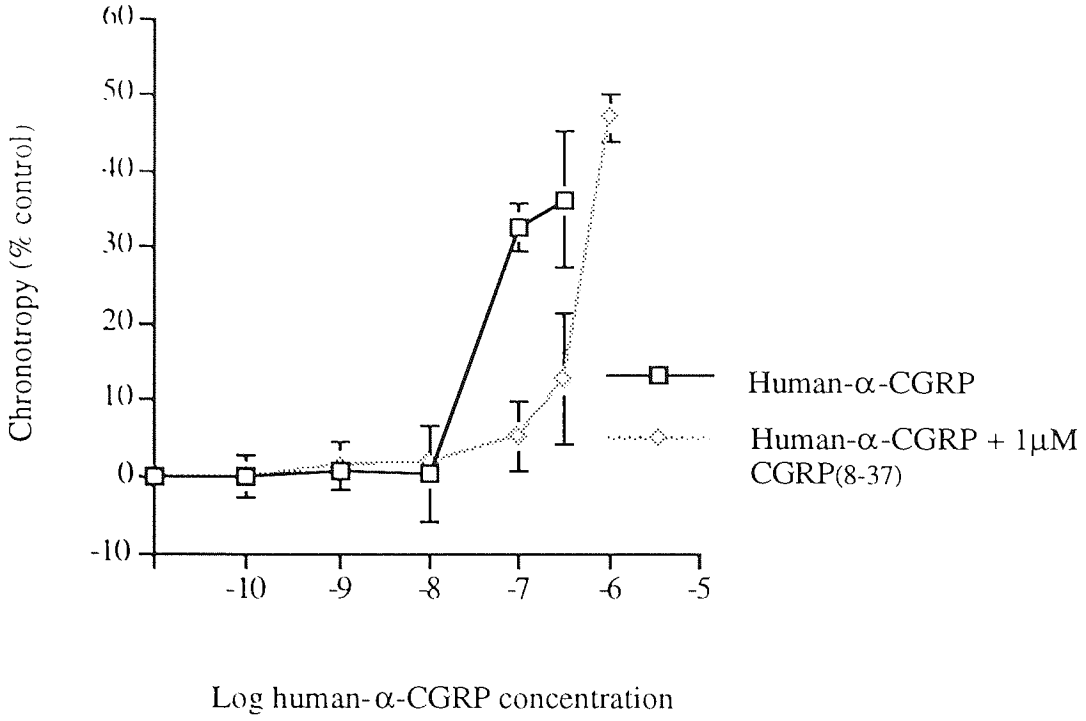


Figure 6.8 *Positive chronotropic effect induced by human-α-CGRP on the guinea-pig isolated spontaneous right atrium (□) and in the presence of 1μM CGRP(8-37) (◇). Values of EC_{50} from fits ± s.e.m were, human-α-CGRP = 78.9 ± 42.9nM, human-α-CGRP + 1μM CGRP(8-37) = 558 ± 37.0nM. s.e.mean shown by the vertical lines. The points represent means of 20 and 4 separate experiments respectively.*

