Dissertation Approved By:

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Dean of the Graduate School
DESIGN, SYNTHESIS AND PHARMACOLOGICAL CHARACTERIZATION
OF NOVEL CALCITONIN GENE-RELATED PEPTIDE RECEPTOR
ANTAGONISTS

By
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A DISSERTATION

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ABSTRACT

Human-α-calcitonin gene-related peptide (CGRP) is a 37 amino acid residue neuropeptide produced from tissue-specific alternative splicing of the primary RNA transcript of the calcitonin gene. CGRP is widely distributed throughout the central and peripheral nervous systems and is involved in wound healing, inflammatory responses, nociception, appetite suppression, gastric emptying, regulation of vascular tone and regional organ blood flow.

The conformation of CGRP, defined in CD and two-dimensional 1H-NMR studies, includes a disulfide bridged N-terminal ring structure between residues 2 and 7, an amphipathic α-helical segment between residues 8 through 18 which ends in a β-turn centered on residues 19 through 22 and a conformationally disordered C-terminus. Molecular modeling studies suggest a turn structure centered on residue 33 exists. Previous studies have shown that the N-terminal loop of CGRP is required for the activation of signal transduction by CGRP receptors. Truncation of the N-terminal ring, resulting in CGRP(8-37), leads to loss of agonist activity while retaining the ability to bind to the receptor and CGRP(8-37) became the primary research tool used to investigate CGRP receptors. The development of CGRP receptor antagonists has progressed slowly since the inception of the CGRP field. Further truncation of the N-terminus of CGRP(8-37) has resulted in antagonists at CGRP receptors with marked reductions in antagonist potency, suggesting that N-terminal structural elements of CGRP(8-37) are important for high affinity binding to CGRP receptors.
Previous studies in my lab described a series of N-terminally modified CGRP(8-37) analogs that were high affinity antagonists of calcitonin gene-related peptide receptors. These analogues, N-α-benzoyl-h-α-CGRP(8-37), N-α-benzyl-h-α-CGRP(8-37) and dibenzyl-h-α-CGRP(8-37), all possess binding affinities for CGRP receptors, similar to the endogenous ligand CGRP, in the low nanomolar range. These studies show that the addition of functional groups to CGRP(8-37), rather than truncation, may be an improved strategy for the development of high affinity antagonists.

The goal of my research was to design, synthesize and pharmacologically characterize novel CGRP receptor antagonists. The antagonists were synthesized using rapid solid phase methods employing in situ neutralization, purified by reversed phase-high performance liquid chromatography, characterized by amino acid analysis and electrospray ionization mass spectrometry and pharmacologically characterized using the mouse thoracic aorta and the human SK-N-MC cell line.

During the synthesis of these antagonists using rapid solid phase methodology, I observed that coupling yields for the synthesis of CGRP(8-37) were highly dependent on the solvent used. I investigated the relationship between solvent properties and solvation of the (para-methylbenzhydrylamine)copoly(styrene-1 % DVB) (resin) and resin covalently bound to the fully protected amino acid sequence of CGRP(8-37) (peptide-resin)
in order to obtain satisfactory coupling yields for the rapid solid phase peptide synthesis of the 30-amino acid residue N-truncated fragment of human-α-calcitonin gene-related peptide, CGRP(8-37). CGRP(8-37) was synthesized manually using rapid solid phase peptide synthesis in various polar aprotic solvents. Also, I measured solvation of the resin and peptide-resin, in solvents with varying hydrogen-bonding ($\delta_h$) and Hildebrand solubility ($\delta$) parameters and contour solvation plots of $\delta_h$ versus $\delta$ were constructed to correlate solvation of the resin or peptide-resin with $\delta_h$ or $\delta$. Maximum resin solvation occurred with NMP, NMP:DMSO (8:2) and DMSO, however, inefficient solvation of the peptide-resin occurred with these solvents and resulted in poor syntheses. Superior peptide-resin solvation was obtained using DMA and DMF, resulting in significantly higher average coupling yields. The region of maximum peptide-resin solvation shifted to solvents with higher $\delta_h$ values. DMF provided the most effective peptide-resin solvation and was the only solvent from which CGRP(8-37) was obtained as a single major product in the crude cleaved material.

Based on the known structure of CGRP(8-37) and the human CGRP receptor I designed an analogue which incorporated hydrophobic benzoyl and benzyl groups into the N-terminus and His$^{10}$ side-chain, respectively, to promote high affinity and selectivity for human CGRP receptors. The affinity ($K_b$) of the putative human selective competitive antagonist at CGRP receptors was determined using the mouse thoracic aorta and the human SK-N-MC cells. In aorta, CGRP(8-37), bzl-CGRP(8-37) and bzl-bn-CGRP(8-37) caused rightward
shifts in the concentration-response relaxation curve for CGRP with $K_B$ values of 1000, 88 and 50 nM, respectively. In human SK-N-MC cells CGRP(8-37), bzl-CGRP(8-37) and bzl-bn-CGRP(8-37) caused rightward shifts in the concentration-response curve for CGRP stimulated cAMP production with $K_B$ values of 797, 15 and 0.63 nM, respectively. Thus, CGRP(8-37) had the same affinity for human and mouse CGRP receptors while bzl-CGRP(8-37) and bzl-bn-CGRP(8-37) displayed 6-fold and 80-fold higher affinity, respectively, for human CGRP receptors. In addition, the selectivity of the antagonists for human CGRP receptors was highly correlated with the antagonist hydrophobicity index. These high affinity, species selective antagonists provide novel tools to differentiate structural and functional features that are unique to the human CGRP receptor. Thus, these analogues may be useful compounds for development of drugs to treat migraine headache and other cardiovascular diseases.

Also, I designed, synthesized and pharmacologically characterized the first irreversible CGRP receptor antagonists. Fluorosulfonyl or bis-(2-chloroethyl)amino moieties were incorporated into para-position of the N-terminal benzoyl group of a potent competitive antagonist, N-$\alpha$-benzoyl-h-$\alpha$-CGRP(8-37). In order to improve potency, a second pair of analogues was synthesized with histidine at position 10 benzylated, at C4 of the imidazole side-chain. All analogues blocked the actions of CGRP on mouse thoracic aorta and SK-N-MC cells and reduced maximal CGRP-mediated responses. The inability to obtain a maximal response to agonist after treatment and removal of either analogue is
consistent with the analogues binding irreversibly to CGRP receptors. The bis-(2-chloroethyl)amino-modified analogues were more potent than the fluorosulfonyl-modified analogues. Benzylation of the His$^{10}$ side-chain increased the potency of the bis-(2-chloroethyl)amino-modified irreversible antagonists but had no effect on the potency of the fluorosulfonyl-modified irreversible antagonist. The most potent analogue was N-$\alpha$-4-[bis-(2-chloroethyl)amino]benzoyl-[His(4-benzyl)$^{10}$]-h-$\alpha$-CGRP(8-37). The addition of electrophillic alkylating moieties to potent CGRP receptor competitive antagonists yielded the first irreversible antagonists of CGRP receptors.
PREFACE

Portions of the work described in this dissertation have been published in:


Dedicated to:

My parents,
Lawrence Randall and Kathleen Jean Taylor.

My Brother,
Lawrence Craig Taylor.

My wife,
Ashley Michelle Taylor.
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I am grateful for having the opportunity to collaborate with the people who have contributed to this work. I would like to thank my advisors Dr. Peter W. Abel and Dr. D. David Smith for their patience, guidance and support throughout my research project as well as fellowship writing and manuscript writing processes. I would also like to acknowledge the members of my committee, Dr. Charles Bockman for endlessly questioning my data and equipping me with the confidence to think scientifically on my feet and Dr. Martin Hulce for use of his laboratory, instrumentation and assistance in the characterization of synthesis products.

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I am also indebted to my brother for always being there to provide a place to escape from graduate school.

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Finally, and most importantly, I would like to thank my parents, for their tremendous, unconditional love and support, encouragement and advice without which I would not have been able to accomplish this.

It would have been arduous to endure this time in my life if not for the significant contributions of family and friends.
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<tr>
<td>BAM</td>
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<td>bn</td>
<td>Benzyl</td>
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<td>Dimethylsulfoxide</td>
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</tr>
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<td>Fourier transform-infrared spectroscopy</td>
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<td>GC-MS</td>
<td>Gas chromatography-mass spectrometry</td>
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<td>G Proteins</td>
<td>Heterotrimeric guanine nucleotide binding proteins</td>
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HBTU  O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate
HCTU  O-(6-Chlorobenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate

k' value  Hydrophobicity Index (Capacity Factor)

K_A  The equilibrium dissociation constant of the ligand-receptor complex.
K_B  Equilibrium dissociation constant determined in functional assays using a single antagonist concentration

MBHA  para-methylbenzhydrylaminecopoly(styrene-1 % DVB) resin
MeOH  Methanol
mp  Melting point
NMP  N-methylpyrrolidone

1H-NMR  1H-Nuclear magnetic resonance spectroscopy
NO  Nitric oxide

pA_2  \(-\log_{10}\) equilibrium dissociation constant determined from Schild regression analysis of functional assays using multiple antagonist concentrations
pK_B  \(-\log_{10}K_B\)

RAMP  Receptor activity-modifying protein
RP-HPLC  Reversed phase-high performance liquid chromatography
SPPS  Solid phase peptide synthesis
TFA  Trifluoroacetic acid
TFMSA  Trifluoromethanesulfonic acid
TLC  Thin layer chromatography
CHAPTER 1

INTRODUCTION

DISCOVERY, BIOSYNTHESIS AND MOLECULAR GENETICS

Calcitonin gene-related peptide (α-CGRP) is a 37-amino acid residue neuropeptide that was discovered by cloning of the rat calcitonin (CT) gene. Alternative tissue-specific processing of the primary messenger ribonucleic acid (mRNA) from the rat-CT gene resulted in generation of two distinct peptides, rat-CT and rat-α-CGRP (Amara et al., 1982; Rosenfeld et al., 1983). A human form of α-CGRP was isolated from thyroid tissue of patients with medullary thyroid carcinoma (Morris et al., 1984). A second CGRP gene was identified from a cosmid library and generates human β-CGRP and rat β-CGRP in humans and rats, respectively (Steenbergh et al., 1985; Alevizaki et al., 1986). CGRP has been established to belong to a family of peptides which includes calcitonin, adrenomedullin, amylin and intermedin.

CT and α-CGRP are derived from the α-CT/CGRP gene which is localized on the short arm of chromosome 11 p13 – p15 qter region between the catalase and the parathyroid hormone genes. Alternative splicing of the primary RNA transcript leads to translation of CT and α-CGRP in a tissue-specific manner. In the central nervous system (CNS) splicing of the α-CT/CGRP gene produces α-CGRP, whereas in the C cells of the thyroid gland, CT is predominantly produced. The β-CGRP gene, also located on chromosome 11, is thought to be
due to gene duplication and it is unclear which gene appeared first (Hoppener et al., 1985). β-CGRP is produced in all tissues which express the gene (Steenbergh et al., 1986). A third CT/CGRP-like gene has also been identified on chromosome 11 which contains nucleotide sequences corresponding to exons II and III of α-CT/CGRP and β-CGRP genes. However, this was found to be a pseudogene as it is not transcribed (Hoppener et al., 1988).

A schematic representation of the human α-CT/CGRP gene structure is illustrated in Figure 1. The CT/CGRP gene consists of six exons. The first three exons are constitutively spliced in both CT and CGRP mRNAs. Exon 1 codes the 5'-untranslated region, exon II codes for the signal peptide and exon III codes for the N-terminal propeptide. Exon IV codes for the CT sequence, katacalcin, a carboxy-terminal flanking peptide, the 3'-untranslated region of CT mRNA and polyadenylation (polyA sites) signal. Exon V codes for the CGRP sequence and a carboxy-terminal flanking tetrapeptide. Exon VI is part of the α-CGRP mRNA and codes for the 3'-untranslated region and polyA signal. Alternative RNA processing of the RNA precursor involves differential recognition of polyA sites on exons III and IV (Amara et al., 1984). Splicing of exon III to exon IV with polyA tail at the 3'-end of exon IV yields CT mRNA and splicing of exon III to exon V with polyA tail at the 3'-end of exon VI yields CGRP mRNA. The mRNA is translated to generate the 127-amino acid residue preproCT and 128-amino acid residue preproCGRP precursor peptides. Posttranslational modifications and proteolytic processing of amino terminus and carboxy terminus flanking peptides
result in release of the C-terminally amidated mature 32-amino acid residue CT and 37-amino acid residue CGRP peptides.

The organization of the β-CGRP gene is similar to the α-CT/CGRP gene. It contains sequences highly homologous to exons III, V and VI of the α-CT/CGRP gene. Exon I codes the 5'-untranslated region, exon II codes for the signal peptide and exon III codes for the N-terminal propeptide. Exon IV, which is 67 % homologous to the region of the α-CT/CGRP gene which gives rise to CT, lacks the polyA signal which prevents alternative splicing. Consequently, transcripts from this gene only produce β-CGRP.
Figure 1. Model of tissue-specific splicing of the calcitonin gene leading to production of calcitonin and CGRP. Exons I – VI (rectangles) are transcribed in neurons and in thyroid C cells. The primary transcript is alternatively spliced to produce discrete mRNA products. Subsequently, mRNA is translated to generate two prepropeptides that are proteolytically processed to produce calcitonin and CGRP. DNA and RNA sequences and amino acid sequences that are common to both pathways are shown in gray. Sequences that are specific to calcitonin and CGRP are shown in white and black, respectively.
STRUCTURE OF CGRP

CGRP belongs to a family of peptides which includes calcitonin, adrenomedullin, amylin and intermedin which are 20 – 50 % similar in sequence. CGRP has been isolated and sequenced from numerous species. Table 1 lists the amino acid sequence of CGRP from seven species (Rosenfeld et al., 1983; Morris et al., 1984; Amara et al., 1985; Steenbergh et al., 1985; Minvielle et al., 1986; Kimura et al., 1987; Collyear et al., 1991; Eysselein et al., 1991; Conlon et al., 1993). All species variants of CGRP have 37 amino acid residues. Human α-CGRP and human β-CGRP differ by three amino acid residues while rat α-CGRP and rat β-CGRP differ by one amino acid residue. The high homology of human CGRP with other species suggests that CGRP is a phylogenetically well conserved peptide.

The secondary structure of human α-CGRP has been defined by circular dichroism (Manning, 1989; Hubbard et al., 1991; Mimeault et al., 1993) and two-dimensional proton nuclear magnetic resonance spectroscopy (1H-NMR) (Breeze et al., 1991; Boulanger et al., 1995). Figure 2 depicts a computational molecular model of the three-dimensional structure of CGRP which correlates well with the structure determined by 1H-NMR. The N-terminus consists of a characteristic disulfide-bridged loop formed between Cys² and Cys⁷. Many peptide hormones contain regions of amphiphilic secondary structures that strongly influence their function. CGRP contains an amphipathic α-helical segment between residues 8 through 18 which ends in a β-turn centered on residues 19 through 22. The C-terminus is amidated and lacks a well defined
secondary structure, although molecular modeling studies suggest a turn structure centered on residue 33 exists (Hakala and Vihinen, 1994).

Table 1. Amino acid sequence of nine calcitonin gene-related peptides from seven species.³

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<tr>
<th>No.</th>
<th>Human CGRPα</th>
<th>Human CGRPβ</th>
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<th>Rat CGRPβ</th>
<th>Porcine CGRP</th>
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³Identical amino acid residues from human-α-CGRP are indicated with (–) and variations are specified.

³Sequences obtained from: Morris et al., 1984; Wimalawansa et al., 1990; Amara et al., 1982; Amara et al., 1985; Kimura et al., 1987; Minvielle et al., 1986; Conlon et al., 1993; Eysselein et al., 1991; and Collyear et al., 1991; respectively.
Figure 2. Molecular model of the 3-dimensional structure of CGRP using HyperChem 7.5. (by Martin Hulce Ph.D. Department of Chemistry, Creighton University). A) Stick model and B) Ribbon model (with the peptide backbone depicted as the green ribbon) of the 3-dimensional structure of CGRP. The N-terminal ring formed by a disulfide bond between Cys$^2$ and Cys$^7$ is shown in yellow followed by an α-helical segment between Val$^6$ through Arg$^{18}$ which ends in a β-turn centered on residues Ser$^{19}$ through Val$^{22}$. The C-terminus is conformationally disordered although a turn structure is suggested to exist centered on Gly$^{33}$. 
BIOLOGICAL ACTIONS

Nervous System. CGRP is one of the most abundant peptides in the nervous system which suggests it is involved in a variety of biological actions. CGRP is extensively distributed in the brain and spinal cord, often coexisting in neurons with other neurotransmitters. In the brain CGRP is present in the nuclei of sensory and motor cranial nerves and in cell bodies of the hypothalamus, preoptic area, ventromedial thalamus, medial amygdala, hippocampus, superior colliculus, lateral lemniscus and dentate gyrus (Goodman and Iversen, 1986; Yamamoto and Tohyama, 1989). In the spinal cord it is concentrated in areas receiving sensory input in the dorsal horn and motor neurons in the ventral horn. In the peripheral nervous system it is found in both sensory and motor nerves. In the autonomic nervous system it is found preganglionically in sympathetic and parasympathetic nerves and postganglionically in nonadrenergic, noncholinergic nerves. Thus, CGRP is found in the skin, heart, blood vessels, gastrointestinal tract, tongue, esophagus, pancreas, salivary glands, lungs, kidney and other organs.

When injected into the CNS, CGRP has a number of actions which indicates that it can activate neuronal pathways. CGRP increases noradrenergic sympathetic outflow, leading to increased heart rate and blood pressure (Fisher et al., 1983), decreased appetite (Tannenbaum and Goltzman, 1985), gastric acid secretion (Tache et al., 1984; Lenz et al., 1985), gastrointestinal motility (Fargeas et al., 1985; Tache et al., 1991) and growth hormone release (Tannenbaum and Goltzman, 1985). It increases rectal temperature, suggesting
a role in thermoregulation (Dennis et al., 1990). It decreases motor activity and causes catalepsy (Clementi et al., 1992). It influences the response to noxious stimuli; intrathecal injection lowers the threshold for mechanical stimulation (Cridland and Henry, 1989), antiserum against CGRP raised the nociception threshold for both thermal and mechanical stimuli (Kawamura et al., 1989) and central administration of CGRP produced antinociception in the mouse measured in the hot plate and formalin tests (Welch et al., 1988; Candeletti and Ferri, 1990).

**Cardiovascular System.** One of the major effects of CGRP is relaxation of vascular smooth muscle. CGRP is one of the most potent vasodilators known. It was first found that intradermal injection of femtomole quantities of CGRP in human and rabbit skin produced an increase in local blood flow which lasted for several hours (Brain et al., 1985; Struthers et al., 1986). The vasodilator activity of CGRP has since been extensively studied *in vitro*; the peptide relaxes almost every vascular preparation studied. CGRP-induced vasodilation has been shown to be mediated through an endothelium-dependent and endothelium-independent mechanism. In most vascular tissue preparations studied *in vitro* CGRP-induced vasodilation has been found to be endothelium-independent, such as in the cat middle cerebral artery (Edvinsson et al., 1985), rat mesentery (Li and Duckles, 1992) porcine coronary artery (Yoshimoto et al., 1998) and mouse aorta (Pomerleau et al., 1997). Vasodilation in the absence of endothelium implies that CGRP acts directly on CGRP receptors expressed on smooth muscle cells and correlated with stimulation of adenylate cyclase and production of 3’,5’-cyclic
adenosine monophosphate (cAMP). Exceptions to this include the rat aorta (Gray and Marshall, 1992a; Gray and Marshall, 1992b), human internal mammary artery (Raddino et al., 1997) and rat pulmonary artery (Wisskirchen et al., 1998) where CGRP-induced vasodilation was found to be dependent on an intact endothelium and responses were blocked by nitric oxide (NO) synthase inhibitors which suggests that CGRP can act directly on CGRP receptors expressed on endothelial cells and is dependent on release of NO.