Controls were taken as heart rate prior to addition of test substance

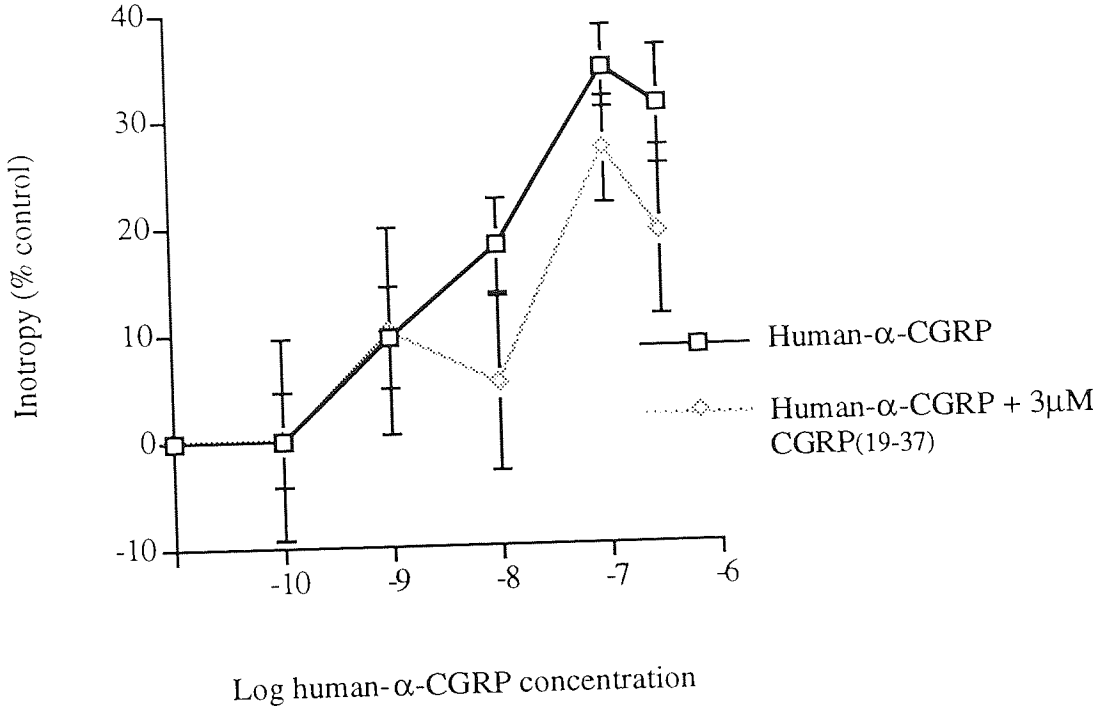


Figure 6.9 *Positive inotropic effect induced by human-α-CGRP on the guinea-pig isolated spontaneous right atrium (□) and in the presence of 3μM CGRP(19-37) (◇). Values of EC50 from fits ± s.e.m were, human-α-CGRP = 10.4 ± 18.2nM, human-α-CGRP + 3μM CGRP(19-37) = 27.7 ± 21.0nM. s.e.mean shown by the vertical lines. The points represent means of 20 and 5 separate experiments respectively. Controls were taken as twitch amplitude prior to addition of test substance.*

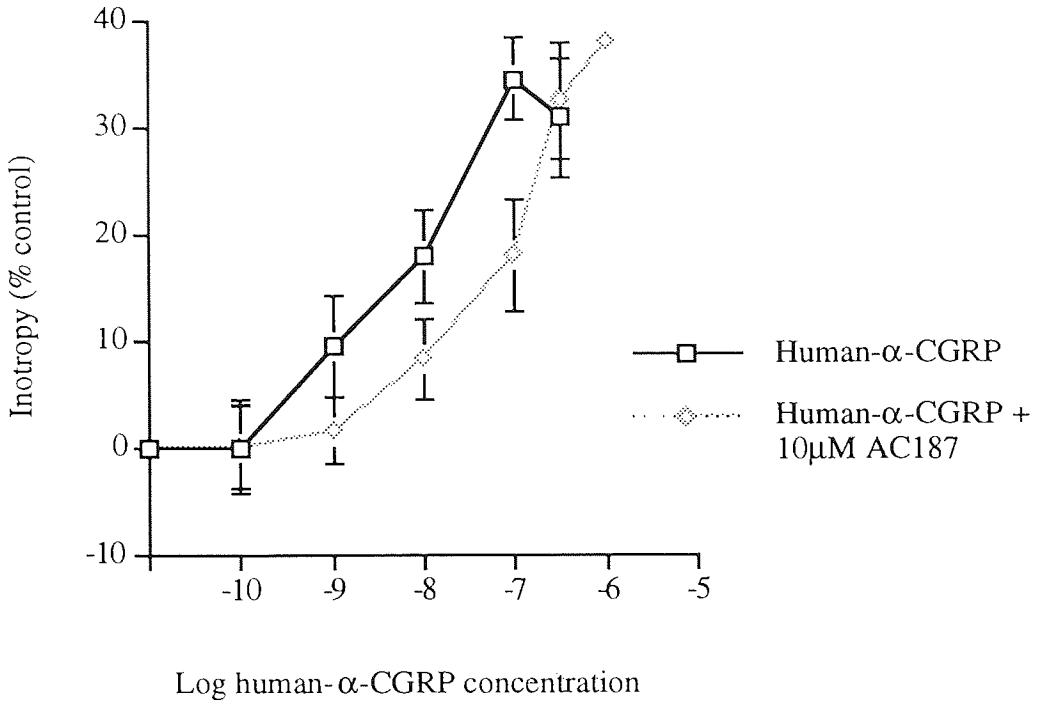


Figure 6.10 *Positive inotropic effect induced by human-α-CGRP on the guinea-pig isolated spontaneous right atrium (□) and in the presence of 10μM AC187 (◇). Values of EC_{50} from fits ± s.e.m were, human-α-CGRP = 10.4 ± 18.2nM, human-α-CGRP + 10μM AC187 = 115 ± 80.0nM. s.e.mean shown by the vertical lines. The points represent means of 20 and 6 separate experiments respectively. Controls were taken as twitch amplitude prior to addition of test substance.*

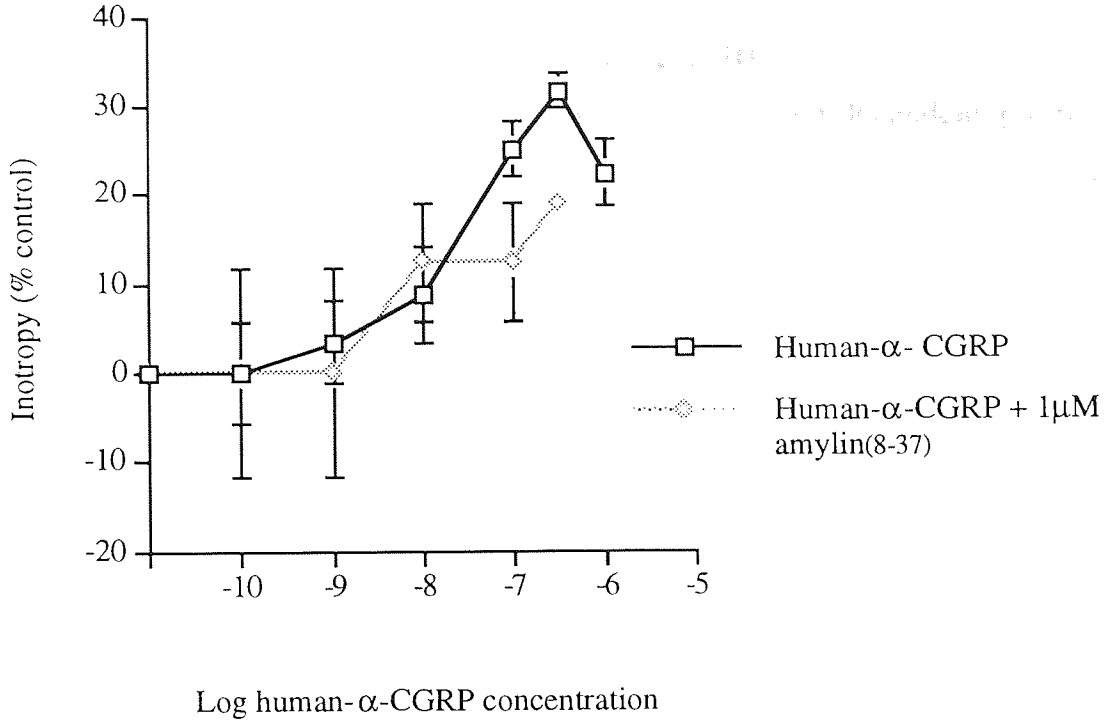


Figure 6.11 *Positive inotropic effect induced by human-α-CGRP on the guinea-pig isolated electrically driven left atrium (□) and in the presence of 1μM amylin(8-37) (◇). Values of EC_{50} from fits ± s.e.m were, human-α-CGRP = 41.4 ± 8.9nM, human-α-CGRP + 1μM amylin(8-37) = 17.2 ± 47.0nM. s.e.mean shown by the vertical lines. The points represent means of 14 and 5 separate experiments respectively. Controls were taken as twitch amplitude prior to addition of test substance.*