CGRP-immunoreactivity has been shown to be broadly distributed in the cardiovascular system. CGRP is localized in a dense peripheral sensory network innervating arteries, veins and heart. CGRP is known to regulate inotropy, chronotropy, microvascular permeability, angiogenesis and also protects the myocardium against ischemic-reperfusion injury (Li et al., 1996). To date, it is unclear whether CGRP acts locally or systemically in the maintenance of basal blood pressure. Intravenous administration of CGRP in the rat decreased mean arterial blood pressure and coronary artery vascular resistance consequently increasing coronary blood flow which was blocked by the antagonist CGRP(8-37). In the guinea pig perfused heart and rat isolated atrium, positive inotropic and chronotropic responses are produced in by CGRP. In contrast, studies with intravenous injection of the antagonist CGRP(8-37) in rodents have shown a lack of effect on blood pressure (Gardiner et al., 1991). To address the role of CGRP on homeostasis of the cardiovascular system, several α-CGRP knockout mice have been generated and the results have only added controversy. The first strain of α-CGRP mice, created by introduction of a translational stop codon
into exon V of the CT/CGRP gene, resulted in normal expression of CT and no expression of α-CGRP. These mice did not show changes in heart rate or blood pressure (Lu et al., 1999). In contrast, a second α-CGRP−/− mouse, created by replacement of exons II – V with a PGK neoBPA cassette resulting in CT and α-CGRP double knockout (CT−/−/α-CGRP−/−), resulted in increased systolic and mean arterial pressure (Gangula et al., 2000). In a third α-CGRP−/− mouse, created by targeted disruption of exon V by replacement with the neomycin resistance gene, mean arterial pressure, heart rate, peripheral vascular resistance and sympathetic nervous activity were elevated (Oh-hashi et al., 2001).

**Gastrointestinal Tract.** CGRP exerts potent central and peripheral action to inhibit gastric acid secretion in rats (Plourde et al., 1993) and dogs (Pappas et al., 1986) and gastric emptying, inhibits contractility and ulcer formation in rats (Holzer et al., 1991). The inhibition of acid secretion is related primarily to the decrease in vagal efferent activity whereas the inhibition of gastric motor functions involves increases in sympathetic outflow. The presence of CGRP-like immunoreactivity and receptors in medullary nuclei receiving visceral information and influencing vagal outflow suggests a role in the vagal regulation of gastric secretion. The peripheral antisecretory effect involves release of gastric somatostatin through interaction with CGRP receptors characterized on D cells. In addition, CGRP induces a decrease in acetylcholine transmission in the enteric nervous system which may contribute to the inhibition of acid. Peripheral CGRP inhibits gastric emptying and motility by a direct action on smooth muscles.
through CGRP receptors. The release of CGRP from spinal afferents innervating the stomach in response to stimulation of capsaicin-sensitive fibers suggests a role in the regulation of gastric function. The central action of CGRP to prevent ethanol-induced lesions is unique to this peptide and not shared by other centrally acting inhibitors of gastric function. It may be related to an increase in gastric mucosal blood flow induced by CGRP (Holzer et al., 1991). Systemic capsaicin treatment led to a complete loss of CGRP immunostaining in the stomach, however, β-CGRP was still present in the intestine. Thus, α-CGRP in the intestine is from an extrinsic site and suggests that a distinct neuronal populations containing β-CGRP. mRNA analysis of the expression of α-CGRP and β-CGRP revealed that only β-CGRP mRNA is expressed in the intestine and it is localized to enteric neurons (Mulderry et al., 1988). In humans, it was found that β-CGRP but not α-CGRP, could inhibit gastric acid secretion (Beglinger et al., 1988).

**Inflammation.** Long lasting vasodilation and increased vascular permeability are responsible for the pro-inflammatory actions of CGRP. In human skin, CGRP produces a potent wheal and flare reaction followed by delayed erythema and surrounding pallor that lasts several hours (Piotrowski and Foreman, 1986). The vasodilation and increased vascular permeability potentiates the actions of inflammatory mediators including histamine, leukotriene B4, N-formyl-methionyl-leucyl-phenylalanine, platelet-activating factor, bradykinin and substance P (Brain and Williams, 1985; Hughes and Brain, 1991; Cruwys et al., 1992). CGRP has also been shown to decrease IL-2 production
from T lymphocytes (Wang et al., 1992), decrease surface immunoglobulin production, inhibit proliferation of B cells (McGillis et al., 1993) and induce granule secretion from neutrophils (Richter et al., 1992). Thus, CGRP may act as a local regulator of the immune response at various levels.

**Pain.** The contribution of CGRP in pain perception has been studied in rat, CGRP−/− and CT−/−/CGRP−/− mice. In rats, CGRP decreased analgesia produced by μ and δ opioid agonists and to a lesser extent by κ opioid agonists (Welch et al., 1989) and morphine was shown to inhibit the release of CGRP in the spinal cord (Pohl et al., 1989). The antagonist CGRP(8-37) was effective in preventing development of tolerance to the antinociceptive effects of μ opioid agonists (Menard et al., 1996). In CGRP−/− and CT−/−/CGRP−/− mice, endogenous CGRP potentiated morphine analgesia in spinal pain pathways, but not higher brain pathways (Salmon et al., 1999) and withdrawal symptoms to morphine and nicotine were reduced (Salmon et al., 2004). Also, CGRP−/− exhibited reduced edema and nociception to chemical pain associated with inflammation. These studies show that CGRP differentially modulates opioid and nicotine evoked analgesia, worsens withdrawal signs after opioid and nicotine use dependence and mediated neurogenic inflammation and pain sensation after neurogenic and arthritic inflammation.

Until recently, CGRP has not been shown to exert any acute sensitizing effects on cutaneous nociceptor endings or to induce heat hyperalgesia. In mice, CGRP gene expression, content, release, and function are all highly strain-dependent and was shown to account for the inherited strain differences in the
thermal response of 11 mice breeds. Recently, it was determined that CGRP does induce heat hyperalgesia depending on the mouse strain studied. Strain differences in behavioral response to noxious heat could be abolished by peripheral injection of CGRP, blockade of cutaneous and spinal CGRP receptors, or long-term inactivation of CGRP with an RNA aptamer (48 oligonucleotide mirror image RNA sequence) that binds CGRP. Furthermore, linkage mapping revealed a candidate gene likely responsible for the strain difference: *Calca*, the gene encoding α-CGRP and CGRP gene expression correlated with differential response to noxious heat. Low CGRP expression led to relative insensitivity to noxious heat (mouse strains: C57BL/6, 129P3, C3H/He, CJ57BL/10, C58, CD-1, and RIIS) while higher CGRP expression results in noxious heat sensitivity (mouse strains: AKR, A, BALK/c, CBA, DBA/2 and SM) (Mogil et al., 2005).

**Bone Metabolism.** CGRP-immunoreactive sensory nerve fibers are localized in bone, bone marrow, periosteum, synovial membranes and soft tissue adjacent to bone (Bjurholm et al., 1988). CGRP mRNA and receptor expression has been detected in osteoblastic and osteoclastic cells (Togari et al., 1997). The presence of CGRP in sensory nerve fibers and expression of CGRP receptor on numerous types of bone cells suggests that CGRP physiologically regulates bone formation and/or metabolism. CGRP has been shown to regulate production of bone-active factors such as, increasing release of interleukin-6 from marrow stromal cells (Sakagami et al., 1993) and insulin-like growth factor-1 from osteoblasts (Vignery and McCarthy, 1996) and decreasing osteoblast production of tumor necrosis factor-α (Millet and Vignery, 1997). CGRP has been shown to
inhibit proliferation and osteoclast differentiation of mice (Owan and Ibaraki, 1994) and human bone marrow cells and human hematopoietic progenitor CD34+ cells (Akopian et al., 2000); and increase proliferation of human osteoblast cells (Villa et al., 2000). CGRP-induced inhibition of bone resorption and osteoclast differentiation are direct effects of CGRP receptor-mediated increases in intracellular cAMP that were blocked by the antagonist CGRP(8-37). In vivo, similar to calcitonin, CGRP was shown to inhibit bone resorption in rats and rabbits, but not bone formation, and was about 100-fold less potent than calcitonin in preventing bone resorption (Valentijn et al., 1997). In transgenic mice, targeted expression of CGRP to osteoblast cells resulted in increased bone volume and density which was associated with increased bone formation rate (Ballica et al., 1999). In CT−/−/CGRP−/− mice, increased bone mass and bone formation rate were observed, rather than the expected osteopenia. Furthermore, ovarectomized CT−/−/CGRP−/− mice maintained bone mass (Hoff et al., 2002). Thus, CT and CGRP-deficiency leads to increased bone mass under basal (non-ovarectomized) and under increased bone resorption (ovarectomized). Thus, CGRP may have multiple roles in bone metabolism: prevention of bone resorption in hypercalcemic states, a regulatory systemic role in bone formation and a local regulator of bone cell function.

Learning and Memory. CGRP-immunoreactive fibers have been found in the caudate-putamen and amygdala which originate in the posterior thalamus and the parabrachial nucleus of the pons, respectively. These regions of the brain are associated with the formation of learned associations between acoustic
stimuli and pain. CGRP was shown to enhance the acquisition, consolidation and retrieval of a passive learning task. CGRP enhanced the latency of passive avoidance behavior in rats (Kovacs and Telegdy, 1992) and administration of CGRP antiserum impaired latency (Kovacs and Telegdy, 1994). In active avoidance behavior tasks, CGRP decreased the avoidance latency (Kovacs and Telegdy, 1995). This suggests that CGRP may be involved in the facilitation of learning and memory processing.

CGRP RECEPTORS AND ACCESSORY PROTEINS

Receptor Isolation, Cloning, Sequencing and Characterization. In 1989 the first CGRP-like receptor was cloned using polymerase chain reaction (PCR). The canine orphan receptor gene RDC-1, when expressed in African green monkey kidney cells (COS-7) increased cAMP (EC$_{50}$ 3 nM) in response to CGRP that was blocked by the antagonist CGRP(8-37) (Kapas and Clark, 1995). The literature has not further supported RDC-1 as a CGRP receptor (McLatchie et al., 1998). The rat calcitonin receptor-like receptor (CL) was cloned in 1993 (Njuki et al., 1993) and human CL cloned in 1995 (Fluhmann et al., 1995) and consists of 461 amino acids with seven-transmembrane domains. However, the receptor did not bind CGRP in COS-7 cells and was considered an orphan receptor. Using an expressed sequence tag analysis, a complementary deoxyribonucleic acid (cDNA) encoding another CGRP-like receptor was cloned from a human synovial tissue cDNA library that had significant peptide sequence homology with CL. Stable expression of this cDNA in human embryonic kidney 293 (HEK293) cells
demonstrated a specific high affinity binding site and in response to CGRP a 60-fold stimulation of cAMP production over basal that was blocked by the antagonist CGRP(8-37) (Aiyar et al., 1996). These results were confirmed using rat (Han et al., 1997) and porcine CL (Elshourbagy et al., 1998). However, Han et al. also reported that in COS-7 cells expressing CL failed to produce cAMP in response to CGRP and they concluded that HEK293 cells must contain an intrinsic factor necessary for formation of the CGRP receptor.

Accessory Proteins. A cDNA library was created from human neuroblastoma cells (SK-N-MC), which endogenously express CGRP receptors. The cDNA library was transcribed in vitro and pools of complementary ribonucleic acid (cRNA) were injected into Xenopus oocytes with cRNA encoding the cystic fibrosis transmembrane regulator (CFTR). CFTR contains a cAMP-activated chloride channel that can be used as a sensitive read-out for receptors positively coupled to adenylate cyclase. Xenopus oocytes endogenously express a CGRP receptor, measured through a small inward current after CGRP stimulation, and a single population of cells was found that demonstrated a significantly larger response than the endogenous response. A single cDNA was isolated that encoded a 148-amino-acid protein called receptor-activity modifying protein 1 (RAMP1). RAMP1 consists of a short cytoplasmic carboxyl terminus, a single transmembrane domain and a large extracellular amino terminus. Two additional RAMP isoforms have been identified, RAMP2 and RAMP3 (McLatchie et al., 1998). RAMP2 and RAMP3 share 55–58% amino acid sequence identity, respectively, to RAMP1. When expressed alone RAMP1 is located in the
endoplasmic reticulum and the golgi as a disulfide-linked homodimer (Hilairet et al., 2001). When expressed at the cell surface RAMP1 interacts noncovalently with CL to form a 1:1 heterodimer that is stable at the cell surface (McLatchie et al., 1998; Poyner et al., 2002). RAMP1 promotes cell surface expression of the CL-RAMP1 complex (Foord and Marshall, 1999), and presents CL at the plasma membrane as a terminally glycosylated mature glycoprotein. Coexpression of CL and RAMP1 is required to produce a functional receptor that displays CGRP receptor pharmacology (Figure 3). Coexpression of CL with RAMP2 or RAMP3 present CL as an immature core glycosylated adrenomedullin receptor. Thus, ligand selectivity of CL is determined by RAMP coexpression and it has been proposed that the amino termini of CL and RAMP1 participate directly in ligand binding or act indirectly to modulate the ligand binding conformation of CL (Hilairet et al., 2001). Subsequently, an intracellular membrane associated protein was identified from *Xenopus* oocytes which was proposed to be part of the CGRP receptor protein complex. Receptor component protein (RCP) was cloned and shown to provide CGRP receptor activity to *Xenopus* oocytes by coupling CGRP receptors to the adenylate cyclase signal transduction pathway (Figure 3). This role of RCP has not been fully examined and whether this protein participates as a necessary component of the functional CGRP receptor complex awaits further study.
Figure 3. CGRP receptor structure and intracellular signaling pathways.
Receptor Distribution. Several studies have demonstrated the anatomically discrete binding sites for CGRP in the CNS of various species including rat and human (Tschopp et al., 1985; Inagaki et al., 1986; Henke et al., 1987). Overall the respective distribution of CGRP mRNA, CGRP-immunoreactivity and CGRP binding sites throughout the brain as well as in various other organs such as the heart, lung, liver and skeletal muscle correlate rather well (Van Rossum et al., 1997). The source of minor discrepancies observed has been suggested to be a result of species differences in the CGRP detection probes used and the sensitivity of various assays.

Intracellular Signaling. CGRP receptors are members of the B family of seven-transmembrane G protein-coupled receptors. In most tissues and cell lines CGRP receptors are coupled to the Gs family of heterotrimeric guanine nucleotide binding proteins (G proteins) and to activation of adenylate cyclase leading to an increase in 3',5' cyclic adenosine monophosphate (cAMP) (Aiyar et al., 1999). Although, CGRP receptors have been shown to couple to other G proteins and activate multiple signal transduction pathways (Figure 3).

In SK-N-MC cells, CGRP receptors couple to Gs, activating adenylate cyclase leading to production of cAMP and activation of protein kinase A (PKA) (Wellman et al., 1998). PKA in turn has been shown to phosphorylate potassium channels (Wellman et al., 1998), decrease intracellular calcium, phosphorylate c-Jun N-terminal kinase (JNK) (Disa et al., 2000; Parameswaran et al., 2000), which was blocked by the PKA inhibitor H89, and phosphorylate CL. Also, CGRP receptors were shown to couple to the Gi G protein. JNK phosphorylation was
not completely blocked by H89 upon CGRP stimulation and was pertussin toxin sensitive.

In HEK293 cells transfected with the porcine CL and human RAMP1, CGRP receptors have been shown to couple to the Gs--adenylate cyclase--cAMP cascade, and to the activation of PKA which subsequently activates p38 mitogen-activated protein kinase (p38) and extracellular signal-regulated kinase (ERK). The mitogen activated protein kinase cascade (MAPK) resulting in ERK activation was also shown to be mediated through the Gs βγ subunit--Src--phosphoinositide 3-kinase (PI3K) cascade, in a wortmannin-sensitive manner. CGRP receptors were also shown to couple to the Gq G protein: CGRP stimulation induced activation of phospholipase C (PLC) which produces inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate (PIP2). DAG was shown to activate Src through PKC and activation of the MAPK cascade resulting in ERK activation.

These various intracellular signaling pathways have been linked to various functions including CGRP-mediated vascular relaxation, angiogenesis and positive inotropic and chronotropic effects in the heart.

CGRP RECEPTOR ANTAGONISTS AND STRUCTURE ACTIVITY STUDIES

Structure and Antagonist Effects of CGRP(8-37). The solution structure of CGRP(8-37) has been defined by CD (Manning, 1989; Hubbard et al., 1991; Matsuura and Manning, 1993; Mimeault et al., 1993) and two-dimensional 1H-NMR (Boulanger et al., 1995) in aqueous, helix-promoting solvent mixtures. All
studies displayed a significant loss of helix structure relative to CGRP. In NMR studies, hydrogen bonding between the amide carbonyl oxygen of Val\textsuperscript{28} and the amide proton of Asn\textsuperscript{31} was shown to exist and determined to be important for antagonist affinity.

The N-terminal loop of CGRP is required for the activation of signal transduction by CGRP receptors. N-terminal fragments, CGRP(1-12), CGRP(1-15) and CGRP(1-22) bind to CGRP receptors in an agonistic manner, although very high concentrations are needed to induce vasodilation (Maggi et al., 1990). Deletion of the N-terminal ring, resulting in CGRP(8-37), leads to loss of agonist activity while retaining the ability to bind to the receptor (Chiba et al., 1989). Further truncation of the N-terminus of CGRP(8-37) has resulted in antagonists at CGRP receptors with marked reductions in antagonist potency. The N-terminal truncated analogue CGRP(9-37) is equipotent to CGRP(8-37), while CGRP(10-37) is less potent and CGRP(11-37) is significantly less potent. These data indicate that Val\textsuperscript{8} may not be important for receptor recognition, while Thr\textsuperscript{9} and His\textsuperscript{10} are important for maintaining high affinity to CGRP receptors. CGRP(12-37), CGRP(19-37), CGRP(23-37), CGRP(28-37) and [Tyr\textsuperscript{0}]CGRP(28-37) are antagonists with weak potency suggesting that N-terminal structural elements of CGRP(8-37) are important for high affinity binding to CGRP receptors (Chakder and Rattan, 1990; Mimeault et al., 1991; Rovero et al., 1992). The \(\alpha\)-helical segment confers high affinity binding to CGRP receptors and residues Thr\textsuperscript{8} through Leu\textsuperscript{12} have been shown to stabilize the helix formation. Increases in the helical content of this segment by replacement of specific residues with the \(\alpha\)-
helix promoting residue Ala, increases the potency of the antagonist CGRP(8-37) (Mimeault et al., 1992). These studies show that both the amino acid sequence and three-dimensional conformation of CGRP(8-37) are important for binding to the receptor and that the addition of functional groups to CGRP(8-37), rather than truncation, may be an improved strategy for the development of high affinity antagonists.

Non-Peptide Antagonists. A potent and selective non-peptide CGRP receptor antagonist has the potential of being a novel and effective treatment for hypertension, cardiac failure, Raynaud's syndrome, migraine, preeclampsia, type 2 diabetes, neurogenic inflammation and other pathophysiological states. A limited number of selective non-peptide CGRP receptor antagonists have been described.

A high throughput screen of the SmithKline Beecham compound collection using porcine lung membranes identified a quinine analogue, originally reported in 1971 as an antimalarial (Yardley et al., 1971), with low micromolar activity (Figure 4). These results were confirmed using SK-N-MC and HEK293 cells. The quinine analogue inhibited specific binding of [125I]-CGRP on membranes from SK-N-MC and HEK293 cells (IC50 6 μM) and inhibited CGRP-induced cAMP production (IC50 6 μM) (Daines et al., 1997).