6.5 Discussion and Conclusions

6.5.1 CGRP receptor classification in the guinea-pig atria

Human-α-CGRP has been shown to produce concentration-dependent positive inotropic and chronotropic effects on the spontaneously beating right atrium, and positive inotropic effects on the electrically driven left atrium. No statistical differences were found between the positive inotropic responses induced by human-α-CGRP on the right and left atria, an observation in agreement with previous studies of this peptide on these preparations (Dennis *et al.*, 1990; Mimeault *et al.*, 1991; Giuliani *et al.*, 1992; Maggi *et al.*, 1991).

A difference in the inotropic responses induced by human-α-CGRP in the right and left atria was highlighted by use of the CGRP receptor antagonist, Tyr^{0}-$CGRP_{(28-37)}$. At 10μM this antagonist caused slight but significant antagonism of the human-α-CGRP-induced inotropy in the right atrium, estimated pA_2 of 5.5, but was much more potent against the human-α-CGRP-induced inotropy in the left atrium (figures 6.5 & 6.6) to the extent that it was not possible to show competitive antagonism with the CGRP concentrations available. This result may imply that human-α-CGRP produces inotropy by acting via different mechanisms in the two halves of the heart. These mechanisms may be mediated via receptor subtypes existing on the two atria which are responsible for the different actions of human-α-CGRP and which may have different affinities for the CGRP amino acid residues 28-37. The putative left atrial receptor subtype binding residues 28-37 with a much greater affinity than the receptor found on the right. Another possible explanation of this result is the existence of different proteases in the two halves of the heart or a difference in distribution of such proteases. More experiments need to be conducted to examine the actions of this CGRP receptor antagonist on this preparation.

$CGRP_{(8-37)}$ (upto 3μM) did not antagonise the human-α-CGRP-induced inotropic responses on the right and left atria, but significantly antagonised the human-α-CGRP-induced positive chronotropic effect on the right atrium (apparent pA_2 value of 6.8). According to the current CGRP receptor classification this indicates the

involvement of $CGRP_1$ receptors in the mediation of the human-α-CGRP responses and correlates well with previous work where pA_2 values of 6.9-7.6 have been obtained (Quirion *et al.*, 1992; Mimeault *et al.*, 1991). The lack of antagonism of the human-α-CGRP-induced inotropy on the right and left atria by $CGRP_{(8-37)}$ (1μM) was in contrast to previous studies where pA_2 values of 7.0 and 7.7 have been found (Dennis *et al.*, 1990; Mimeault *et al.*, 1991). There seems to be no apparent explanation for this discrepancy, apart from the differing sources of $CGRP_{(8-37)}$ used in experiments shown in this thesis and those by other investigators. The $CGRP_{(8-37)}$ used here was obtained from Sigma chemicals whereas other groups have synthesised their own fragment. Further experiments need to be performed to clarify these discrepancies. A possible, albeit highly unlikely, explanation could be that addition of human-α-CGRP to the atrial preparations used here could have stimulated $CGRP_2$ receptors, causing either direct inotropic effects or via the release of other transmitters. However, no other workers have reported the presence of $CGRP_2$ receptors on this preparation.

The other CGRP receptor antagonist utilised in this study was $CGRP_{(19-37)}$. This receptor antagonist did not affect the human-α-CGRP-induced inotropic or chronotropic effects on the guinea-pig right and left atria (figure 6.9 & table 6.1). This observation was in general agreement with Rovero *et al* (1992) who obtained a pA_2 value of 5.39, indicative of a low potency of action of $CGRP_{(19-37)}$ against human-α-CGRP. Amino acid residues 19-37 do not appear to have a high affinity for the receptor responsible for the actions of human-α-CGRP on the guinea-pig atria.

The amylin receptor antagonists, AC187 and $amylin_{(8-37)}$ did not antagonise the human-α-CGRP-induced responses in the guinea-pig atria implying that CGRP may act at its own specific receptors in these tissue preparations (figures 6.10 & 6.11 and table 6.1). In the vas deferens (chapter 5), AC187 only antagonised rat amylin-induced responses, taken together with its inability to antagonise CGRP-mediated responses here, this may imply that it is selective. Further work examining the actions

the actions of amylin on these tissue preparations would be required in order to investigate the selectivity of these amylin receptor antagonists in greater detail.

6.5.2 Conclusions

These experiments have made some preliminary observations regarding the classification of CGRP receptors in the guinea-pig atria. They concur with some previous studies by other workers and have also produced some initial observations regarding the actions of CGRP receptor antagonists on the human-α-CGRP-induced effects on this preparation.

These experiments utilising Tyr°-$CGRP_{(28-37)}$, indicate that slight differences may exist between the human-α-CGRP-induced inotropic responses on the guinea-pig right and left atria. $CGRP_1$ receptors could be involved in the positive chronotropic response induced by human-α-CGRP on the spontaneously beating right atrium, as deduced from the experiments utilising $CGRP_{(8-37)}$.

Further experiments need to be conducted on the guinea-pig isolated right and left atria to clarify the classification of CGRP receptors present and to obtain a clear picture of the effects of the available CGRP receptor antagonists in these preparations.

Chapter 7

General Discussion and Conclusions

This study extends the known pharmacology of CGRP and amylin receptors. The results support the current subdivision of CGRP receptors into types 1 and 2 and also support the existence of distinct amylin receptors, sensitive to the receptor antagonist AC187. Novel observations using CGRP and amylin receptor antagonists may provide evidence to suggest further subdivision of the current CGRP receptor classification is required.

7.1 CGRP receptor classification

The current receptor classification for CGRP and amylin appears to show that species specific receptors exist, which are distinct for these two peptides but show some degree of cross-reactivity.

As explained in section 1.2.1, CGRP receptors are currently sub-divided into 2 classes; $CGRP_1$ and $CGRP_2$. $CGRP_1$ receptors are typified by those found on guinea-pig atria and are sensitive to the receptor antagonist $CGRP_{(8\text{-}37)}$. $CGRP_2$ receptors are typified by those found on the rat vas deferens and are of low sensitivity to $CGRP_{(8\text{-}37)}$ but are sensitive to the linear CGRP analogue [Cys $(ACM)_{2,7}$]-human CGRP (Dennis *et al.*, 1989, 1990; Mimeault *et al.*, 1991; Maggi *et al.*, 1991).

Binding sites have been identified at which CGRP is capable of cross-reactivity with other peptides, for example, amylin and sCT-like peptides have been shown to cross-react at binding sites in rat forebrain: C1 = sCT binding, C2 = CGRP binding and C3 = CGRP, sCT and amylin binding (Sexton *et al.*, 1988; Veale *et al.*, 1994). In rat skeletal muscle CGRP is reported to act at an amylin receptor (Beaumont *et al.*, 1995). $AM_{(13\text{-}52)}$ and CGRP also cross-react at receptors on cultured vascular smooth muscle (Eguchi *et al.*, 1994).

The general pattern of CGRP receptor classification has been made more complex by this discovery of cross-reactivity of between distinct peptides but CGRP has been reported to have its own distinct receptors as well as having the ability to bind to receptors reported to be distinct for amylin, $AM_{(13\text{-}52)}$ and sCT. Some of the binding sites appear to have equal affinities for more than one of these peptides.

Due to discrepancies seen with the present CGRP receptor classification it was important to attempt to identify receptor subtypes within the same animal species and to compare results obtained with previous work on that animal and on other animal preparations in order to examine inter-species variations.