The first high affinity small molecule antagonist, BIBN4096BS, was reported in 2000 (Doods et al., 2000). A high throughput screen of the Boehringer Ingelheim compound library led to the identification of dipeptide-like compounds ((R)-Tyr-(S)-Lys) that inhibited specific binding of [125I]-CGRP on
membranes from SK-N-MC cells with micromolar affinity. Lead optimization indicated that interaction with the CGRP receptor was stereospecific (R,S). Structure activity studies (SAR) on the S-Lys segment identified incorporation of a rigidifying element bearing a negatively polarized group at the end of the aromatic fragment as favorable for CGRP receptor interaction. SAR on the R-Lys segment identified a urea bridge, a rigidifying piperazinyl group and the presence of bicyclic quinazolinone system at a certain distance from the dibromo-Tyr as favorable for CGRP receptor interaction (Figure 4) (Rudolf et al., 2005). BIBN4096BS possessed 14 pM affinity for the human CGRP receptor in SK-N-MC cells, higher affinity for the human CGRP receptor than the endogenous ligand, 150-fold higher affinity than CGRP(8-37) and 200-fold higher affinity for primate CGRP receptors compared to rat, dog, guinea pig and rabbit CGRP receptors (Doods et al., 2000). Receptor chimera studies determined that the amino acids 66 – 112 in the amino terminus of RAMP1 were responsible for modulating the affinity of BIBN4096BS. Protein sequence alignment of RAMP1 between human, marmoset, rat, mouse, dog and rabbit revealed that amino acid 74 was the least conserved between species; human and marmoset sequences contained a tryptophan, while rat, mouse, pig and rabbit contained a basic lysine residue. Site-directed mutagenesis of Lys$^{74}$ to Trp$^{74}$ in RAMP1, in non-human species, and cotransfection with CL revealed human CGRP pharmacology. These studies determined that the affinity and species selectivity of BIBN4096BS is influenced by the RAMP1 protein, specifically the amino acid at position 74.
Subsequently, two more non-peptide antagonists were reported in 2001, Compound 1 (WO98/11128) and SB(+)273779 (Figure 4). Compound 1 inhibited specific binding of \[^{125}\text{I}]\text{-CGRP} on membranes from SK-N-MC cells (pK\text{,} 7.8) and antagonized CGRP-induced cAMP production (pA\text{,} 7.7). In human cerebral arteries Compound 1 antagonized CGRP-induced vasodilation (pA\text{,} 8.1) (Edvinsson et al., 2001). In contrast, Compound 1 only weakly antagonized CGRP-induced vasodilation in the guinea pig basilar artery (pA\text{,} 5.7) (Edvinsson et al., 2001) and had no antagonistic effect in the porcine left anterior descending coronary artery (Hasbak et al., 2001). These studies suggest that Compound 1 may have some human selectivity. Receptor chimera studies have identified amino acid residues 37 – 63 in the amino terminus of CL (Salvatore et al., 2006) and amino acids 66 – 112 in the amino terminus of RAMP1 (Mallee et al., 2002) as critical for modulating the affinity of Compound 1. Furthermore, as observed with BIBN4096BS, the amino acid at position 74 of RAMP1 is responsible for modulating the affinity and species selectivity of Compound 1 (Mallee et al., 2002).

Another high throughput screen of the SmithKline Beecham compound collection using SK-N-MC membranes identified a class of benzanilides (SB-211973) with low micromolar activity (3 \(\mu\text{M}\)) (Aiyar et al., 2001). The lead structure SB-211973 was optimized and resulted in a racemic compound, thiazolylsulfoxide nitrobenzanilide. Enantiomeric separation by HPLC resulted in SB(+)-273779 and SB(-)273780 (Figure 4). SB(+)-273779 was at least 5-fold more potent in functional assays and selected for further evaluation. SB(+)-
273779 inhibited specific binding of $[^{125}\text{I}]-\text{CGRP}$ on membranes from SK-N-MC and HEK293 cells (pK$_i$ 6.5). However, SB(+)273779 only weakly inhibited specific binding of $[^{125}\text{I}]-\text{CGRP}$ on membranes from rat or porcine lung and also recombinant porcine CGRP receptor expressed in HEK293 cells, but inhibited CGRP-induced cAMP production in all systems studied. Prolonged treatment (>30 min) of SK-N-MC and HEK293 cells decreased maximal CGRP-induced cAMP production suggesting that this antagonist may have irreversiblle binding characteristics. In addition, SB(+)273779 antagonized CGRP-induced calcium release in HEK293 cells, vasodilation in rat pulmonary artery and decrease in blood pressure in anesthetized rats. Although, it was found that SB(+)273779 precipitated at concentrations required for in vivo studies and preliminary pharmacokinetic studies revealed poor bioavailability and short half-life. Thus, SB(+)273779 may only be suitable as a research tool for in vitro studies. This antagonist serves as the first “cross species” non-peptide antagonist.

Compound 3, an analogue of BIBN4096BS, was reported in 2005 by Merck Research Laboratories (Hershey et al., 2005) (Figure 4). Compound 3 inhibited specific binding of $[^{125}\text{I}]-\text{CGRP}$ on membranes from SK-N-MC cells and rhesus cerebellum (pK$_i$ 11.1 and 10.8) and 100-fold lower on membranes from rat brain (pK$_i$ 8.9). Receptor chimera studies have identified amino acid residues 37 – 63 of CL as critical for receptor recognition by Compound 3 (Salvatore et al., 2006). Furthermore, as observed with BIBN4096BS and Compound 1, the amino acid at position 74 of RAMP1 is responsible for modulating the affinity and species selectivity of Compound 3 (Hershey et al., 2005).
Bristol-Myers Squib used molecular modeling to design conformationally restricted analogues with the aim of obtaining high affinity CGRP receptor antagonists. Compound 1 was used a template in molecular modeling studies to design Compound 2a (Figure 4) (Zuev et al., 2005). In binding assays, Compound 2a weakly inhibited specific binding of $[^{125}I]$-CGRP on membranes from SK-N-MC cell (pK, 5.4).

In 2006, Merck Research Laboratories reported a novel antagonist, Compound 4, which was structurally unrelated to the other non-peptide CGRP receptor antagonists (Salvatore et al., 2006). Compound 4 (Figure 4) was determined to be non-selective for human compared to rat CGRP receptors (pK, 5.4, 5.2, respectively) as well as non-selective between CGRP and adrenomedullin receptors (pK, 5.4, 5.1, respectively). Receptor chimera studies determined that Compound 4 binds to the TM7 domain of CL. Thus, this antagonist is designed RAMP-independent because it displayed similar affinity for human and rat CGRP receptors as well as similar affinity for adrenomedullin receptors. The inhibition of CGRP and adrenomedullin binding to CGRP receptors (CL/RAMP1) and adrenomedullin receptors (CL/RAMP2) by Compound 4 suggests that CGRP and adrenomedullin may have contact points on CL or that CL is involved in modulation of the binding pocket for CGRP and adrenomedullin.
Figure 4. Structure of non-peptide CGRP receptor antagonists.
CHAPTER 2

SOLVENT EFFECTS ON COUPLING YIELDS DURING RAPID SOLID PHASE SYNTHESIS OF CGRP(8-37) EMPLOYING IN SITU NEUTRALIZATION

ABSTRACT

The success of solid phase peptide synthesis is often dependent upon solvation of the resin and the growing resin-bound peptide chain. I investigated the relationship between solvent properties and solvation of the resin and peptide-resin in order to obtain satisfactory coupling yields for the rapid solid phase peptide synthesis, using N-α-tert-butyloxycarbonyl (Boc)-amino acid derivatives, of human-α-calcitonin gene-related peptide(8-37) (CGRP(8-37)).

Solvation of (para-methylbenzhydrylamine)copoly(styrene-1 % divinylbenzene) resin (resin) and resin covalently bound to the fully protected amino acid sequence of CGRP(8-37) (peptide-resin) was correlated to solvent Hildebrand solubility (δ) and hydrogen-bonding (δh) parameters. Contour solvation plots of δh versus δ revealed maximum solvation regions of MBHA-resin and peptide-resin. The maximum resin solvation occurred with NMP, NMP:DMSO (8:2) and DMSO. Inefficient solvation of the peptide-resin occurred with these solvents and resulted in poor syntheses with average coupling yields of 78.1, 88.9 and 91.8 %, respectively. Superior peptide-resin solvation was obtained using DMA and DMF, resulting in significantly higher average coupling yields of 98.0 and 99.5 %, respectively. Thus, the region of maximum peptide-resin solvation shifts to
solvents with higher $\delta_n$ values. DMF provided the most effective peptide-resin solvation and was the only solvent from which CGRP(8-37) was obtained as a single major product in the crude cleaved material.
INTRODUCTION

The success of solid phase peptide synthesis (SPPS) is dependent upon efficient peptide chain assembly on the resin (Merrifield, 1963). Efficient peptide chain assembly relies upon accessibility of free amino termini on resin-bound peptide chains to incoming acylation species. The use of polar aprotic solvents is critical to resin solvation (Sarin et al., 1980) as well as to suppression of interchain (Narita et al., 1984) and intrachain (Merrifield et al., 1988) aggregation of the resin-bound peptide chains. Addition of salt in high concentrations to the solvent also reduces aggregation of resin-bound peptide chains and is useful with difficult couplings (Stewart et al., 1990). The extent of underivatized resin solvation alone is not sufficient to achieve acceptable coupling yields since, peptide-polystyrene resins are expected to have different physiochemical properties compared to the initial underivatized resin due to the accumulation of the polar peptide backbone, residue side-chains and protecting groups (Fields et al., 1991) as the synthesis progresses. Thus, consideration of effects of the peptide component upon solvation, particularly as peptide chains elongate and physiochemical properties of the peptide start to dominate, is prudent. Decreased peptide-resin solvation leads to incomplete couplings, which results in the production of deletion peptides. This reduces the overall yield and separation of the desired product from the deletion peptides becomes increasingly difficult.

Rapid SPPS by Kent is a highly efficient method that allows for the synthesis of large peptides within twenty four hours (Schnolzer et al., 1992). This is achieved using 100% TFA for deprotection of the N-α-Boc group, washing the
resin by flowing the solvent continuously through the resin bed, neutralization of the resin during the coupling reaction \( \textit{in situ} \), and high concentrations of activated Boc-amino acid derivatives for single couplings to the resin. During the application of rapid SPPS to the synthesis of the 30-amino acid residue N-truncated fragment of human-\( \alpha \)-calcitonin gene-related peptide, CGRP(8-37), I observed that coupling yields are highly dependent on the solvent used. I investigated solvent effects during the synthesis of CGRP(8-37), which revealed a correlation between solvation of the resin-bound side-chain protected peptide, solvent properties and coupling yields.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents.**

Boc-amino acid derivatives were purchased from Bachem Inc. (Torrance, CA) and Chem-Impex International (Wood Dale, IL), O-(Benzotriazol-1-yl)-\( N,N,N',N' \)-tetramethyluronium hexafluorophosphate (HBTU) was obtained from Chem-Impex International (Wood Dale, IL), trifluoroacetic acid (TFA), diisopropylethylamine (DIEA), diethyl ether, N-methylpyrrololidone (NMP), dimethylformamide (DMF), dimethylacetamide (DMA), dimethylsulfoxide (DMSO), acetonitrile (ACN), and dichloromethane (DCM) were purchased from Fisher Scientific (Pittsburgh, PA). A quantitative ninhydrin test kit was obtained from Anaspec (San Jose, CA), trifluoromethanesulfonic acid (TFMSA) was obtained from Fluka Chemical Co. (Milwaukee, WI), \((\text{para-methylbenzhydrylamine})\text{copoly(styrene-1 % divinylbenzene)}\) resin (substitution
level 1.16 mmol/g) was obtained from Bachem Inc., 1,2-ethanedithiol (EDT), and thioanisole were obtained from Sigma-Aldrich (St. Louis, MO), and a fritted-glass buret was custom-made by Ace Glass Inc. (Vineland, NJ).

**Peptide Synthesis.**

CGRP(8-37) was synthesized manually using solid phase peptide synthesis employing *in situ* neutralization. Reactive side-chains were protected as follows: Asn, xanthine; Thr, Ser, benzyl ether; His, benzyloxymethyl; Arg, mesitylene-2-sulfonyl; Lys, 2-chlorocarbobenzoxy. The solvents used for syntheses were NMP, NMP:DMSO (8:2), DMSO, DMA and DMF. Syntheses were carried out in a 15 mL glass reaction vessel at a 0.1 mmol scale. MBHA resin (88.0 mg, 1.16 mmol/g) was placed in the reaction vessel and subjected to the following protocol (**Figure 5**). 1) Boc-amino acid derivative (0.4 mmol) was dissolved in a 0.38 M solution of HBTU in DMF (1.0 mL, 0.38 mmol), DIEA (0.105 mL, 0.6 mmol) was added and the solution was mixed vigorously on a Fisher Scientific “Touch Mixer Model 231” (Pittsburgh, PA) vortexer 3 times over 2 min. 2) The resin was washed with solvent (45 sec continuous flow wash) and drained to the level of the resin bed. 3) The activated amino acid derivative solution was transferred to the reaction vessel and rocked on a wrist-arm shaker for 10 min. 4) The resin was washed with solvent (45 sec continuous flow wash), drained completely and a resin sample removed (5 - 10 mg). 5) The Boc protecting group was removed with 100 % TFA (5 mL, 2 x 1 min) with rocking and the resin drained. This protocol was repeated until the sequence of CGRP(8-37) was assembled on the resin in the C-terminal to N-terminal direction (F³⁷ to V⁸). The
final peptide-resin was washed with DCM (45 sec continuous flow wash), MeOH (45 sec continuous flow wash) and dried in vaccuo. Resin samples (5-10 mg) removed after each coupling were used to determine coupling yields by the quantitative ninhydrin test (Sarin et al., 1981).

**Peptide Cleavage.**

The peptide was cleaved from the resin and freed of its side-chain protecting groups using TFMSA. Peptide-resin (100 mg) was suspended in thioanisole:ethanedithiol (150 μL, 2:1, v/v) for 10 min. TFA (1 mL) was added with stirring and the flask was cooled to 0° C for 10 min. TFMSA (100 μL) was added dropwise while stirring and the reaction was left to stir for 2 h at room temperature. The peptide was precipitated with cold diethyl ether and together with the resin, filtered using a medium porosity sintered-glass funnel. The peptide was dissolved in a minimum of TFA (3 x 1 mL), the TFA solution passed through the sintered-glass funnel under suction and dripped directly into the cold diethyl ether (100 mL) present in the Buchner funnel to precipitate the crude product. This was repeated two more times with fresh aliquots of cold diethyl ether. The precipitate was removed by filtration through a fine porosity sintered-glass funnel, dissolved in 5 % acetic acid and lyophilized to yield the crude product as a fluffy white powder.

**Analytical RP-HPLC.**

A sample (10 μL, 1 mg/mL) of the crude cleavage solution was subjected to analytical RP-HPLC on a Vydac 218TP5415 C18 column (0.46 cm x 15 cm). Buffers for RP-HPLC were 0.1 % TFA in water (buffer A) and 0.05 % TFA in
acetonitrile (buffer B). The crude product was eluted with a gradient from 5 – 60 % buffer B in buffer A over 30 min at a flow rate of 1 mL/min and detected at 214 nm.

**Resin and Peptide-Resin Solvation.**

Solvation studies were carried out with the use of a custom-made 15 mL glass-fritted buret from Ace Glass Inc. (Vineland, NJ) with 0.1 mL graduations. Resin or peptide-resin (250 mg) was washed and mixed five times with solvent (15 mL) and allowed to swell in the same solvent for 30 min prior to determining solvation volume.

**RESULTS**

**Coupling Yields for the Synthesis of CGRP(8-37) using Different Solvents.**

To my astonishment, low coupling yields were obtained for the synthesis of CGRP(8-37) using N-methylpyrrololidone (NMP) as the solvent. The lowest yield observed was 41.9 % for the coupling of S^{17} to R^{18} (Figure 6), determined by the quantitative ninhydrin method (Sarin et al., 1981). The average coupling yield for the entire synthesis was 78.1 % (Table 2). No desired product was observed by analytical reversed-phase high performance liquid chromatography (RP-HPLC) (Figure 13) in the crude material after treatment of the resin with a mixture of trifluoromethanesulfonic acid and trifluoroacetic acid (TFMSA:TFA, 1:9) in the presence of thiol scavengers (Tam et al., 1986). Consequently, I investigated the cause of the low coupling yields. In two experiments, the activation time for the reaction of Boc-amino acid derivatives with O-(Benzotriazol-1-yl)-N,N,N',N'-

*Numbers refer to positions in the full length peptide CGRP.*
tetramethyluronium hexafluorophosphate (HBTU) was increased from 2 minutes to 5 minutes with a 10 minute coupling time and activation of Boc-amino acid derivatives was held at 2 minutes while the coupling time was increased from 10 minutes to 20 minutes. This reduced the average coupling yield, for the entire synthesis, to 61.9 % and 71.7 % (Table 2), respectively. The lowest yield observed was 24.0 % for coupling V8 to T9 (Figure 7) and 36.2 % for coupling to V8 to T9 (Figure 8), respectively. These data suggest that incomplete Boc-amino acid activation and inadequate coupling time are not the basis for the poor coupling yields.

The use of DMSO has been shown to increase the coupling yields of difficult sequences (Geiser et al., 1988; Forest et al., 1990; Hyde et al., 1992). To examine the ability of DMSO to increase coupling yields for the rapid SPPS of CGRP(8-37) I used a mixture of NMP and DMSO (8:2) and DMSO alone as the synthesis solvents. Indeed, with the addition of 20 % DMSO to NMP, the average coupling yield increased significantly to 88.9 % (Table 2) and the lowest yield observed increased to 61.9 % for coupling L16 to S17 (Figure 9). When DMSO was used as the sole solvent, the lowest yield increased to 66.8 % for coupling L16 to S17 (Figure 10) and the average yield improved to 91.8 % (Table 2). In spite of significant improvements in coupling yields obtained by changing addition of DMSO as a co-solvent or single solvent, no desired product was observed by analytical RP-HPLC in the crude cleaved product.

Use of dimethylacetamide (DMA) as the solvent resulted in coupling yields >98.2 % for coupling of the first twenty two amino acid derivatives. Low coupling
yields were obtained for the N-terminal eight amino acid derivatives with the lowest yield of 83.1 % for coupling L\textsuperscript{15} to L\textsuperscript{16} (Figure 11). The average coupling yield improved substantially to 98.0 % (Table 2) and CGRP(8-37) was present in the crude cleaved material, although significant amounts of impurities were also present (Figure 14). Use of dimethylformamide (DMF) as the solvent resulted in excellent coupling yields, >98.3 % through the entire synthesis with an average coupling yield of 99.5 % (Table 2) and the lowest yield of 98.3 % for coupling H\textsuperscript{10} to R\textsuperscript{11} (Figure 13). Synthesis using DMF resulted in a single major product present in the crude cleaved material (Figure 15).