7.1.1 The Rat CGRP receptor

The aims of this project were to characterise CGRP receptors in rat tissues previously reported to possess CGRP receptor populations. Work using CGRP and its analogues; Tyr°-CGRP and [Cys $(ACM)_{2,7}$]-human CGRP and a panel of 15 monoclonal antibodies raised against CGRP, showed that few significant differences existed between the CGRP receptors found in rat liver, spleen and cerebellum. The results highlighted some possible CGRP receptor differences exist between tissues which would need to be investigated in future studies. Technical difficulties which arose during the course of this study, producing large experimental errors, meant conclusive results could not be produced.

The CGRP receptors found on rat liver, splenic and cerebellar membrane preparations have been reported to be the same, based upon results obtained from binding studies. However, functional assays measuring adenylate cyclase activity have suggested a degree of receptor heterogeneity (Stangl *et al.*, 1993).

The results presented using [^{125}I]-α-human CGRP radioligand binding (chapter 3) did not highlight any conclusive differences between CGRP receptors on rat liver, splenic and cerebellar membrane preparations. Receptor antagonists showed a distinct order of potency for each of the tissues if experimental variations could be ignored. $CGRP_{(8\text{-}37)}$ appeared to show high affinity binding in the spleen and cerebellum but not in the liver. [Cys $(ACM)_{2,7}$]-human CGRP showed higher affinity binding in the liver, compared to the spleen and cerebellum. Based upon these results, the rat liver

preparation could contain $CGRP_2$ receptors and the rat spleen and cerebellum could contain $CGRP_1$ receptors.

Small differences in the binding characteristics for CGRP in the rat tissue preparations were highlighted by the panel of monoclonal antibodies (Mabs). All the tissues showed [^{125}I]-α-human CGRP binding to be inhibited by some common Mabs but each tissue had a specific Mab which also inhibited binding. Though it would be inadvisable to draw any real conclusions from this data, it would appear that the liver and cerebellar CGRP receptors have similar binding characteristics but that the splenic CGRP receptor requires slightly different regions of the CGRP molecule for successful high affinity binding.

Further radioligand binding studies would be required before any conclusive evidence regarding the CGRP receptor types in rat liver, spleen and cerebellum could be obtained.

Due to the aforementioned technical difficulties which arose during the course of these experiments, it was decided to investigate the characterisation and classification of CGRP receptors in a second, less well documented species, the guinea-pig.

As previously explained the present CGRP receptor classification is based upon results obtained using two animal species and one receptor antagonist. Reports of cross-reactivity with receptors of structurally related peptides also complicates the current classification.

Following the preliminary studies using rat tissues, the guinea-pig ileum, vas deferens and atria were chosen to examine the effects of CGRP, amylin and a number of CGRP and amylin receptor antagonists. The main findings of these studies showed that the guinea-pig ileum and vas deferens possess $CGRP_1$ and $CGRP_2$ receptors respectively but that subdivisions of this classification system may be required. The presence of distinct amylin receptors on the ileum and vas deferens was also

purported. The classification of CGRP receptors present on the guinea-pig atria require more extensive investigation.

7.1.2 The Guinea-pig CGRP receptor

The results presented in chapters 4-6 indicate that the guinea-pig ileum, vas deferens and atria express different CGRP receptor subtypes. The guinea-pig atria and ileum both show $CGRP_1$ receptors to be present but the receptors do not demonstrate the same pattern of antagonism by the CGRP and amylin fragments used here. The vas deferens appears to possess mainly $CGRP_2$ receptors (see table 7.1).

The guinea-pig ileum preparations showed that human-α-CGRP and rat-α-CGRP produced equipotent relaxant effects on the stimulated twitch contractions produced in this preparation. These agonist effects were antagonised by $CGRP_{(8\text{-}37)}$ (1μM). Human-β-CGRP was less potent than human-α-CGRP and rat-α-CGRP at inhibiting twitch contractions and was not significantly antagonised by $CGRP_{(8\text{-}37)}$ (1μM). In the guinea-pig basilar artery, $CGRP_{(8\text{-}37)}$ has been shown to antagonise human-α-CGRP-induced responses but not those induced by human-β-CGRP (Jansen *et al.*, 1992), providing further evidence that the two forms of human CGRP can differentiate between receptor subtypes in this species. Work using the opossum internal anal sphincter showed no difference in the ability of human-α-$CGRP_{(8\text{-}37)}$ to antagonise responses to human-α and β-CGRP and it was suggested that the two forms of human CGRP act at the same receptor in this preparation. These results clearly suggest some degree of interspecies difference between CGRP receptors (Chadker & Rattan, 1991). In the guinea-pig ileum, both $CGRP_1$ and $CGRP_2$ receptors have been previously identified based upon results obtained with $CGRP_{(8\text{-}37)}$ (Bartho *et al.*, 1992; Dennis *et al.*, 1990). Based upon results presented in chapter 4, it is proposed that $CGRP_1$ and $CGRP_2$ receptors are present on the ileal preparations with human-α-CGRP acting at a $CGRP_1$ receptor, sensitive to antagonism by $CGRP_{(8\text{-}37)}$ and human-β-CGRP acting possibly acting via a $CGRP_2$ receptor, insensitive to antagonism by this fragment.

Results from experiments using AC187, $CGRP_{(19-37)}$ and $CGRP_{(8-37)}$ highlighted differences between the effects of human-α-CGRP and rat-α-CGRP on the guinea-pig ileum. The human-α-CGRP-induced effects were more potently antagonised by AC187 and $CGRP_{(8-37)}$ whereas rat-α-CGRP-induced effects were more potently antagonised by $CGRP_{(19-37)}$. These results suggest that human-α-CGRP and rat-α-CGRP act at different types of $CGRP_1$ receptors in the guinea-pig ileum which are capable of distinguishing between the two types of CGRP, to be referred to as $CGRP_{1\alpha}$ and $CGRP_{1\beta}$ (see table 7.1).

In the guinea-pig vas deferens, results also suggested the existence of receptors capable of distinguishing between human-α-CGRP and rat-α-CGRP. The human-α-CGRP and rat-α-CGRP-induced inhibition of stimulated twitch contractions were not antagonised by $CGRP_{(8-37)}$ thereby indicating that the CGRP receptor present on the guinea-pig vas deferens was broadly type 2 (Dennis *et al.*, 1990; Poyner *et al.*, 1992). The effects induced by the two types of CGRP showed distinct patterns of antagonism mediated by Tyr°-$CGRP_{(28-37)}$, $CGRP_{(19-37)}$ and $amylin_{(8-37)}$. The human-α-CGRP-mediated responses were antagonised by Tyr°-$CGRP_{(28-37)}$ and $CGRP_{(19-37)}$ whereas the rat-α-CGRP-induced effects were antagonised by $amylin_{(8-37)}$ alone. These observations, along with the results obtained using $CGRP_{(8-37)}$ implied that $CGRP_2$ receptor subtypes existed on the guinea-pig vas deferens which were capable of distinguishing between human-α-CGRP and rat-α-CGRP and which can be referred to as $CGRP_{2\alpha}$ and $CGRP_{2\beta}$ (see table 7.1).

Experiments studying the effects of human-α-CGRP and $CGRP_{(8-37)}$ on the guinea-pig atria showed human-α-CGRP was not antagonised by $CGRP_{(8-37)}$ and therefore may act at a $CGRP_2$ receptor. This observation did not agree with previous studies (Dennis *et al.*, 1990; Mimeault *et al.*, 1991) and further experiments need to be performed to establish the subtype of CGRP receptors present on the guinea-pig atria.