Correlation of Resin-Peptide Solvation to Solvent Hildebrand Solubility Parameter (\(\delta\)) and hydrogen-bonding parameters (\(\delta_h\)). Clearly, low coupling yields were related to the choice of solvent used in synthesis. Therefore, I measured solvation of both native MBHA-resin (resin) and MBHA-resin covalently bound to the fully protected amino acid sequence of CGRP(8-37) (peptide-resin) in solvents with varying hydrogen-bonding (\(\delta_h\)) and Hildebrand solubility (\(\delta\)) parameters and constructed contour solvation plots of \(\delta_h\) versus \(\delta\) to determine if there was a correlation between solvation of resin or peptide-resin and \(\delta_h\) or \(\delta\). Maximum resin solvation occurred in NMP, NMP:DMSO (8:2), DMSO and DMA while solvation by DMF was significantly lower (Table 3). Conversely, maximum peptide-resin solvation occurred in DMA and DMF while solvation by NMP, NMP:DMSO (8:2) and DMSO was significantly lower. The contour solvation plot of \(\delta_h\) versus \(\delta\) shows a region of maximum resin solvation occurring with NMP, NMP:DMSO (8:2), DMSO and DMA, solvents having \(\delta = 10.8 - 12.9\)
and $\delta_n = 3.5 - 5.0$ (Figure 16, A). The contour solvation plot for the peptide-resin shows a region of maximum solvation occurring with solvents having $\delta = 10.8 - 12.1$ and $\delta_n \geq 5.0$ (Figure 16, B). The addition of the peptide chain shifts the region of maximum solvation to solvents with higher $\delta_n$ values compared to resin alone. The shift in peptide-resin solvation may be explained by the increased ability of DMA and DMF to solvate the resin-bound peptide. Presumably, increased solvation makes the free amino termini more accessible resulting in the significantly higher coupling yields observed when DMA or DMF is used as the solvent.
1) BocPhe-OH (0.4mmol)
HCTU (0.38mmol)
DIEA (0.6mmol)
2) DMF Flow wash

3) TFA
l^eprotectioj
Repeat protocol
for 29 couplings

1) Boc-aa-OH (0.4mmol)
HCTU (0.38mmol)
DIEA (0.6mmol)
2) DMF Flow wash

Figure 5. Rapid SPPS protocol employing in situ neutralization for the synthesis of CGRP(8-37).
Figure 6. Coupling yields for the rapid synthesis of CGRP(8-37) using NMP as the solvent.
Figure 7. Coupling yields for the rapid synthesis of CGRP(8-37) using NMP as the solvent with increased activation time.
Figure 8. Coupling yields for the rapid synthesis of CGRP(8-37) using NMP as the solvent with increased coupling time.
Figure 9. Coupling yields for the rapid synthesis of CGRP(8-37) using NMP:DMSO (8:2) as the solvent.
**Figure 10.** Coupling yields for the rapid synthesis of CGRP(8-37) using DMSO as the solvent.
Figure 11. Coupling yields for the rapid synthesis of CGRP(8-37) using DMA as the solvent.
Figure 12. Coupling yields for the rapid synthesis of CGRP(8-37) using DMF as the solvent.
Table 2. Quantitative Ninhydrin Results for the Rapid Synthesis of CGRP(8-37)-resins.\(^a\)

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| Avg. Yield (%) | 78.1 | 61.9 | 71.7 | 88.9 | 91.8 | 98.0 | 99.5 |

\(^a\)Lowest yield for coupling X to X in each synthesis are specified by shaded boxes.
Figure 13. Reversed-phase high performance liquid chromatography of crude cleaved product using NMP as the solvent. Retention time of CGRP(8-37) is 19.1 min. Conditions: Vydac 218TP5415 C\textsubscript{18} column (0.46 cm x 15 cm); buffer A, 0.1 \% TFA in H\textsubscript{2}O, buffer B, 0.05 \% TFA in ACN; 5 – 60 \% buffer B over 30 min; flow rate 1 mL/min; detected at 214 nm.
Figure 14. Reversed-phase high performance liquid chromatography of crude cleaved product using DMA as the solvent. Retention time of CGRP(8-37) is 19.1 min. Conditions: Vydac 218TP5415 C_{18} column (0.46 cm x 15 cm); buffer A, 0.1 % TFA in H_{2}O, buffer B, 0.05 % TFA in ACN; 5 – 60 % buffer B over 30 min; flow rate 1 mL/min; detected at 214 nm.
Figure 15. Reversed-phase high performance liquid chromatography of crude cleaved product using DMF as the solvent. Retention time of CGRP(8-37) is 19.1 min. Conditions: Vydac 218TP5415 C18 column (0.46 cm x 15 cm); buffer A, 0.1 % TFA in H2O, buffer B, 0.05 % TFA in ACN; 5 – 60 % buffer B over 30 min; flow rate 1 mL/min; detected at 214 nm.
Table 3. Properties of solvents and solvation of resin and peptide-resin.

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<th>Solvation(^c)</th>
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<td></td>
<td>(\delta)</td>
<td>(\delta_h)</td>
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<td>8</td>
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\(^a\) Solvent \(\delta\) and \(\delta_h\) values were obtained from Barton AFM (1975) Solubility Parameters. *Chemical Reviews* **75**: 731-753.

\(^b\) \(\delta\) and \(\delta_h\) are in \([(\text{cal/cm}^3)^{1/2}]\).

\(^c\) Resin and peptide-resin solvation are in (mL/g resin).
Figure 16. Contour solvation plots of A) resin and B) peptide-resin as a function of Hildebrand solubility ($\delta$) and hydrogen-bonding ($\delta_h$) parameters. Each numbered point represents data from the different solvents identified in Table 3.
DISCUSSION

Previous solvation studies showed the polystyrene resin and the protected peptide exert complementary solubilizing effects on each other. These effects mutually determine swelling behavior of the peptide-resin, which is maximized in polar aprotic solvents. More recently, NMP was found to be a superior solvent for solvation of peptide-resins containing tert-butyl side-chain protecting groups (Fields and Fields 1991). This suggests that NMP is the appropriate solvent for SPPS using Fmoc/tert-butyl protecting group strategy. NMP, DMSO, DMA and DMF were all superior solvents for solvation of peptide-resins containing benzyl side-chain protecting groups suggesting that these solvents would be appropriate for SPPS using the Boc/benzyl protecting group strategy (Fields et al., 1991). The peptide-resin used in these studies was comprised of the fully-protected, tridecapeptide chain of [Lys^9]-α-conotoxin G1 and the peptide content of benzyl-side-chain protected peptide-resin was 1.22 g of peptide/g of polystyrene resin based on the reported resin substitution. I evaluated the use of these solvents for the rapid SPPS of CGRP(8-37) using Boc-amino acid derivatives with benzyl-type side-chain protection when necessary. Excellent coupling yields of >98.5 % were observed for the addition of the first eight amino acid derivatives in all solvents, resulting in a peptide-resin with 1.55 g of peptide/g of polystyrene resin. Clearly, these data are in good agreement with the [Lys^9]-α-conotoxin G1 synthesis.

However, as the peptide chain grew longer, coupling yields plummeted when NMP and DMSO were the solvent, with coupling yields as low as 41 %
observed during the addition of the last twenty amino acid derivatives. Solvation studies also revealed that swelling of the peptide-resin containing the fully protected 30-amino acid sequence of CGRP(8-37) was significantly reduced in NMP and DMSO when compared to DMA and DMF. This “CGRP(8-37)”-peptide-resin has a substantially increased peptide content of 5.71 g of peptide/g of polystyrene resin when compared to the previously reported “conotoxin”-peptide-resin. Since NMP and DMSO are superior solvents to DMF for swelling the native MBHA-resin (Table 2) the poor swelling of the “CGRP(8-37)”-peptide-resin can be attributed to (i) poor solvation of the much larger protected peptide chain and (ii) a smaller solubilizing effect derived from the resin due to its substantially reduced content. These data suggest that NMP and DMSO are only suitable solvents for SPPS when the peptide content of the peptide-resin does not exceed 55-60 % by weight, whereas peptide-resins containing >85 % peptide content can be assembled using DMF.

In summary, DMF was the only solvent suitable for the assembly of the fully protected peptide chain of CGRP(8-37) on the solid phase using previously published, rapid solid phase methods employing the Boc-protection strategy and in situ neutralization. These data suggest that DMF is a superior solvent for solvation of peptide-resins with high peptide content making it the solvent of choice for the synthesis of large peptides (15 to 50 amino acid residues in length).
CHAPTER 3

PHARMACOLOGICAL CHARACTERIZATION OF NOVEL HIGH AFFINITY CGRP RECEPTOR ANTAGONISTS THAT ARE SELECTIVE FOR HUMAN CGRP RECEPTORS

ABSTRACT

Human α-calcitonin gene-related peptide (CGRP) is a 37-residue neuropeptide that produces a variety of cardiovascular and other effects via activation of specific CGRP receptors that produce 3',5' cyclic adenosine monophosphate (cAMP). Functional CGRP receptors are a heterodimeric complex comprised of the heptahelical calcitonin receptor-like receptor and the single transmembrane receptor activity-modifying protein 1. Based on the known structures of the antagonist CGRP(8-37) and the human CGRP receptor, I designed novel CGRP receptor peptide antagonists with modifications to promote high affinity and selectivity for human CGRP receptors. Antagonist affinity (K_B) at CGRP receptors was determined using the mouse thoracic aorta and human SK-N-MC cells. In aorta, CGRP(8-37), bzl-CGRP(8-37) and bzl-bn-CGRP(8-37) caused rightward shifts in the concentration-response relaxation curve for CGRP with \( K_B \) values of 1000, 88 and 50 nM, respectively. In human SK-N-MC cells CGRP(8-37), bzl-CGRP(8-37) and bzl-bn-CGRP(8-37) caused rightward shifts in the concentration-response curve for CGRP stimulated cAMP production with \( K_B \) values of 797, 15 and 0.63 nM, respectively. Thus, CGRP(8-
37) had the same affinity for human and mouse CGRP receptors while bzl-
CGRP(8-37) and bzl-bn-CGRP(8-37) displayed 6-fold and 80-fold higher affinity,
respectively, for human CGRP receptors. In addition, the selectivity of the
antagonists for human CGRP receptors correlated highly with the antagonist
hydrophobicity index. These relatively high affinity, species selective peptide
antagonists provide novel tools to differentiate structural and functional features
that are unique to the human CGRP receptor. Thus, these analogues may be
useful compounds for the development of drugs to treat migraine headache and
other cardiovascular diseases.
INTRODUCTION

Calcitonin gene-related peptide (CGRP) belongs to a family of structurally and biologically related peptides that include calcitonin, amylin, adrenomedullin and intermedin all of which share 20-43 % sequence similarity. CGRP is an endogenous 37-residue neuropeptide that exists in two forms, denoted α and β. These peptides are produced from separate genes; α-CGRP being produced from tissue-specific alternative splicing of the primary RNA transcript of the calcitonin gene while β-CGRP is produced by a separate gene (Amara et al., 1982; Steenbergh et al., 1985). α-CGRP is the more abundant of the two forms. α-CGRP is widely distributed throughout the central and peripheral nervous systems and is involved in wound healing (Engin, 1998), inflammatory responses (Smith et al., 1993), nociception (Yu et al., 1998), appetite suppression (Tannenbaum and Goltzman, 1985), gastric emptying (Raybould, 1992), regulation of vascular tone (Gangula et al., 2000) and regional organ blood flow (Brain et al., 1985) in both humans and mouse models. In humans, excessive CGRP-mediated cerebrovascular dilation plays an important role in the pathophysiology of headache. Currently BIBN4096BS, a non-peptide CGRP receptor antagonist, is in clinical trials for the treatment of migraine headache (Doods et al., 2000).

CGRP produces its effects by activation of specific G protein-coupled receptors located at the cell surface. Functional CGRP receptors are a 1:1 heterodimeric protein complex comprised of the heptahelical calcitonin receptor-like receptor (CL) and an accessory protein termed receptor activity-modifying
protein 1 (RAMP1) (McLatchie et al., 1998; Poyner et al., 2002). RAMP1 promotes cell surface expression of the CL-RAMP1 complex (Foord and Marshall, 1999) and co-expression of CL with RAMP1 is necessary to produce a functional CGRP receptor. CGRP receptors in most tissues and cell lines are coupled to the Gs family of heterotrimeric G proteins and to an increase in 3',5' cyclic adenosine monophosphate (cAMP) (Aiyar et al., 1999). In some systems, CGRP receptor activation has also been reported to cause intracellular calcium mobilization (Aiyar et al., 1999), potassium channel activation (Wellman et al., 1998), activation of extracellular signal-regulated kinase, P38 mitogen-activated protein kinase and c-Jun N-terminal kinase (Disa et al., 2000; Parameswaran et al., 2000). These signaling pathways have been linked to various functions including CGRP-mediated vascular relaxation and positive inotropic and chronotropic effects in the heart.

The exact mechanism of how the CGRP receptor, composed of CL and RAMP1, binds CGRP and/or CGRP antagonists is unknown. It has been proposed that the extracellular domain of RAMP1 may participate directly in ligand binding or act indirectly to modulate the ligand binding conformation of CL (Hilairet et al., 2001). The affinity of small molecule antagonists for CGRP receptors has been shown to be modulated by amino acid residues residing in the extracellular domain of RAMP1 which provides evidence that RAMP1 directly participates in antagonist binding (Mallee et al., 2002). Based on the amino acid composition and analysis of hydropathy plots of CL and RAMP1, I hypothesized that RAMP1 forms a hydrophobic binding pocket with CL. The mouse CL shares
90% amino acid residues with human CL (Miyauchi et al., 2002) (Figure 17) and hydropathy plots of human and mouse CL are nearly identical (Figure 19). In contrast, mouse RAMP1 shares only 71% amino acid residues with human RAMP1 (Husmann et al., 2000) (Figure 18) and most of the amino acid sequence dissimilarity resides in the extracellular domain of RAMP1. Furthermore, hydropathy plots of human and mouse RAMP1 are markedly different particularly in the N-terminus of the extracellular domain (Figure 19) and indicate that human RAMP1 is more hydrophobic. Based on amino acid hydrophobicity differences between human and mouse RAMP1, I designed and synthesized a novel competitive antagonist, N-α-benzoyl-[His(4-benzyl)\(^{10}\)]-hα-CGRP(8-37) (bzl-bn-CGRP(8-37)). This antagonist was designed to favor interaction with human RAMP1 by hydrophobic modifications I predicted would confer species selectivity due to the more hydrophobic amino acid composition of human RAMP1.

Knockout mice and other mouse assays are gaining increasing importance as model systems to understand human cardiovascular functions. Therefore, I characterized the standard CGRP receptor antagonist CGRP(8-37), our previously reported high affinity competitive antagonist N-α-benzoyl-hα-CGRP(8-37) (bzl-CGRP(8-37)) and the new antagonist, bzl-bn-CGRP(8-37) at mouse CGRP receptors, using the mouse thoracic aorta, and at human CGRP receptors, using the human SK-N-MC cell line. Comparison of antagonist affinities showed that bzl-bn-CGRP(8-37) is a relatively high affinity competitive antagonist that is selective for human compared to mouse CGRP receptors.
Correlations between antagonist affinities and the hydrophobicity index (k' value) of these antagonists suggest that hydrophobicity of the antagonist is a key factor in human CGRP receptor selectivity. This study may facilitate the design of high affinity human selective CGRP receptor antagonists for treatment of cardiovascular and other diseases.

EXPERIMENTAL PROCEDURES

Drugs and Chemicals.

CGRP(8-37), bzl-CGRP(8-37) and bzl-bn-CGRP(8-37) were synthesized as described below. CGRP, N-α-tert-butyloxycarbonyl (Boc) amino acids and para-methylbenzhydrylamine (MBHA) resin (substitution level 1.16 mmol/g) were purchased from Bachem Inc. (Torrance, CA). Norepinephrine bitartrate, isoproterenol bitartrate, forskolin and Sigmacote were purchased from Sigma-Aldrich (St. Louis, MO). Isobutylmethylxanthine was purchased from EMD Biosciences Inc. (San Diego, CA). O-(6-Chlorobenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HCTU) was obtained from Chem-Impex International (Wood Dale, IL). Trifluoroacetic acid (TFA), other solvents and chemicals were purchased from Fisher Scientific (Pittsburgh, PA). Dubelco’s modified Eagle’s medium, fetal bovine serum, and antibiotic/antimycotic (containing 10,000 units/mL penicillin G, 10,000 μg/mL streptomycin sulfate and 25 μg/mL amphotericin B) were purchased from Invitrogen (Carlsbad, CA).
Hydropathy analysis of human and mouse CL and RAMP1 proteins.

Hydropathy analysis of human and mouse CL and RAMP1 proteins was carried out according to the Kyte-Doolittle method (window length 19) by use of the Protein Identification and Analysis Tool, ProtScale, on the Expert Protein Analysis System (ExPASy) server (Gasteiger et al., 2003; Gasteiger et al., 2005). The calculated hydropathy score was used to determine the hydrophobicity of the RAMP1 proteins.

Solid Phase Peptide Synthesis, Purification and Characterization.

Peptides were synthesized by Merrifield’s solid-phase methods (Merrifield, 1965) employing in situ neutralization (Schnolzer et al., 1992; Taylor et al., 2005) using Boc amino acids and MBHA resin. Reactive side-chains of Boc-amino acid derivatives were protected as follows: Asp, benzyl ester; Thr, Ser, benzyl ether; His, benzyloxymethyl (BOM); Arg, mesitylene-2-sulfonyl (Mts); Lys, 2-chlorocarbobenzoxy (2CI-CBZ). Synthesis was carried out manually in a 30 mL glass reaction vessel on a 0.5 mmol scale. MBHA-resin (435 mg, 1.16 mmol/g) was placed in the reaction vessel and subjected to the following protocol: 1) Boc-Phe (0.53 g, 2 mmol) was dissolved in a 0.38M solution of HCTU in DMF (5 mL, 1.90 mmol), DIEA (0.523 mL, 3.0 mmol) was added and the solution was mixed vigorously on a Fisher Scientific “Touch Mixer Model 231” vortexer 3 times over 2 min. 2) The resin was washed with DMF (45 sec continuous flow wash) and drained to the level of the resin bed. 3) The activated-Boc-Phe solution was transferred to the reaction vessel and rocked on a wrist-arm shaker for 10 minutes. 4) The resin was washed with DMF (45 sec continuous flow wash),
drained completely and a resin sample (5 – 10 mg) removed. 5) The Boc-
protecting group was removed with 100 % TFA (10 mL, 2 x 1 min) with rocking
and the resin drained completely to yield protonated Phe-MBHA-resin. This
procedure was repeated for coupling of the next 29 residues. The activation of
the next residue was started during the first TFA deprotection to alleviate any
delay in the activation and coupling. Once the sequence of CGRP(8-37) was
assembled on the resin, the peptide-resin was washed with DMF (30 sec
continuous flow wash), DCM (30 sec continuous flow wash), MeOH (30 sec
continuous flow wash) and dried in vacuo for 2 h. Resin samples removed after
each coupling were used to determine coupling yields by the quantitative
ninhydrin test (Sarin et al., 1981). Single couplings were performed for addition of
all amino acid derivatives. Peptides were purified to >98 % by semi-preparative
reversed-phase high performance liquid chromatography (RP-HPLC) on a Vydac
218TP510 C_{18} column (1 cm x 25 cm) from the Separations Group (Hesperia,
CA). Buffers for chromatography were either 100 mM triethylammoniumphosphate, pH 2.5 (buffer A) and a mixture of acetonitrile and
buffer A (60/40, v/v) (buffer B) or 0.1 % TFA in water (buffer C) and 0.05 % TFA
in acetonitrile (buffer D). Analytical RP-HPLC was performed on a Vydac
218TP5415 C_{18} column (0.46 cm x 15 cm). All peptides were structurally
characterized by amino acid analysis and electrospray-ionization mass
spectrometry (ESI-MS). Amino acid analyses were performed using an AccQTag
system from Waters (Milford, MA) after samples were hydrolyzed using constant
boiling 6 M HCl at 110 °C for 24 h. ESI-MS was performed on an API150EX instrument from PE-SCIEX (Foster City, CA).

**Synthesis of h-α-CGRP(8-37).**

The amino acid sequence of CGRP(8-37) was assembled on MBHA-resin using Boc-amino acid derivatives as described above. Synthesis was carried out manually in a 30 mL glass reaction vessel on a 0.5 mmol scale. The peptide was cleaved from the resin using TFMSA and the crude product was purified using RP-HPLC.

**Synthesis of N-α-benzoyl-h-α-CGRP(8-37).**

The amino acid sequence of CGRP(8-37) was assembled on MBHA-resin using Boc-amino acid derivatives as described above. Synthesis was carried out manually in a 30 mL glass reaction vessel on a 0.5 mmol scale. The N-terminal was benzoylated as previously described (Smith et al., 2003). The peptide was cleaved from the resin using TFMSA and the crude product was purified using RP-HPLC.

**Synthesis of N-α-benzoyl(His\textsuperscript{10}-benzyl)-h-α-CGRP(8-37).**

The amino acid sequence of CGRP(8-37) was assembled on MBHA-resin using Boc-amino acid derivatives as described above. A portion of the fully protected peptide-resin (100 mg, 0.02 mmol) was benzylated by addition of benzyl bromide (24 μL, 0.2 mmol) and diisopropylethylamine (35 μL, 0.2 mmol) in dimethylformamide (300 μL) and rocked on a wrist-arm shaker for 24 h. The N-α-Boc-protecting group was removed with TFA (2 x 1 min), flow-washed with dimethylformamide and the peptide-resin benzoylated by addition of benzoic
anhydride (45 mg, 0.2 mmol) and diisopropylethylamine (35 μL, 0.2 mmol) in 750 μL dimethylformamide. After further washing with DMF, the peptide was cleaved from the resin using TFMSA and the crude product was purified using RP-HPLC.