In general, the results described in this thesis indicates the possible existence of upto four CGRP receptor subtypes. This conclusion is based on current receptor

	Ileum		Vas deferens	
	Human-α-CGRP	Rat-α-CGRP	Human-α-CGRP	Rat-α-CGRP
Tyr°-CGRP-(28-37)	√	√	√	x
CGRP(19-37)	√	√	√	x
CGRP(8-37)	√	√	x	x
Human amylin (8-37)	√	x	x	√
Rat amylin (8-37)	-	-	x	-
AC187	√	√	x	x
AM (22-52)	-	-	x	x

Table 7.1 *Summary of the pattern of antagonism of human and rat-α-CGRP by peptide fragments in the guinea-pig ileum and vas deferens. √indicates statistically significant antagonism of the agonist control response, x indicates no antagonism occurred,- indicates not determined. Details of the antagonist concentrations can be found in chapters 4 and 5.*

classification which divides the receptors into types 1 and 2 and possible further division based upon novel findings reported here. These receptors appear to be both species and tissue dependent ;

$CGRP_1$ receptors (Guinea-pig ileum)	- (α) Activated by human-α-CGRP. Sensitive to human-α-$CGRP_{(8-37)}$ less sensitive to $CGRP_{(19-37)}$ than 1β - (β) Activated to rat-α-CGRP. Less sensitive to human-α-$CGRP_{(8-37)}$
$CGRP_2$ receptors (Guinea-pig vas deferens)	- (α) Activated by human-α-CGRP. Insensitive to human-α-$CGRP_{(8-37)}$ - (β) Activated by rat-α-CGRP. Insensitive to human-α-$CGRP_{(8-37)}$

The current receptor classification does not account for putative subtypes and may be required to do so if future studies support the findings reported here. The present classification relies almost entirely upon the effects of a single CGRP fragment; $CGRP_{(8-37)}$. As more peptide fragments have become available with both agonistic and antagonistic properties, the current classification needs to be updated to account for the potency of these compounds and the receptors with which they interact.

7.1.3 The Guinea-pig amylin receptor

Results shown in chapters 4-6 suggest the existence of high affinity binding sites for amylin; specific amylin receptors.

In the ileum, rat amylin-induced inhibition of stimulated twitch contractions was shown to be of lower potency than CGRP and this effect was not antagonised by any of the CGRP or amylin receptor antagonists utilised. Based upon these observations it is proposed that amylin may not be working via a CGRP receptor in the guinea-pig ileum. Due to the low potency of action seen with rat amylin it may be possible that

the level of amylin receptor expression may be low in this preparation or it may not be acting via a distinct amylin receptor. Further experiments using more amylin receptor antagonists would be required before valid conclusions regarding the receptor at which rat amylin was working in this preparation can be made.

In the guinea-pig vas deferens, human and rat amylin-induced responses were antagonised by $CGRP_{(8-37)}$ (3μM and 1μM respectively). The putative amylin receptor antagonist, AC187, $AM_{(22-52)}$ and $amylin_{(8-37)}$ antagonised rat amylin-induced responses but not those induced by CGRP. These results imply that amylin is acting via a receptor distinct to that used by CGRP. Human amylin and rat amylin-induced effects exhibited distinct patterns of sensitivity to antagonism by the peptide fragments. Rat amylin-induced effects were antagonised by $CGRP_{(19-37)}$, AC187 and $amylin_{(8-37)}$, whereas the human amylin-induced effects were not. These results implied that two amylin receptors may be present on the guinea-pig vas deferens, capable of distinguishing between these two forms of amylin.

In general, the results presented here may suggest that the CGRP receptors present in rat liver, spleen and cerebellum may be of different subtypes which require different areas of the CGRP molecule for successful ligand: receptor interactions but more conclusive evidence needs to be obtained.

In the guinea-pig ileum and vas deferens distinct CGRP and amylin receptors were identified which may be able to differentiate between their endogenous receptor ligands. These receptors could be tissue specific and based upon previous studies using the rat vas deferens (Giuliani *et al.*, 1992), species specific. CGRP receptors were identified in the guinea-pig atria.

Future studies need to investigate the selectivity of the CGRP and amylin receptor antagonists. Further studies on tissue preparations from the guinea-pig and other

animal species need to be carried out in order to lend support to the theory of further subdivisions of the current CGRP receptor classification and examine the species specificity of CGRP and amylin receptors.

Chapter 8

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