**Peptide-Resin Cleavage.**

The peptides were cleaved from the resin and freed of side-chain protecting groups using TFMSA. For CGRP(8-37) and bzl-CGRP(8-37) the cleavage was split into two cleavage reactions (718 mg and 552 mg each cleavage, respectively). For bzl-bn-CGRP(8-37) 100 mg of peptide-resin was cleaved. Peptide-resin was suspended in thioanisole:EDT (1.073 mL, 828 μL and 150 μL; 2:1, v/v) for 10 min. TFA (7.15 mL, 5.52 mL and 1 mL) was added with stirring and the reaction mixture was cooled to 0 °C with stirring for 10 min. TFMSA (715 μL, 552 μL and 100 μL) was added dropwise and the reaction mixture was left to stir at room temperature for 2 h. The peptide was precipitated with cold diethyl ether and together with the resin, filtered through a medium porosity sintered-glass funnel. To separate the peptide from the resin the peptide was dissolved in a minimum of TFA (3 x 1 mL), the TFA solution passed through the sintered-glass funnel under suction and dripped directly into the cold diethyl ether (100 mL) present in the Büchner funnel to precipitate the crude product. This was repeated two more times with fresh batches of cold diethyl ether in the flask. The precipitate was removed from the ether by filtration through a fine porosity sintered-glass funnel, dissolved in 5 % aqueous acetic acid and subjected to RP-HPLC.
Hydrophobicity Index.

Quantitation of the hydrophobicity of each peptide was determined by the retention time of the peptide on a RP-HPLC column. The hydrophobicity index, k' value, was measured using three different RP-HPLC columns: column 1 was a Vydac C\textsubscript{18} monomeric 238TP54 column (250 x 4.6 mm), column 2 was a Waters Symmetry C\textsubscript{18} 300 Å column (250 x 4.6 mm) and column 3 was a Kromasil C\textsubscript{8} column (250 x 4.6 mm). Peptides were isocratically eluted with 0.1 % TFA in water/acetonitrile 69/31 (v/v) at a flow rate of 1 mL/min and detected at 214 nm. The k' value was calculated for each peptide by the formula:

$$k' = \frac{t_R - t_0}{t_0}$$

where $t_R$ is the retention time of the peptide and $t_0$ is the retention time of unretained material.

Measurement of Thoracic Aorta Relaxation.

Male albino mice (CF1, 25-35g) were obtained from Charles River Laboratories (Wilmington, MA). Mice were euthanized using CO\textsubscript{2} and thoracic aortas were removed and placed in Krebs' solution (composition in mM: NaCl, 118; KCl, 4.7; CaCl\textsubscript{2}, 2.5; KH\textsubscript{2}PO\textsubscript{4}, 1.2; MgSO\textsubscript{4}, 1.2; NaHCO\textsubscript{3}, 25; dextrose, 11.1; Na\textsubscript{2}Ca EDTA, 0.029; pH 7.4). The aorta was cleaned of adhering connective tissue, endothelium was removed by gentle rubbing of the vessel lumen and the aorta cut into 3-mm long ring segments. Ring segments were mounted between two stainless steel pins passed through the vessel lumen and placed in water-jacketed organ baths maintained at 37° C which contained Krebs' solution gassed with 95% O\textsubscript{2}/5% CO\textsubscript{2}, pH 7.4. The glass organ baths were coated with
Sigmacote to reduce the binding of peptides to the glass surfaces. One pin was attached to a Grass FT.03 isometric force transducer (Grass Instruments, Quincy, MA) for measurement of isometric tension while the other pin was held in a fixed position. Ring segments were equilibrated in Krebs' solution for 30 min at a resting tension of 300 mg, contracted with 60 mM KCl followed by KCl washout and a 30 min equilibration period. Ring segments were contracted a second time with 60 mM KCl and absence of endothelium was assessed by lack of relaxation caused by 1 μM of the endothelium-dependent vasodilator acetylcholine. The ring segments were then thoroughly washed for 30 min with Krebs' solution.

CGRP analogues were tested for both agonist and antagonist activity. For agonist studies analogues were tested for their ability to change resting tone and to alter the tone of aortic ring segments precontracted to a stable level of tone with 1 μM norepinephrine. For antagonist studies, aortic ring segments were contracted with 1 μM norepinephrine for 20 min to obtain a stable amount of contractile tone and cumulative CGRP concentration-response relaxation curves were obtained. Ring segments were then incubated with CGRP(8-37), bzl-CGRP(8-37) or bzl-bn-CGRP(8-37) for 60 minutes, contracted with 1 μM norepinephrine and cumulative CGRP concentration-response relaxation curves were repeated. Thus, two relaxant concentration-response curves to CGRP were generated in each aortic ring, a CGRP control and CGRP in the presence of antagonist. The spontaneous decline in tension of aortic rings contracted with norepinephrine averaged 12 ± 3 % during the time course of CGRP relaxation experiments. To test for non-specific effects and receptor specificity of the
agonism, the same analogue treatment protocol described above was used except that isoproterenol, rather than CGRP, was employed as the relaxant agonist.

**Measurement of cAMP Production.**

SK-N-MC cells were seeded and grown to confluence in CellStar 24-well plates (Greiner Bio-One Inc., Longwood, FL) in Dubelco’s Modified Eagles Medium (DMEM) supplemented with fetal bovine serum (10 %), penicillin G (100 units/mL), streptomycin (100 µg/mL) and amphotericin B (0.25 µg/mL). DMEM culture media was removed and the cells washed 3 times with 200 µL of HEPES-Krebs’ buffer (composition in mM: NaCl, 118; KCl, 4.7; CaCl₂, 2.5; KH₂PO₄, 1.3; MgSO₄, 1.2; NaHCO₃, 15; Dextrose, 11.1; HEPES acid, 12.4; HEPES-Na, 7.5; isobutylmethylxanthine, 0.5; pH 7.4). HEPES-Krebs’ buffer (900 µL) was added to each well and the plates incubated for 10 min in a humidified atmosphere at 37° C. CGRP, 0.3 µM forskolin or 3 nM isoproterenol was added to various wells and the plates incubated for 30 min in a humidified atmosphere (95 % air/5 % CO₂) at 37° C. The cells were then lysed by addition of 150 µL of 95 % ethanol and the cell lysate was dried in an oven at 40° C. cAMP production was measured by radioassay according to the manufacturer’s protocol (Diagnostics Products Corporation, Los Angeles, CA). Radioactivity was measured using a Beckman LS 6000IS Scintillation counter.

To test for agonist activity, the CGRP analogues alone were added to wells for 15 min. To generate CGRP concentration-response curves, different concentrations of CGRP were added to various wells. To test for antagonist
activity other wells were incubated with a single concentration of CGRP(8-37), bzl-CGRP(8-37) or bzl-bn-CGRP(8-37) for 15 min, followed by addition of various concentrations of CGRP. After drug treatments the plates were incubated for 30 min in a humidified atmosphere at 37° C and cAMP production measured. To test for non-specific effects and receptor specificity of the antagonism, the same treatment protocol described above was used except that 0.3 μM forskolin or 3 nM isoproterenol, rather than CGRP, was used to stimulate cAMP production.

**Calculation of Equilibrium Dissociation Constants and Data Analysis.**

pA₂ values for CGRP(8-37), bzl-CGRP(8-37) and bzl-bn-CGRP(8-37) were determined as described by Arunlakshana and Schild (Arunlakshana and Schild, 1959). In mouse aorta three adjacent aortic ring segments from each animal were treated with different concentrations of CGRP(8-37), bzl-CGRP(8-37) or bzl-bn-CGRP(8-37). In separate experiments using different groups of SK-N-MC cells, cells were treated with different concentrations of bzl-bn-CGRP(8-37). For each concentration of antagonist used, dose ratios were calculated as the EC₅₀ value of CGRP in the presence of antagonist divided by the EC₅₀ value of CGRP in the absence of antagonist. EC₅₀ values were calculated using all points on the relaxation or cAMP production concentration-response curves using least sum of squares nonlinear regression curve fitting with Graphpad Prism 4.0 (San Diego, CA). Schild plots were constructed by plotting log dose ratio – 1 versus the log of the antagonist concentration. Linear regression of the plotted points was used to determine the x-intercept (pA₂). In other experiments
$K_B$ values for antagonists were determined using a single concentration of antagonist from the following equation:

$$\log K_B = \log[\text{Antagonist}] - \log[(\text{DoseRatio} - 1)]$$

Individual $pA_2$ or $pK_B$ values were averaged and compared statistically as log values. For convenience, they are listed in the text and table as arithmetic mean values ± S.E.M. after conversion to their antilogs.

Differences in maximal force generated by norepinephrine, isoproterenol $EC_{50}$ values, basal, forskolin-stimulated and isoproterenol-stimulated cAMP production, in the absence and presence of bzl-bn-CGRP(8-37), were compared using a Student's $t$-test with a $p < 0.05$ level of probability accepted as a significant difference.

Differences in antagonist affinity values (mean $pK_B$ values and mean $pA_2$ values) within an assay and between assays were determined using analysis of variance followed by Bonferroni post test with a $p < 0.05$ level of probability accepted as a significant difference. The slopes of the Schild regressions were analyzed for departure from linearity using one-way analysis of variance followed by Dunnett's post test with a $p < 0.05$ level of probability accepted as a significant different from a slope of 1.

Correlation plots were constructed to compare antagonist affinities ($pK_B$) determined in the mouse thoracic aorta with antagonist affinities ($pK_B$) determined in human SK-N-MC cells. These data are expressed as the mean of individual log $K_B$ values or their reciprocals ± 95 % confidence interval. Correlation plots were also constructed to compare antagonist affinity values ($\log$
determined in the mouse thoracic aorta and human SK-N-MC cells with antagonist hydrophobicity index (k' value). Data are expressed as the mean of the log of the reciprocal of individual K₆ values ± 95 % confidence interval and mean of individual log k' values ± 95 % confidence interval. The antagonist k' value was determined using three different RP-HPLC systems.

RESULTS

Hydropathy analysis of human and mouse CL and RAMP1 proteins. Using ProtScale on the ExPASy server I constructed hydropathy plots to compare hydrophobicity between human and mouse CL and between human and mouse RAMP1. The human and mouse CL share high amino acid sequence identity (90 %) and, as illustrated by the hydropathy plot Figure 19, are nearly identical in their hydrophobicity across the entire protein. In contrast, human and mouse RAMP1 share only 71 % amino acid sequence identity and most of the non-conserved amino acids reside in the extracellular domain. The hydrophobicity of the transmembrane and intracellular domains of RAMP1 are nearly identical while the extracellular domains are markedly different as illustrated by the hydropathy plot, Figure 19. Analysis of the hydrophobicity of human and mouse RAMP1 revealed that the extracellular domain of human RAMP1 is 62 % more hydrophobic than mouse RAMP1. Furthermore, most of the increased hydrophobicity occurred in the extreme N-terminus of the extracellular domain of human RAMP1.
Peptide Synthesis and Characterization. Figure 20 depicts assembly of novel analogues of the antagonist CGRP(8-37), containing modifications to the N-terminus and the imidazole side-chain of histidine at position 10, on the polystyrene resin. Briefly, the amino acid sequence of h-α-CGRP(8-37) was assembled on MBHA-resin using my previously published methods (Taylor et al., 2005) employing Boc-amino acid derivatives and in situ neutralization (Schnolzer et al., 1992). For bzl-CGRP(8-37), after the N-terminal Boc group was removed the N-terminus was acylated with benzoic anhydride. For bzl-bn-CGRP(8-37), prior to removal of the N-terminal Boc group, the histidine imidazole side-chain, at position 10, of the resin bound peptide was benzylated at C4 using benzyl bromide (Smith et al., 2003), then the N-terminal Boc group was removed and the N-terminus was acylated with benzoic anhydride. Peptides were cleaved from the resin and simultaneously freed of side-chain protecting groups using trifluoromethanesulfonic acid:trifluoroacetic acid (TFMSA:TFA) in the presence of thiol scavengers (Tam et al., 1986) and immediately purified by semi-preparative RP-HPLC. Peptide purity was assessed to be >98 % by analytical RP-HPLC under isocratic elution conditions using three different RP-HPLC columns. Purified products had correct masses as determined by electrospray ionization-mass spectrometry (Table 4) and satisfactory amino acid composition (Table 5). As predicted, low recovery of valine was found in the composition of bzl-CGRP(8-37) and bzl-bn-CGRP(8-37) which is consistent with benzylation of the N-terminal valine residue. No histidyl residues were found in the amino acid
composition of bzl-bn-CGRP(8-37), which is consistent with irreversible benzylolation of this analogue in position 10 (Smith et al., 2003).

**h-α-CGRP(8-37).** The crude product from was loaded onto a C18 RP-HPLC column previously equilibrated with a mixture of buffer A (77 %) and buffer B (23 %). The product was eluted by raising the concentration of buffer B from 23 % to 53 % over 75 min. Fractions containing the desired product were identified by analytical RP-HPLC, pooled and loaded onto the same column previously equilibrated with a mixture of buffer C (83 %) and buffer D (17 %). The product was eluted by raising the concentration of buffer D from 17 % to 29 % over 50 min. Fractions were detected isocratically using a mixture of buffer C (76 %) and buffer D (24 %) by analytical RP-HPLC. Fractions containing only the desired product were pooled and lyophilized to yield 184.6 mg (16 %) as a fluffy white powder. The results of amino acid analysis and ESI-MS are shown in Table 4 and Table 5. The k' values are shown in Table 6.

**N-α-benzoyl-h-α-CGRP(8-37).** The crude product was loaded onto a C18 RP-HPLC column previously equilibrated with a mixture of buffer C (79 %) and buffer D (21 %). The product was eluted by raising the concentration of buffer D from 21 % to 33 % over 50 min. Fractions were detected isocratically using a mixture of buffer C (71 %) and buffer D (29 %) by analytical RP-HPLC. Fractions containing only the desired product were pooled and lyophilized to yield 35.7 mg (4 %) as a fluffy white powder. The results of amino acid analysis and ESI-MS are shown in Table 4 and Table 5. The k' values are shown in Table 6.
**N-α-benzoyl-His\(^{10}\)-benzyl-h-α-CGRP(8-37).** The crude product was loaded onto a C\(_{18}\) RP-HPLC column previously equilibrated with a mixture of buffer A (70 %) and buffer B (30 %). The product was eluted by raising the concentration of buffer B from 30 % to 60 % over 75 min. Fractions containing the desired product were identified by analytical RP-HPLC, pooled and loaded onto the same column previously equilibrated with a mixture of buffer C (78 %) and buffer D (22 %). The product was eluted by raising the concentration of buffer D from 22 % to 52 % over 75 min. Fractions were detected isocratically using a mixture of buffer C (68 %) and buffer D (32 %) by analytical RP-HPLC. Fractions containing only the desired product were pooled and lyophilized to yield 4 mg (6 %) as a fluffy white powder. The results of amino acid analysis and ESI-MS are shown in Table 4 and Table 5. The \(k'\) values are shown in Table 6.

**Hydrophobicity of h-α-CGRP(8-37), N-α-benzoyl-h-α-CGRP(8-37) and N-α-benzoyl-[His(4-benzyl)\(^{10}\)]-h-α-CGRP(8-37).** The hydrophobicity index, \(k'\) value, of the peptide analogues was measured by RP-HPLC using three different RP-HPLC columns. The \(k'\) value of CGRP(8-37), bzl-CGRP(8-37) and bzl-bn-CGRP(8-37) using column 1 (Vydac) was 0.1, 0.7 and 2.1, respectively (Figure 21 A, B and C, respectively); column 2 (Waters Symmetry) was 0.1, 0.9 and 3.1, respectively; column 3 (Kromasil) was 0.5, 1.0 and 2.7, respectively. The rank order of hydrophobicity was bzl-bn-CGRP(8-37) > bzl-CGRP(8-37) > CGRP(8-37) and was the same among all RP-HPLC columns used, Table 6.

**Evaluation of Agonist Activity and Non-Specific Effects of N-α-benzoyl-[His(4-benzyl)\(^{10}\)]-h-α-CGRP(8-37) in Mouse Aorta.** Using mouse aorta I tested
the novel CGRP receptor antagonist, bzl-bn-CGRP(8-37), for agonist activity to cause relaxation by precontracting aorta with 1 μM norepinephrine and determining the effects of addition of 1 μM of this antagonist. bzl-bn-CGRP(8-37) (1 μM) had no significant effect on norepinephrine induced tone of aorta. In addition, 1 μM bzl-bn-CGRP(8-37) had no effect on the resting level of tone in this tissue. I also determined if bzl-bn-CGRP(8-37) had non-specific effects to interfere with norepinephrine-mediated contraction by measuring the amount of tension produced by 1 μM norepinephrine tone in the absence and presence of this antagonist. There was no significant difference in the amount of norepinephrine-induced contractile tone in the absence (185 ± 15 mg, n = 12) compared to the presence (195 ± 17 mg, n = 12) of 1 μM of bzl-bn-CGRP(8-37).

Affinity of h-α-CGRP(8-37), N-α-benzoyl-h-α-CGRP(8-37) and N-α-benzoyl-[His(4-benzyl)¹⁰]-h-α-CGRP(8-37) for CGRP Receptors in Mouse Aorta. Mean concentration-response curves for CGRP induced relaxation of mouse aorta in the absence and presence of antagonists are shown in Figure 22. CGRP produced concentration-dependent relaxation to the baseline level of tone with an EC₅₀ value of 10 ± 1.0 nM. In the presence of CGRP(8-37) (10 μM), bzl-CGRP(8-37) (3 μM) and bzl-bn-CGRP(8-37) (1.5 μM) the CGRP concentration-response curves were shifted to the right in a parallel manner consistent with competitive antagonism. EC₅₀ values for CGRP in the presence of CGRP(8-37), bzl-CGRP(8-37) and bzl-bn-CGRP(8-37) were 158 ± 52, 1945 ± 645 and 530 ± 126 nM, respectively. Using these data the affinities (Kᵦ values) of the
antagonists were calculated and are listed in Table 7. The rank order of antagonist affinity was bzl-bn-CGRP(8-37) = bzl-CGRP(8-37) > CGRP(8-37).

The affinity of bzl-bn-CGRP(8-37), for CGRP receptors mediating relaxation of mouse aorta was further evaluated by generating concentration-response relaxation curves for CGRP in the absence and presence of various concentrations of bzl-bn-CGRP(8-37). As shown in Figure 23, bzl-bn-CGRP(8-37) inhibited CGRP-induced relaxation and caused concentration-dependent rightward shifts in the CGRP concentration-response curve. These data were used to construct Schild plots (Figure 27) from which the affinity (pA2 value) for bzl-bn-CGRP(8-37) in inhibiting CGRP-induced relaxation was calculated. The mean pA2 value for bzl-bn-CGRP(8-37) calculated from the Schild regressions was 7.3 with a mean slope of 1.3 ± 0.1, Table 7.

Specificity of N-α-benzoyl-[His(4-benzyl)10]-h-α-CGRP(8-37) Interaction with CGRP Receptors in Mouse Aorta. bzl-bn-CGRP(8-37) was also evaluated for the specificity of its CGRP receptor antagonism effect. After precontraction of mouse aorta with 1 μM norepinephrine, isoproterenol concentration-response relaxation curves in the absence and presence of 1 μM of bzl-bn-CGRP(8-37) were generated. There was no significant effect on maximal isoproterenol-mediated relaxation of the aorta in the absence (100 ± 1 %, n = 4) compared to the presence (98 ± 3 %, n = 4) of bzl-bn-CGRP(8-37) or on isoproterenol EC50 values in the absence (36 ± 12 μM, n = 4) compared to the presence (46 ± 5 μM, n = 4) of bzl-bn-CGRP(8-37). Thus, bzl-bn-CGRP(8-37) does not cause non-specific inhibition of aortic relaxation at this concentration.
Evaluation of Agonist Activity, Non-Specific Effects and Receptor Specificity of N-α-benzoyl-[His(4-benzyl)₁₀]-h-α-CGRP(8-37) in Human SK-N-MC Cells. Mean basal, forskolin-stimulated and isoproterenol-stimulated cAMP production in SK-N-MC cells in the absence and presence of 1 μM of bzl-bn-CGRP(8-37) is shown in Figure 24. There was no significant effect of bzl-bn-CGRP(8-37) on the basal level of cAMP production or on forskolin-stimulated cAMP production. This indicates that bzl-bn-CGRP(8-37) lacks intrinsic agonist activity and does not cause non-specific inhibition of adenylyl cyclase at this concentration. In addition, there was no significant effect of bzl-bn-CGRP(8-37) on isoproterenol-stimulated cAMP production, indicating that 1 μM of this antagonist does not non-specifically inhibit G protein-coupled receptor-mediated cAMP production, Figure 24.

Affinity of h-α-CGRP(8-37), N-α-benzoyl-h-α-CGRP(8-37) and N-α-benzoyl-[His(4-benzyl)₁₀]-h-α-CGRP(8-37) for CGRP Receptors in Human SK-N-MC Cells. Mean concentration-response curves for CGRP-stimulated cAMP production in the absence and presence of antagonists are shown in Figure 25. CGRP produced concentration-dependent increases in cAMP production with an EC₅₀ value of 5.7 ± 0.5 nM. In the presence of CGRP(8-37) (1 μM), bzl-CGRP(8-37) (0.025 μM) and bzl-bn-CGRP(8-37) (0.1 μM) the CGRP concentration-response curves were shifted to the right in a parallel manner. EC₅₀ values for CGRP in the presence of CGRP(8-37), bzl-CGRP(8-37) and bzl-bn-CGRP(8-37) were 31 ± 14, 20 ± 1 and 133 ± 29 nM, respectively. The EC₅₀ values from these concentration-response curves were used to calculate antagonist affinity values.
which are listed in Table 7. The rank order of antagonist affinity was bzl-bn-CGRP(8-37) > bzl-CGRP(8-37) > CGRP(8-37).

The affinity of the most potent antagonist, bzl-bn-CGRP(8-37), for CGRP receptors mediating cAMP production in SK-N-MC cells was further evaluated by generating concentration-response curves for CGRP in the absence and presence of various concentrations of bzl-bn-CGRP(8-37). As shown in Figure 26, bzl-bn-CGRP(8-37) inhibited CGRP-induced cAMP production and caused concentration-dependent parallel rightward shifts in the CGRP concentration-response curve. These data were used to construct Schild plots (Figure 27) from which the affinity (pA2 value) for bzl-bn-CGRP(8-37) in inhibiting CGRP-induced cAMP production was calculated. The mean pA2 value for bzl-bn-CGRP(8-37) calculated from the Schild regression was 9.2 with a mean slope of 0.9 ± 0.1, Table 7.

Comparison of Antagonist Affinities in Mouse Aorta and Human SK-N-MC Cells. Figure 27, shows mean Schild plots for the antagonist bzl-bn-CGRP(8-37) derived from the data shown in Figure 23 and Figure 26. The mean pA2 value was 80-fold lower (higher affinity) in human SK-N-MC cells (pA2 = 9.2) compared to the mean pA2 value in the mouse aorta (pA2 = 7.3), Figure 27.

I also compared antagonist affinity in inhibiting CGRP-induced relaxation in mouse aorta with antagonist affinity in inhibiting CGRP-induced cAMP production in human SK-N-MC cells for all antagonists using the data shown in Figure 22 and Figure 25. There was no difference in the affinity of CGRP(8-37) in the aorta compared to that in SK-N-MC cells (Table 7). In contrast, affinities of
bzl-CGRP(8-37) and bzl-bn-CGRP(8-37) were significantly higher in human SK-N-MC cells compared to the mouse aorta (Table 7). Figure 28 shows the correlation, for all antagonists, of their affinities in mouse aorta compared to their affinities in human SK-N-MC cells. The 95% confidence interval of mean pK₉ values for CGRP(8-37) included the line of identity while mean pK₉ values for bzl-CGRP(8-37) and bzl-bn-CGRP(8-37) did not. Thus, there was a statistically significant decrease in the slope of the regression line correlating affinities in human SK-N-MC cells and mouse aorta compared to the line of identity. These data show that bzl-CGRP(8-37) and bzl-bn-CGRP(8-37) both have a higher affinity for CGRP receptors in human SK-N-MC cells.

Correlation of Antagonist Affinities in Mouse Aorta and Human SK-N-MC Cells with Antagonist Hydrophobicity Index. I also correlated antagonist affinity to inhibit CGRP-induced relaxation in mouse aorta and cAMP production in human SK-N-MC cells, with antagonist hydrophobicity index, k' value (Figure 29). Increasing the hydrophobicity of the antagonists correlated with increases in the affinity of CGRP(8-37), bzl-CGRP(8-37) and bzl-bn-CGRP(8-37) in both the mouse aorta and human SK-N-MC cells. In human SK-N-MC cells, increasing antagonist hydrophobicity caused a linear, proportional increase in affinity. Thus, the linear regression line correlating these parameters in human SK-N-MC cells was not different from the line of identity. In contrast, in the mouse aorta the correlation between antagonist affinity and hydrophobicity was not proportional. Thus, in mouse aorta, there was a significant decrease in the slope of the
regression line correlating affinity and hydrophobicity index compared to the line of identity.
<table>
<thead>
<tr>
<th>Mouse CL</th>
<th>Human CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDKKHILCFL</td>
<td>VLLPLNMALI</td>
</tr>
<tr>
<td>MEKKCTLYFL</td>
<td>VLLPPFMILV</td>
</tr>
<tr>
<td>KIMQDPIQQA</td>
<td>EGLYCNRTWD</td>
</tr>
<tr>
<td>KIMQDPIQQA</td>
<td>EGVCNRTWD</td>
</tr>
<tr>
<td>VTKICDQDGH</td>
<td>WFRHPDSNRT</td>
</tr>
<tr>
<td>VTKICDQDGN</td>
<td>WFRHPASNRT</td>
</tr>
<tr>
<td>LSIASLIISL</td>
<td>IFFYFKSLS</td>
</tr>
<tr>
<td>LSIASLLISL</td>
<td>GIFFYFKSLS</td>
</tr>
<tr>
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<td>SCKVSQFIHI</td>
</tr>
<tr>
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<td>SCKVSQFIHI</td>
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<tr>
<td>HLMWYYFLGW</td>
<td>GFPLLPCAIH</td>
</tr>
<tr>
<td>HLMWYYFLGW</td>
<td>GFPLLPCAIH</td>
</tr>
<tr>
<td>ALLVNLFFLL</td>
<td>NIVRVLITKL</td>
</tr>
<tr>
<td>ALLVNLFFLL</td>
<td>NIVRVLITKL</td>
</tr>
<tr>
<td>LFPWRPEGKV</td>
<td>AEEVYDYVMH</td>
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<td>AEEVYDYIMH</td>
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<tr>
<td>Sequence</td>
<td>Relative Position</td>
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<td>-------------------</td>
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<td>TAELEESPED</td>
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<tr>
<td>GWLCWNDVAA</td>
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<tr>
<td>WTNYTLCNNS</td>
<td>200</td>
</tr>
<tr>
<td>WTNYTQCNVN</td>
<td>250</td>
</tr>
<tr>
<td>CQRITLHKNL</td>
<td>300</td>
</tr>
<tr>
<td>CQRITLHKNL</td>
<td>350</td>
</tr>
<tr>
<td>YLMGCNYFWM</td>
<td>400</td>
</tr>
<tr>
<td>YLMGCNYFWM</td>
<td>450</td>
</tr>
<tr>
<td>AIARSLYYND</td>
<td>50</td>
</tr>
<tr>
<td>NCWISSDTHL</td>
<td>100</td>
</tr>
<tr>
<td>AIARSLYYND</td>
<td>150</td>
</tr>
<tr>
<td>KVTHQVESNL</td>
<td>200</td>
</tr>
<tr>
<td>KVTHQAESNL</td>
<td>250</td>
</tr>
<tr>
<td>IMLHFDGLLV</td>
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</tr>
<tr>
<td>IMLHFDGLLV</td>
<td>350</td>
</tr>
<tr>
<td>YTVSTISDMQ</td>
<td>400</td>
</tr>
<tr>
<td>YTVSTISDGP</td>
<td>450</td>
</tr>
</tbody>
</table>

The table above shows a sequence of amino acids and their corresponding relative positions in the document. The sequences are presented in a tabular format with columns indicating the relative position.
Figures 17. Comparison of amino acid sequence homology between human and mouse CL. Conserved amino acid residues are indicated with shading. ( ) Signal peptide sequence. ( ) Transmembrane domains 1 - 7.
| Mouse RAMP1 | MAPGLRCLPR | CGLWLLLAAHH | LFMVTACRDP | DVGTLIQELC | LSRFKENMET | 50 |
| Human RAMP1 | MARALCRLPR | CGLWLLLAAHH | LFMTTACQEA | NYGALLRELQ | LTQFQVDMEA |    |

| IGKTLWCDWG | KTIQSYGELT | YCTKHVIAHTI | GCFWPNEVD | RFFIAVHHRY | 100 |
| VGETLWCDWG | RTIRSYRELQ | DCTWHMAEKL | GCFWPNAEVD | RFFLAVHGQY |    |

| FSKCPISGRA | LRDPPNSILC | PFIAPITVT | LLMTALVVWR | SKRTEGIV | 148 |
| FRSCPISGRA | VRDPFSGILY | PFIVVIPITV | LLVTALVVWR | SKRTEGIV |    |

**Figure 18.** Comparison of amino acid sequence homology between human and mouse RAMP1. Conserved amino acid residues are indicated with light shading and variations in hydrophobic amino acid residues are indicated with dark shading. (___) Signal peptide sequence. (---) Amino acid residues 66 - 112 modulates affinity of BIBN 4096BS and Compound 1. (‡) Single amino acid residue responsible for human selectivity of BIBN 4096BS. (___) CGRP binding and signal transduction. (*) Single amino acid residue involved in regulating cell surface expression. (___) Transmembrane domain and RAMP1/CL dimerization domain. (^) PKC phosphorylation site. (....) Intracellular retention signal sequence.
Figure 19. Hydropathy plot overlays of predicted human and mouse CL Panel A and RAMP1 Panel B amino acid sequences. The regions of the hydropathy plots on the left represent extracellular N-termini and the regions on the right represent transmembrane (TM) domains and intracellular C-termini. The hydropathy plot of human and mouse CL are nearly identical while the hydropathy plot of human and mouse RAMP1 are markedly different, particularly at the end of the N-terminus of the extracellular domains. The region that is more hydrophobic in human RAMP1, where hydrophobic peptide antagonists are proposed to interact, is labeled Human > Mouse. The more hydrophobic region in mouse RAMP1 and where non-peptide antagonists interact is labeled Mouse > Human, amino acids 66 – 112 and position 74. Positive numbers indicate hydrophobic regions while negative numbers indicate hydrophilic regions.
**Figure 20.** Synthesis of high affinity competitive CGRP receptor antagonists N-α-benzoyl-h-α-CGRP(8-37) and N-α-benzoyl-[His(4-benzyl)^10]-h-α-CGRP(8-37). Rapid SPPS protocol employing *in situ* neutralization for the synthesis of CGRP(8-37), benzylation of the imidazole side-chain of His at position 10 and benzoylation of the N-terminus of resin-bound side-chain protected sequences of CGRP(8-37) and [His(4-benzyl)^10]-CGRP(8-37).
Table 4. Observed masses of N-terminally and His^{10} modified analogues of h-α-CGRP(8-37).

<table>
<thead>
<tr>
<th>Analogue</th>
<th>ESI-MS (amu)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Found</td>
</tr>
<tr>
<td>h-α-CGRP(8-37)</td>
<td>3125.7</td>
</tr>
<tr>
<td>N-α-benzoyl-h-α-CGRP(8-37)</td>
<td>3230.5</td>
</tr>
<tr>
<td>N-α-benzoyl-[His(4-benzyl)^{10}]h-α-CGRP(8-37)</td>
<td>3319.9</td>
</tr>
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</table>
Table 5. Amino acid composition of CGRP(8-37) and N-terminal and His\textsuperscript{10} modified analogues of CGRP(8-37).

<table>
<thead>
<tr>
<th>Analogue</th>
<th>Asp</th>
<th>Ser</th>
<th>Gly</th>
<th>His\textsuperscript{a}</th>
<th>Arg</th>
<th>Thr</th>
<th>Ala</th>
<th>Pro</th>
<th>Val\textsuperscript{b}</th>
<th>Lys</th>
<th>Leu</th>
<th>Phe</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGRP(8-37)</td>
<td>3.08(3)</td>
<td>2.86(3)</td>
<td>4.16(4)</td>
<td>1.03(1)</td>
<td>2.01(2)</td>
<td>1.94(2)</td>
<td>2.02(2)</td>
<td>1.09(1)</td>
<td>4.88(5)</td>
<td>1.86(2)</td>
<td>3.00(3)</td>
<td>1.98(2)</td>
</tr>
<tr>
<td>bzl-CGRP(8-37)</td>
<td>3.04(3)</td>
<td>2.99(3)</td>
<td>4.10(4)</td>
<td>1.04(1)</td>
<td>1.97(2)</td>
<td>2.10(2)</td>
<td>2.12(2)</td>
<td>1.11(1)</td>
<td>3.90(5)</td>
<td>1.87(2)</td>
<td>2.85(3)</td>
<td>1.86(2)</td>
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<tr>
<td>bzl-bn-CGRP(8-37)</td>
<td>3.02(3)</td>
<td>2.81(3)</td>
<td>4.11(4)</td>
<td>0.03(1)</td>
<td>2.06(2)</td>
<td>1.84(2)</td>
<td>2.01(2)</td>
<td>1.00(1)</td>
<td>4.03(5)</td>
<td>1.86(2)</td>
<td>3.01(3)</td>
<td>1.92(2)</td>
</tr>
</tbody>
</table>

Theoretical values are shown in parentheses.

\textsuperscript{a} Benzylation of His at position 10 results in loss of the histidyl residue in the composition.

\textsuperscript{b} Benzoylation of the N-terminus results in low recovery due to incomplete hydrolysis of the benzoyl-Val\textsuperscript{8} bond.
Table 6. k’ values for CGRP(8-37) and N-terminally and His^{10} modified analogues of CGRP(8-37)^a.

<table>
<thead>
<tr>
<th>Analogue</th>
<th>System 1</th>
<th>k’ values^b</th>
<th>System 2</th>
<th>System 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>h-α-CGRP(8-37)</td>
<td>0.1</td>
<td>0.1</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>N-α-benzoyl-h-α-CGRP(8-37)</td>
<td>0.7</td>
<td>0.9</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>N-α-benzoyl-[His(4-benzyl)^{10}]-h-α-CGRP(8-37)</td>
<td>2.1</td>
<td>3.1</td>
<td>2.7</td>
<td></td>
</tr>
</tbody>
</table>

^a Columns used were **System 1**, Vydac C_{18} monomeric 238TP54 column (250 x 4.6 mm); **System 2**, Waters Symmetry C_{18} 300A column (250 x 4.6 mm); **System 3**, Kromasil C_{8} column (250 x 4.6 mm).

^b Peptides were eluted under isocratic conditions using 0.1 % TFA in water/acetonitrile 69/31 (v/v) at a flow rate of 1 mL/min and detected at 214nm.
Figure 21. Representative RP-HPLC chromatograms of isocratic elution of CGRP(8-37) Panel A, bzI-CGRP(8-37) Panel B and bzI-bn-CGRP(8-37) Panel C from which the hydrophobicity index ($k'$ value) was determined. The hydrophobicity index was calculated as (analogue retention time minus solvent front time) divided by solvent front time.
Figure 22. Effect of the antagonist peptides, CGRP(8-37) (10 μM), bzl-CGRP(8-37) (3 μM) and bzl-bn-CGRP(8-37) (1.5 μM), on concentration-response curves for CGRP-induced relaxation of mouse aorta. Aortic rings were pre-contracted with norepinephrine and CGRP concentration-response curves were obtained. The rings were then incubated with the antagonist for one hour and CGRP concentration-response curves were repeated. Concentration-response curves are plotted as percent relaxation to the baseline tone present before contraction with 1 μM norepinephrine. Points are the mean ± S.E.M. of responses of four to six individual thoracic aortas, each taken from different animals.
Figure 23. Effect of different concentrations of bzI-bn-CGRP(8-37) on concentration-response curves for CGRP-induced relaxation of mouse aorta. Aortic rings were pre-contracted with norepinephrine and CGRP concentration-response curves were obtained. The rings were then incubated with the antagonist for one hour and CGRP concentration-response curves were repeated. Concentration-response curves are plotted as percent relaxation to the baseline tone present before contraction with 1 μM norepinephrine. Points are the mean ± S.E.M. of responses of four to six individual thoracic aortas, each taken from different animals.
Figure 24. Effect of bzl-bn-CGRP(8-37) on basal, forskolin-stimulated and isoproterenol-stimulated cAMP production in SK-N-MC cells. Cells were incubated with vehicle (basal), 300 nM forskolin or 3 nM isoproterenol (open bars) for 30 min at 37 °C and cAMP production measured or pretreated with 1 μM bzl-bn-CGRP(8-37) (solid bars) for 15 min at 37 °C then stimulated with vehicle (basal), 300 nM forskolin or 3 nM isoproterenol and cAMP production measured. Bars are the mean ± S.E.M. generated from three to fourteen individual experiments performed in duplicate, with each experiment using cells grown in different culture plates on different days.
Figure 25. Effect of the antagonist peptides, CGRP(8-37) (1 µM), bzl-CGRP(8-37) (25 nM) and bzl-bn-CGRP(8-37) (25 nM), on concentration-response curves for CGRP-stimulated cAMP production in SK-N-MC cells. Untreated control cells were stimulated with CGRP and cAMP production was measured. Treated cells were incubated with antagonist for 15 minutes, the cells were then stimulated with CGRP and cAMP production was measured. Concentration-response curves are plotted as percent of the maximal CGRP-stimulated cAMP production. Points are the mean ± S.E.M. of responses from three individual experiments performed in duplicate, with each experiment using cells grown in different culture plates on different days.
Figure 26. Effect of different concentrations of bzl-bn-CGRP(8-37) on concentration-response curves for CGRP-stimulated cAMP production in SK-N-MC cells. Untreated control cells were stimulated with CGRP and cAMP production was measured. Treated cells were incubated with a different concentration of bzl-bn-CGRP(8-37) for 15 minutes, the cells were then stimulated with CGRP and cAMP production was measured. Concentration-response curves are plotted as percent of the maximal CGRP-stimulated cAMP production. Points are the mean ± S.E.M. of responses from three individual experiments performed in duplicate, with each experiment using cells grown in different culture plates on different days.
Table 7. Antagonist equilibrium dissociation constants (K_B and pA_2) and Schild slopes for CGRP(8-37), bzl-CGRP(8-37) and bzl-bn-CGRP(8-37) in the mouse thoracic aorta and human SK-N-MC cells.

<table>
<thead>
<tr>
<th></th>
<th>Mouse Thoracic Aorta</th>
<th>Human SK-N-MC Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K_B (nM)</td>
<td>pA_2</td>
</tr>
<tr>
<td>CGRP(8-37)</td>
<td>1000.3 ± 0.19^d</td>
<td>6.4^d</td>
</tr>
<tr>
<td>bzl-CGRP(8-37)</td>
<td>88.9 ± 0.25^c</td>
<td>7.1^c</td>
</tr>
<tr>
<td>bzl-bn-CGRP(8-37)</td>
<td>22.9 ± 0.21^c</td>
<td>7.3^c</td>
</tr>
</tbody>
</table>

^a Each value is the mean ± S.E.M. of four to six experiments using separate aortas, each taken from different animals.

^b Each value is the mean ± S.E.M. of three experiments performed in duplicate, each using different groups of cells.

^c Significantly different from CGRP(8-37) within a tissue or cell line (p < 0.05).

^d Significantly different from bzl-CGRP(8-37) within a tissue or cell line (p < 0.05).

^e Significantly different from the value in mouse thoracic aorta (p < 0.05).
Figure 27. Mean Schild plots of the data from Figure 22 using mouse aorta and from Figure 25 using human SK-N-MC cells. The affinity (pA₂) of bzI-bn-CGRP(8-37) for CGRP receptors in the mouse aorta and human SK-N-MC cells was calculated as the x-intercept of the Schild plot which was 7.3 and 9.2, respectively.
Figure 28. Correlation plot of the affinity values (pK$_B$) determined from the data for CGRP receptor-mediated responses in the mouse aorta (Figure 22) and SK-N-MC cells (Figure 25). The dashed line is the line of identity while the solid line is the Deming regression of the affinity of the antagonists in mouse aorta versus human SK-N-MC cells. Data points are mean ± 95% confidence intervals. The slope and correlation coefficient ($r^2$) of the linear regression of the data points are indicated. Note that the points for bzl-CGRP(8-37) and bzl-bn-CGRP(8-37) deviate from the line of identity while the point for CGRP(8-37) does not.
Figure 29. Correlation plot of the affinity values (log (1/pK\textsubscript{B})) determined using functional assays for CGRP receptor-mediated responses in the mouse aorta (data from Figure 22) or SK-N-MC cells (data from Figure 25) and the log of the hydrophobicity index (k') of the antagonists. The dashed line is the line of identity. The solid line is the Deming regression of the affinity of the antagonists in mouse aorta (solid symbols) or human SK-N-MC cells (open symbols) versus the hydrophobicity of the individual antagonist. Data points are mean ± 95% confidence intervals. Note that in mouse aorta, the points for bzl-CGRP(8-37) and bzl-bn-CGRP(8-37) deviate from the line of identity while the points for these antagonists in human SK-N-MC cells do not.
CGRP is a 37-residue neuropeptide that is widely distributed in the central and peripheral nervous systems. This peptide acts as a neurotransmitter and neuromodulator with important cardiovascular actions, which include regulation of peripheral vascular tone and force and rate of cardiac contraction. The actions of CGRP are mediated through CGRP receptors which are family B members of the G protein-coupled receptor superfamily. CGRP(8-37) is the primary pharmacological tool used to characterize CGRP receptors and the differential affinity of this antagonist in different tissues provides evidence for CGRP receptor heterogeneity. At this time, only one CGRP receptor, the CGRP1 receptor, has been cloned. The CGRP1 receptor consists of a heterodimer comprised of the calcitonin receptor-like receptor (CL) and receptor activity-modifying protein 1 (RAMP1) (McLatchie et al., 1998). To date, the International Union of Pharmacology (IUPHAR) and most other authorities recognize the CGRP1 receptor subtype and a second heterogeneous population of CGRP receptors that may contain more than one pharmacologically distinct CGRP receptor subtype (Poyner et al., 2002).

The mouse is an important experimental model system used to identify new agents for the treatment of cardiovascular disease. Homozygous CGRP knockout mice have been developed to examine the role of CGRP in the cardiovascular system. In homozygous CGRP knockout mice, mean arterial blood pressure, heart rate, peripheral vascular resistance and sympathetic nerve activity were elevated (Gangula et al., 2000; Oh-hashi et al., 2001), which
suggest that endogenous CGRP plays an important role in regulation of the cardiovascular system. Effective use of the mouse as a model for human CGRP receptor-mediated effects requires an understanding of the pharmacological tools used to examine CGRP receptor-mediated responses and if these tools differentiate human CGRP receptors from CGRP receptors in other species. Despite the establishment of the mouse as a sensitive system to study the vasodilatory effects of CGRP (Pomerleau et al., 1997; Chan and Fiscus, 2001), there are no functional studies that quantify the affinity of the prototypical CGRP receptor antagonist CGRP(8-37) in mouse blood vessels to identify the CGRP receptor type causing vascular relaxation. In my studies, I found that CGRP(8-37) had micromolar affinity (pA$_2$ = 6.4) in blocking CGRP induced relaxation of mouse aorta consistent with this response being mediated by CGRP1 receptors. In addition, the affinity of CGRP(8-37) to inhibit relaxation was the same as its affinity to inhibit cAMP production in human SK-K-MC cells, which are routinely used as a model system to study CGRP1 receptors. Thus, unlike blood vessels from some other species (Wisskirchen et al., 1999), I found that the mouse has typical CGRP1 receptor mediated vascular relaxation as has been reported in isolated blood vessels from humans (Hasbak et al., 2003).

In addition to the prototypical CGRP receptor antagonist CGRP(8-37), I also quantitatively characterized a high affinity CGRP receptor antagonist, bzI-CGRP(8-37), previously identified by my laboratory, and a novel antagonist bzI-bn-CGRP(8-37). These three antagonists all acted in a competitive manner in blocking functional responses in mouse aorta and in human SK-N-MC cells and
the rank order of affinity at CGRP receptors in both of these assay systems was bzl-bn-CGRP(8-37) ≥ bzl-CGRP(8-37) > CGRP(8-37). Furthermore, the new antagonist, bzl-bn-CGRP(8-37), had no agonist activity or non-specific effects in both the aorta and SK-N-MC cell line at concentrations up to 1 μM. Correlations among the affinities of CGRP(8-37), bzl-CGRP(8-37) and bzl-bn-CGRP(8-37) in the mouse aorta compared to human SK-N-MC cells showed that CGRP(8-37) does not discriminate between mouse and human CGRP receptors. In contrast, bzl-CGRP(8-37) and bzl-bn-CGRP(8-37), have increased affinity for human CGRP receptors in SK-N-MC cells. I also measured antagonist hydrophobicity and found the same rank order of antagonist hydrophobicity of bzl-bn-CGRP(8-37) > bzl-CGRP(8-37) > CGRP(8-37), as for the rank order of antagonist affinity. These correlations suggest that increasing the hydrophobic characteristics of these CGRP(8-37) based peptide antagonists provides progression toward human CGRP receptor selectivity.

I cannot rule out the possibility that tissue mediated peptide degradation, the binding of peptides to glass surfaces, or tissue barriers to diffusion of peptides into receptor compartments could all reduce the concentration of these antagonists at CGRP receptors leading to an underestimation of their affinities in functional assays using mouse aorta. However, my new antagonist peptides differ importantly from the standard antagonist CGRP(8-37) because they are N-terminally acylated. It is well established that derivatization of the N-terminus of peptides protects them from degradation by aminopeptidases (Drapeau et al., 1993) which suggest that antagonist metabolism is minimal in my experiments. In
addition, the organ chambers are coated with organosilanes which limits the binding of peptides and other agents to glass surfaces. This treatment would also reduce peptide loss in our experiments. I also found that the standard peptide antagonist CGRP(8-37), had the same affinity in mouse aorta compared to SK-N-MC cells which indicates that, at least for this antagonist, diffusion barriers do not confound my affinity measurements in the mouse aorta. Although these experimental factors could contribute to differences in antagonist affinity among these assays, I believe that these factors are not likely to explain the 80-fold difference in the affinity of bzl-bn-CGRP(8-37) between mouse aorta and SK-N-MC cells.

The N-terminus of family B G protein-coupled receptors plays a key role in ligand binding to receptors. For the CGRP receptor the extracellular domains of CL and RAMP1 are thought to form the binding site for peptide ligands (Fraser et al., 1999; Koller et al., 2002), with RAMP1 being the more important component. The extracellular domain of RAMP1 is the least conserved region among species and amino acids 66 – 112 of this region are important for determining the affinity of the non-peptide antagonists BIBN4096BS and Compound 1 for CGRP receptors (Mallee et al., 2002). These non-peptide antagonists show selectivity for the human compared to the rodent CGRP receptor and a single amino acid at position 74 in the extracellular domain of RAMP1 has been reported to explain this species selectivity. The fact that my new peptide antagonist bzl-bn-CGRP(8-37), also showed relatively high affinity and human CGRP receptor selectivity suggests that the extracellular domain of RAMP1 may be necessary for CGRP
receptor interaction with both peptide and non-peptide antagonists. In my studies, I found that bzl-CGRP(8-37) and bzl-bn-CGRP(8-37) have up to 80-fold higher affinity for the human compared to the mouse CGRP receptor. I propose that this species difference in affinity may be accounted for by sequence dissimilarity in the extracellular domain between human and mouse RAMP1. In fact, comparison of the extracellular domain amino acid composition between human and mouse RAMP1, using the Kyte-Doolittle hydrophobicity scale (Kyte and Doolittle, 1982), showed numerous amino acid differences resulting in an extracellular domain of human RAMP1 that is 62% more hydrophobic compared to mouse RAMP1. These findings indicate that the CGRP receptor binding pocket formed by the extracellular domains of human CL and RAMP1 may be more hydrophobic than the binding pocket formed between the extracellular domains of mouse CL and RAMP1.

Important information about binding sites on the native CGRP receptor can be gained by comparing the interaction of peptide and non-peptide antagonists which can be used to guide modeling of receptor-ligand interaction. Although there is a significant size difference between the low molecular weight non-peptide antagonists BIBN4096BS and Compound 1 compared to my new peptide antagonists, both interact with RAMP1 and their human selectivity is directed by RAMP1. While non-peptide antagonists require only a single amino acid residue on RAMP1 to confer human selectivity, peptide antagonists appear to require a wider amino acid region for this interaction. Hydropathy plots of human and mouse RAMP1 show that the region encompassing position 74, the
amino acid responsible for human selectivity of non-peptide antagonists, is more hydrophobic in mouse RAMP1 (Figure 19). This shows that human selective non-peptide antagonists have a greater affinity for the extracellular domain of human RAMP1 which is less hydrophobic than mouse RAMP1. Thus, for non-peptide antagonists decreasing hydrophobic interactions between ligand and receptor are correlated with human selectivity of these antagonists. My new peptide antagonist bzl-bn-CGRP(8-37) was designed with hydrophobic modifications to the region of this peptide that is thought to interact with the hydrophobic extreme N-terminus of the extracellular domain of human RAMP1. My correlations between antagonist affinities and their hydrophobicity indicate that increasing hydrophobicity of peptide antagonists is a key factor in human CGRP receptor selectivity. Based on a hydrophobic interaction between our new peptide antagonists and RAMP1 I predict they interact with the region of human RAMP1 that is more hydrophobic than the mouse, residues 55 to the N-terminus. Thus, peptide and non-peptide antagonists appear to interact in a different manner with RAMP1 and there may be a difference in the structural determinants within the CGRP receptor that contribute to their species selectivities. These human selective peptide antagonists provide novel tools to study the integrative processes that cause functional responses in native cells, tissues and animals, the functionome.

In summary, I designed, synthesized and measured the affinity of CGRP(8-37), and the more hydrophobic antagonists bzl-CGRP(8-37) and bzl-bn-CGRP(8-37) at mouse and human CGRP receptors. I found that CGRP(8-37),
does not discriminate between mouse and human CGRP receptors, while bzl-CGRP(8-37) possesses some preference for human CGRP receptors. In contrast, the most hydrophobic antagonist, bzl-bn-CGRP(8-37), possessed the highest affinity and greatest selectivity for human CGRP receptors. The modification of CGRP(8-37) with bulky hydrophobic groups resulting in relatively high affinity peptide antagonists at human receptors suggests that human CGRP receptor selectivity is directed by the extreme N-terminus of the extracellular domain of RAMP1 which participates as a unique entity for peptide antagonists forming a hydrophobic binding pocket with CL. I propose that the species selectivity of these peptide antagonists is due to increased hydrophobic ligand-receptor interactions. Thus, the N-terminus of the extracellular domain of human RAMP1 is a critical target to exploit for development of human selective CGRP receptor agents.
CHAPTER 4

SYNTHESIS AND PHARMACOLOGICAL CHARACTERIZATION OF N-TERMINAL MODIFIED ANALOGUES OF CALCITONIN GENE-RELATED PEPTIDE(8-37) CONTAINING bis-(2-Chloroethyl)amino and Fluorosulfonyl ALKYLATING MOIETIES

ABSTRACT

The synthesis of the first irreversible CGRP receptor antagonists is described. Fluorosulfonyl or bis-(2-chloroethyl)amino moieties were incorporated at the 4-position of the N-terminal benzoyl group of a potent competitive antagonist, N-α-benzoyl-h-α-CGRP(8-37). In order to improve potency, a second pair of analogues was synthesized with histidine at position 10 benzylated, at C4 of the imidazole side-chain. Analogues blocked the actions of CGRP on mouse thoracic aorta and SK-N-MC cells and reduced maximal CGRP-mediated responses which is consistent with the analogues binding irreversibly to CGRP receptors. The bis-(2-chloroethyl)amino-modified analogues were more potent than the fluorosulfonyl-modified analogues. Benzylation of the His\textsuperscript{10} side-chain increased the potency of the bis-(2-chloroethyl)amino-modified irreversible antagonists but had no effect on the potency of the fluorosulfonyl-modified irreversible antagonist. The most potent analogue is N-α-4-[bis-(2-chloroethyl)amino]benzoyl-[His(4-benzyl)\textsuperscript{10}]-h-α-CGRP(8-37). These selective
irreversible antagonists will prove invaluable for fully characterizing the actions of CGRP in cells, organs and whole animals.
INTRODUCTION

Irreversible receptor antagonists are important tools that permit the study of receptor structure and functions, including ligand binding site mapping (Dickinson et al., 1985), receptor occupancy response relationships (Nelson et al., 1986) and physiological functions (Minneman, 1988). Desirable characteristics of an irreversible antagonist include a pharmacophore that provides affinity and selectivity for the receptor and a reactive moiety that results in covalent bonding to the receptor, such as an electrophilic group or a group which can be activated toward bond formation by photolysis. Irreversible antagonists bind to receptors in a two-step process, with the ligand first binding reversibly followed by the reactive moiety making a covalent bond at or near the ligand binding site of the receptor protein. Development of irreversible CGRP receptor antagonists would provide novel pharmacological tools to characterize the interaction of CGRP with its receptors and investigate the physiological role of CGRP receptors in vivo.

Calcitonin gene-related peptide (CGRP) belongs to a family of structurally and biologically related peptides that include calcitonin, amylin, adrenomedullin and intermedin all of which share 20-43 % sequence similarity. CGRP is a 37-amino acid residue neuropeptide peptide that exists in two forms, denoted α and β, which differ at positions 3, 22 and 25 in the human peptide. They are produced from separate genes; α-CGRP being produced from tissue-specific alternative splicing of the primary RNA transcript of the calcitonin gene while β-CGRP is produced by a separate gene (Amara et al., 1982; Steenbergh et al., 1985). α-
CGRP is widely distributed throughout the central and peripheral nervous system and has been implicated in the regulation of vascular tone (Gangula et al., 2000), wound healing (Engin, 1998), inflammatory responses (Smith et al., 1993), nociception (Yu et al., 1998), appetite suppression (Tannenbaum and Goltzman, 1985) and gastric emptying (Raybould, 1992).

The actions of CGRP are mediated through two cell surface receptors, the CGRP1 receptor, a member of the family B G protein-coupled receptor superfamily, and the CGRP2 receptor. Removal of the N-terminal loop of CGRP produces a competitive antagonist, CGRP(8-37) (Chiba et al., 1989), which is the primary pharmacological tool used to characterize CGRP receptors. CGRP1 receptor-mediated responses are inhibited by CGRP(8-37) with high affinity while CGRP2 receptor-mediated responses are inhibited by CGRP(8-37) with low affinity (Dennis et al., 1990; Poyner et al., 2002). The CGRP1 receptor has been cloned and consists of a heterodimeric membrane bound protein complex comprised of the calcitonin receptor-like receptor (CL) and the receptor activity-modifying protein-1 (RAMP1) (McLatchie et al., 1998). No such correlate has been identified for the CGRP2 receptor subtype.

Recently, Smith et al. reported significantly increased potency and affinity of N-terminally modified analogues of h-α-CGRP(8-37) (Smith et al., 2003) which possessed a benzyoylated or benzylated N-terminus and benzylated C4 of the imidazole side-chain of His10 (Smith et al., 2003). Consequently, I envisioned the incorporation of electrophilic bis-(2-chloroethyl)amino and fluorosulfonyl groups, into the para-position of N-terminally benzyoylated CGRP(8-37) to produce
irreversible CGRP receptor antagonists. The bis-(2-chloroethyl)amino moiety is highly selective and only undergoes substitution with nitrogen nucleophiles (e.g., NH₂, NH, C(NH)=NH₂) (Panthananickal et al., 1978), while the fluorosulfonyl moiety is less selective undergoing substitution with a variety of nucleophilic receptor groups (e.g., NH₂, OH, SH) (Bruncel, 1970). I now describe the use of these electrophilic moieties in the design of irreversible antagonists of CGRP to identify amino acid residues present within the binding pocket.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents.

Boc-amino acids were purchased from Bachem Inc. (Torrance, CA) and Chem-Impex International (Wood Dale, IL), HCTU was obtained from Chem-Impex International, TFA, DIEA, diethyl ether, DMF, ACN, DCM, benzene, thionyl chloride and HCl were purchased from Fisher Scientific (Pittsburgh, PA). Ninhydrin test kit was obtained from Anaspec (San Jose, CA), TFMSA was obtained from Fluka Chemical Co. (Milwaukee, WI), p-MBHA resin (substitution level 1.16 mmol/g) was obtained from Bachem Inc., EDT, benzyl bromide, 4-(fluorosulfonyl)benzoyl chloride, ethylene oxide and thioanisole were obtained from Sigma-Aldrich (St. Louis, MO). HCl (6 M) was obtained from Pierce (Rockford, IL). Silica gel (230 – 400 mesh, 60 Å) was obtained from Merck (San Diego, CA). TLC plates (2.5 x 5 cm, Fluorescent) were obtained from Whatman (Florham Park, NJ). Norepinephrine bitartrate, isoproterenol bitartrate and forskolin were purchased from Sigma-Aldrich (St. Louis, MO).
Isobutylmethylxanthine was purchased from EMD Biosciences Inc. (San Diego, CA). Dulbecco’s modified eagles medium, fetal bovine serum, and antibiotic/antimycotic (containing 10,000 units/mL penicillin G, 10,000 μg/mL streptomycin sulfate and 25 μg/mL amphotericin B) were purchased from Invitrogen (Carlsbad, CA).

**Ethyl p-[N,N-bis(2-hydroxyethyl)amino]benzoate (1).**

To a chilled suspension of ethyl-p-aminobenzoate (16 g, 96 mmol) in 25 % aqueous acetic acid (200 mL) was added ethylene oxide (55 mL, d = 0.882, 1.10 mol) with stirring. The reaction was left to stir for 24 h at room temperature to yield a clear solution. The solution was made basic by addition of sodium bicarbonate (70 g) and extracted with ethyl acetate (200 mL x 3). Pooled organic extracts were dried over anhydrous calcium sulfate, filtered and excess solvent removed under reduced pressure to yield the crude product as a white cake. The crude product was dissolved in ethyl acetate, loaded onto a flash chromatography column and eluted with ethyl acetate. Fractions containing the desired product were identified by TLC, pooled and concentrated under reduced pressure. The desired product was purified by recrystallization from ethyl acetate.

**p-[N,N-bis-(2-chloroethyl)amino]benzoic acid (2).**

To a solution of compound 1 (8.57 g 33.8 mmol) in benzene (100 mL), was added thionyl chloride (10 mL, 137 mmol), the solution was heated at reflux for 2 h and after cooling to room temperature, the solvent was removed under reduced pressure to yield a yellow oil. To the resulting oil, HCl (conc.) (70 mL) was added and the solution was heated at reflux for 3 h, during which the product
precipitated as white flakes. The reaction mixture was allowed cool overnight, the precipitate was filtered, washed with cold 50 % aqueous ethanol, air dried and purified by recrystallization from cold 95 % ethanol.

\( p-[N,N\text{-bis-}(2\text{-chloroethyl})\text{amino}]\text{benzoyl} \text{ chloride (3).} \)

To a solution of compound 2 (104 mg, 0.4 mmol) in benzene (10 mL) was added thionyl chloride (207 \( \mu \text{L} \), 2.86 mmol) and the solution was heated at reflux for 6 h. Aliquots (20 \( \mu \text{L} \)) of the reaction mixture were removed at 1 h intervals, mixed with 2-propanol (100 \( \mu \text{L} \)) and vortexed, then analyzed by TLC to monitor the reaction. When complete, the solvent was removed under reduced pressure, dried in vacuo overnight and crystallized from benzene/hexane.

**Solid Phase Peptide Synthesis.**

CGRP(8-37) was synthesized by Boc-SPPS (Merrifield, 1965) employing in situ neutralization (Schnolzer et al., 1992; Taylor et al., 2005). Reactive side-chains of Boc-amino acid derivatives were protected as follows: Asp, benzyl ester; Thr, Ser, benzyl ether; His, benzyloxymethyl (BOM); Arg, mesitylene-2-sulfonyl (Mts); Lys, 2-chlorocarbobenzoxy (2Cl-CBZ). Synthesis was carried out manually in a 30 mL glass reaction vessel on a 0.5 mmol scale. MBHA-resin (435 mg, 1.16 mmol/g) was placed in the reaction vessel and subjected to the following protocol: 1) Boc-Phe (0.53 g, 2 mmol) was dissolved in a 0.38M solution of HCTU in DMF (5 mL, 1.90 mmol), DIEA (0.523 mL, 3.0 mmol) was added and the solution was mixed vigorously on a Fisher Scientific “Touch Mixer Model 231” vortexer 3 times over 2 min. 2) The resin was washed with DMF (45 sec continuous flow wash) and drained to the level of the resin bed. 3) The
activated-Boc-Phe solution was transferred to the reaction vessel and rocked on a wrist-arm shaker for 10 minutes. 4) The resin was washed with DMF (45 sec continuous flow wash), drained completely and a resin sample (5 – 10 mg) removed. 5) The Boc-protecting group was removed with 100 % TFA (10 mL, 2 x 1 min) with rocking and the resin drained completely to yield protonated Phe-MBHA-resin. This procedure was repeated for coupling of the next 29 residues. The activation of the next residue was started during the first TFA deprotection to alleviate any delay in the activation and coupling. Once the sequence of CGRP(8-37) was assembled on the resin, the peptide-resin was washed with DMF (30 sec continuous flow wash), DCM (30 sec continuous flow wash), MeOH (30 sec continuous flow wash) and dried in vacuo for 2 h. Resin samples removed after each coupling were used to determine coupling yields by the quantitative ninhydrin test (Sarin et al., 1981). Single couplings were performed for addition of all amino acid derivatives.

N-α-4-[N,N-bis-(2-chloroethyl)amino]benzoyl-h-α-CGRP(8-37) (4).

The fully protected amino acid sequence of CGRP(8-37) was assembled on MBHA-resin using Boc-amino acid derivatives as described above. A portion of the peptide-resin (100 mg, 0.02 mmol) was flow-washed with DMF (45 sec continuous flow wash), the N-α-Boc-protecting group removed with TFA (5 mL, 2 x 1 min), flow-washed with DMF (45 sec continuous flow wash) and reacted with compound 3 (100 mg, 0.36 mmol) and DIEA (35 µL, 0.2 mmol) in DMF (600 µL) overnight. The crude product from cleavage reaction was purified using RP-HPLC.
N-α-4-[N,N-bis-(2-chloroethyl)amino]benzoyl-[His(benzyl)\(^{10}\)]-h-α-CGRP(8-37) (5).

The fully protected amino acid sequence of CGRP(8-37) was assembled on MBHA-resin using Boc-amino acid derivatives as described above. A portion of the peptide-resin (100 mg, 0.02 mmol) was benzylated with benzyl bromide (24 µL, 0.2 mmol) and DIEA (35 µL, 0.2 mmol) in DMF (300 µL) for 24 h. The peptide-resin was flow-washed with DMF (45 sec continuous flow wash), the N-α-Boc-protecting group removed with TFA (5 mL, 2 x 1 min) and flow-washed with DMF (45 sec continuous flow wash). Then, reacted with compound 3 (100 mg, 0.36 mmol) and DIEA (35 µL, 0.2 mmol) in DMF (600 µL) overnight. The crude product from cleavage reaction was purified using RP-HPLC.

N-α-4-[fluorosulfonyl]benzoyl-h-α-CGRP(8-37) (6).

The fully protected amino acid sequence of CGRP(8-37) was assembled on MBHA-resin using Boc-amino acid derivatives as described above. A portion of the peptide-resin (100 mg, 0.02 mmol) was flow-washed with DMF (45 sec continuous flow wash), the N-α-Boc group removed with TFA (5 mL, 2 x 1 min), flow-washed with DMF (45 sec continuous flow wash) and reacted with 4-(fluorosulfonyl)benzoyl chloride (45 mg, 0.2 mmol), HCTU (74 mg, 0.18 mmol) and DIEA (35 µL, 0.2 mmol) in DMF (600 µL) for 1 h. The crude product from cleavage reaction was purified using RP-HPLC.

N-α-4-[fluorosulfonyl]benzoyl-[His(benzyl)\(^{10}\)]-h-α-CGRP(8-37) (7).

The fully protected amino acid sequence of CGRP(8-37) was assembled on MBHA-resin using Boc-amino acid derivatives as described above. A portion
of the peptide-resin (300 mg, 0.06 mmol) was benzylated with benzyl bromide (72 μL, 0.6 mmol) and DIEA (105 μL, 0.6 mmol) in DMF (800 μL) for 24 h. The peptide-resin was flow-washed with DMF (45 sec continuous flow wash), the N-α-Boc group removed with TFA (5 mL, 2 x 1 min) and flow-washed with DMF (45 sec continuous flow wash). Then, reacted with 4-(fluorosulfonyl)benzoyl chloride (135 mg, 0.6 mmol), HCTU (241.5 mg, 0.58 mmol) and DIEA (105 μL, 0.6 mmol) in DMF (600 μL) for 1 h. The crude product from cleavage reaction was purified using RP-HPLC.

**Peptide-Resin Cleavage.**

The peptide was cleaved from the resin and freed of side-chain protecting groups using TFMSA. Peptide-resin (200 mg) was suspended in thioanisole:EDT (300 μL, 2:1, v/v) for 10 min. TFA (2 mL) was added with stirring and the reaction mixture was cooled to 0 °C with stirring for 10 min. TFMSA (200 μL) was added dropwise and the reaction mixture was left to stir at room temperature for 2 h. The peptide was precipitated with cold diethyl ether and together with the resin, filtered through a medium porosity sintered-glass funnel. To separate the peptide from the resin the peptide was dissolved in a minimum of TFA (3 x 1 mL), the TFA solution passed through the sintered-glass funnel under suction and dripped directly into the cold diethyl ether (100 mL) present in the Büchner funnel to precipitate the crude product. This was repeated two more times with fresh batches of cold diethyl ether in the flask. The precipitate was removed from the ether by filtration through a fine porosity sintered-glass funnel, dissolved in 5 % aqueous acetic acid and subjected to C_{18} and/or C_{4} RP-HPLC immediately.
Chromatography:

Flash.

Flash chromatography was performed using a 580 mL glass column (5 cm x 30 cm) containing a coarse porosity glass frit (40 – 60 micron) and a 250 mL solvent reservoir. Silica gel (300 g) (230 – 400 mesh, 60Å) was made into a slurry with ethyl acetate (300 mL) and poured into the column in a single portion to give a depth of 12.5 – 15.0 cm. Ethyl acetate was then carefully poured over the silica bed to completely fill the column and solvent reservoir. The solvent reservoir was then equipped with a flow control adaptor connected to a nitrogen gas cylinder, and low pressure was used to remove all the air bubbles from the silica gel. Crude ethyl p-[N,N-bis(2-hydroxyethyl)amino]benzoate was dissolved in 15 mL ethyl acetate and carefully applied by pipette onto the adsorbent bed of the silica gel. The flow controller was placed onto the column to push the sample into the silica gel. Then, 3-5 mL of ethyl acetate was used to wash the column walls and the washings was pushed into the silica gel as before. The column and reservoir was filled with ethyl acetate while not disturbing the adsorbent bed. The flow controller was placed onto the column and the sample was eluted under low pressure. Fractions were collected with 15 mL test tubes and the ethyl p-[N,N-bis(2-hydroxyethyl)amino]benzoate was identified by analytical TLC.

Analytical TLC.

A solvent mixture was identified to develop each sample that gave an Rf value of approximately 0.2. Generally, the sample was dissolved in a minimum amount of chloroform and transferred to TLC plates (2.5 x 5 cm, 250 micron silica
gel GF plates) with a glass micropipette. Plates were put into a chamber pre-saturated with the solvent mixture and developed. The plates were removed from the developing chamber when the solvent front had reached approximately 1 cm from the top of the plate. The solvent front was marked on the plate and the plates were air dried in a fume hood. The chromatogram was visualized under UV light (254 nm) and the sample location identified and marked on the plate. Rf values were calculated by measuring the distance of sample migration from the point of application on the plate, divided by the solvent front distance.

**Semi-preparative RP-HPLC.**

Analytical RP-HPLC, using a Vydac 218TP5415 C18 column (0.46 cm x 15 cm) from Waters (Milford, MA), was first used to determine the percentage of buffer B or buffer D, X % specified for each peptide, that elutes the desired product with a k’ of 4 under isocratic conditions at a flow rate of 1 mL/min. Peptides were purified by semi-preparative RP-HPLC on a Vydac 218TP510 C18 column (1 cm x 25 cm) and/or Vydac 214TP510 C4 column (1 cm x 25 cm) from the Separations Group (Hesperia, CA). Buffers for chromatography were either 100 mM TEAP, pH 2.5 (buffer A) and a mixture of ACN and buffer A (60/40, v/v) (buffer B) or 0.1 % TFA in water (buffer C) and 0.05 % TFA in ACN (buffer D). Generally, the crude cleavage product was dissolved in either buffer A or buffer C and loaded onto the Vydac C18 semi-preparative column that had been previously equilibrated with X-10 % buffer A or buffer C, specified for each peptide, for 15 min. After the unretained material was removed from the column, a shallow gradient, X-10 % to X+10 % buffer B or buffer D, at a flow rate of 7
mL/min was used to elute the peptide. The fractions collected (0.5 min/tube) were analyzed for desired peptide product by analytical RP-HPLC.

**Analytical RP-HPLC.**

Analytical RP-HPLC was used to determine capacity factor values (k') for each peptide, to analyze semi-preparative RP-HPLC fractions for desired peptide product and to determine peptide homogeneity. A Vydac 218TP5415 C\textsubscript{18} column (0.46 cm x 15 cm) was used to determine the percentage of buffer B or buffer D that elutes the desired product with a k' of 4 under isocratic conditions at a flow rate of 1 mL/min with a detection wavelength of 214 nm. Semi-preparative RP-HPLC fractions were analyzed for desired peptide product using a Vydac 218TP5415 C\textsubscript{18} column (0.46 cm x 15 cm) under isocratic conditions, specified for each peptide, with buffer C and buffer D at a flow rate of 1 mL/min and detection wavelength of 214 nm. Peptide homogeneity was determined using a Vydac C\textsubscript{18} monomeric 238TP54 column (250 x 4.6 mm), Waters Symmetry C\textsubscript{18} 300Å column (250 x 4.6 mm) from Waters (Milford, MA) and Kromasil C\textsubscript{8} column (250 x 4.6 mm) from AkzoNobel (Dobbs Ferry, NY) under isocratic conditions at a flow rate of 1 mL/min and detection wavelength of 214 nm.

**Fourier Transform Infrared Spectroscopy.**

Infrared spectroscopy of ethyl \( p-[\text{N,N-bis-(2-hydroxyethyl)amino}]\text{benzoate (1)}, \) \( p-[\text{N,N-bis-(2-chloroethyl)amino}]\text{benzoic acid (2)} \) and \( p-[\text{N,N-bis-(2-chloroethyl)amino}]\text{benzoyl chloride (3)} \) were obtained as KBr pellets on a Nicolet Avatar 360 instrument from Thermo Electron Corp. (Madison, WI).
1H-Nuclear Magnetic Resonance Spectroscopy.

1H-NMR spectra of ethyl $p$-[N,N-bis-(2-hydroxyethyl)amino]benzoate (1), $p$-[N,N-bis-(2-chloroethyl)amino]benzoic acid (2) and $p$-[N,N-bis-(2-chloroethyl)amino]benzoyl chloride (3) in CDCl$_3$ were obtained by Dr. Martin Hulce in the Department of Chemistry on an Inova Unity 300 MHz spectrometer from Varian Inc. (Palo Alto, CA). Proton peak positions are given in parts per million ($\delta$) and $J$ values in hertz.

Gas Chromatography-Mass Spectrometry.

Mass spectrometry of ethyl $p$-[N,N-bis-(2-hydroxyethyl)amino]benzoate (1), $p$-[N,N-bis-(2-chloroethyl)amino]benzoic acid (2) and $p$-[N,N-bis-(2-chloroethyl)amino]benzoyl chloride (3) was performed by Dr. Martin Hulce in the Department of Chemistry using an HP5988A mass spectrometer from Hewlett Packard (Palo Alto, CA).

Melting Point.

Melting points of ethyl $p$-[N,N-bis-(2-hydroxyethyl)amino]benzoate (1), $p$-[N,N-bis-(2-chloroethyl)amino]benzoic acid (2) and $p$-[N,N-bis-(2-chloroethyl)amino]benzoyl chloride (3) were determined on a digital melting point apparatus from Electrothermal Engineering Ltd. (SouthEnd on Sea, UK) and are uncorrected.

Elemental Analysis.

Elemental analyses of ethyl $p$-[N,N-bis-(2-hydroxyethyl)amino]benzoate (1), $p$-[N,N-bis-(2-chloroethyl)amino]benzoic acid (2) and $p$-[N,N-bis-(2-chloroethyl)amino]benzoyl chloride (3) was performed by Atlantic Microlab Inc.
(Norcross, GA) and the results were within ±0.3 % of the theoretical values for C, H, N and Cl.

**Amino Acid Analysis.**

Amino acid analyses were performed on an AccQTag system from Waters (Milford, MA) after peptides were hydrolyzed in 6 M constant boiling HCl under vacuum at 110 °C for 24 h. Amino acid analyses were performed by Dr. Donald Babin in the Department of Biomedical Sciences.

**Electrospray Ionization-Mass Spectrometry.**

Peptides were dissolved in water (1 mg/mL) and filtered through a 0.22 μM membrane. ESI-MS was performed by Eva Lovas in the Department of Biomedical Sciences and performed on an API150EX mass spectrometer from PE-SCIEX (Foster City, CA).

**Thoracic Aorta Relaxation.**

Male albino mice (CF1, 25-35 g) were obtained from Charles River Laboratories (Wilmington, MA). Mice were euthanized using CO₂ and thoracic aortas were removed and placed in Krebs’ solution (composition in mM: NaCl, 118; KCl, 4.7; CaCl₂, 2.5; KH₂PO₄, 1.2; MgSO₄, 1.2; NaHCO₃, 25; dextrose, 11.1; Na₂Ca EDTA, 0.029; pH 7.4). The aorta was cleaned of connective tissue, and the endothelium removed by gentle rubbing of the vessel lumen. The aorta was cut into 3-mm long ring segments which were mounted between two stainless steel pins passed through the vessel lumen and placed in water-jacketed organ baths maintained at 37 °C which contained Krebs’ solution gassed with 95 % O₂/5 % CO₂, pH 7.4. One pin was attached to a Grass FT.03 isometric force
transducer from Grass Instruments (Quincy, MA) for measurement of isometric tension while the other pin was held in a fixed position. Ring segments were equilibrated in Krebs’ solution for 30 min at a resting tension of 300 mg, contracted with 60 mM KCl followed by a 30 min washout with Krebs’ solution. Ring segments were contracted a second time with 60 mM KCl and the presence or absence of endothelium assessed by the ability of the endothelium-dependent vasodilator acetylcholine. Rings segments were then thoroughly washed for 30 min with Krebs’ solution.

All analogues were tested for both agonist and antagonist activity. For agonist studies, antagonists were tested for their ability to change resting tone of aortic ring segments and to alter the tone of aortic ring segments precontracted to a stable level of tone with 1 μM norepinephrine. For antagonist studies, aortic ring segments were sub-maximally contracted with 1 μM norepinephrine for 20 min to obtain a stable amount of contractile tone and cumulative CGRP concentration-response relaxation curves were obtained. Rings were then incubated with 4 or 5 for 15 min or 6 or 7 for 45 min and then the ring segments were thoroughly washed for 45 min. Cumulative concentration-response curves for h-α-CGRP were then repeated.

To test for receptor specificity of antagonism, the same protocol was used except that isoproterenol was employed as the relaxant agonist.

cAMP Assay.

Cells were seeded and grown in Cellstar 24-well plates from Greiner Bio-One Inc. (Longwood, FL) in Dubelco’s Modified Eagles Medium (DMEM)
supplemented with fetal bovine serum (10 %), penicillin G (100 units/mL), streptomycin (100 μg/mL) and amphotericin B (0.25 μg/mL). DMEM culture media was removed and the cells washed 3 times with 200 μL of HEPES-Krebs' buffer composed of (in mM): NaCl, 118; KCl, 4.7; CaCl₂, 2.5; KH₂PO₄, 1.3; MgSO₄, 1.2; NaHCO₃, 15; Dextrose, 11.1; HEPES acid, 12.4; HEPES-Na, 7.5; isobutylmethylxanthine, 0.5; pH 7.4. HEPES-Krebs' buffer (900 μL) was added to each well and the cells incubated for 10 min in a humidified atmosphere (95 % air/5 % CO₂) at 37 °C.

All analogues were tested for both agonist and antagonist activity. For agonist studies, antagonists were tested for their ability to change the basal level of cAMP production in SK-N-MC cells. Analogues were added to wells alone for 15 min, and the cells washed 3 times with 200 μL of HEPES-Krebs' buffer, the plates incubated for 30 min in a humidified atmosphere (95 % air/5 % CO₂) at 37 °C and cAMP formation measured.

To generate CGRP concentration-response curves, different concentrations of CGRP were added to various wells and, the plates incubated for 30 min in a humidified atmosphere (95 % air/5 % CO₂) at 37 °C and cAMP formation measured. Other wells were incubated with a single concentration of 4, 5, 6, or 7 for 15 minutes, washed 3 times with HEPES-Krebs' buffer, followed by addition of various concentrations of CGRP. The plates were incubated for 30 min in a humidified atmosphere (95 % air/5 % CO₂) at 37 °C and cAMP formation measured.
To test for non-specific effects and receptor specificity of the antagonism, the same protocol as described above was used except that either forskolin or isoproterenol, respectively, was used to stimulate cAMP formation.

Data Analysis.

Functional data were analyzed by nonlinear regression curve fitting using Graphpad Prism 4.0 (San Diego, CA). Cumulative concentration-response curves were constructed and EC$_{50}$ values and % maximal responses were calculated using all points on the concentration-response curve. Data are expressed as the mean ± SEM.

RESULTS

N-terminally derivatized peptides used in this study are shown in Figure 30. All peptides were assembled on the solid phase and derivatized with an appropriate benzoyl chloride derivative containing bis-(2-chloroethyl)amino or fluorosulfonyl affinity labels. p-[N,N-bis-(2-chloroethyl)amino]benzoyl chloride was synthesized from benzocaine by modifying a published route (Elderfield and Liao, 1961), outlined in Figure 31, while 4-(fluorosulfonyl)benzoyl chloride was readily available.

Ethyl p-[N,N-bis(2-hydroxyethyl)amino]benzoate (1). Reaction of ethyl p-aminobenzoate with ethylene oxide gave p-[N,N-bis-(2-hydroxyethyl)-amino]benzoate (1) and a previously unreported impurity that was readily removed by flash chromatography on silica. Compound 1 was obtained in an improved overall yield of 82 % and an increased melting point of 96 – 97 °C. The
product from purification yielded 19.9 g (82 %) as clear prisms. m.p. 96 – 97 °C (lit 93 – 94 °C (Elderfield and Liao, 1961)); Rf = 0.24 (ethyl acetate); FTIR (KBr) 3355, 1674 cm⁻¹ (Figure 32); 1H NMR (CDCl₃) δ: 1.4 (t, 3H, J = 8.3, OCH₂CH₃); 3.4 – 4.4 (s, 2H, N(CH₂CH₂OH)₂); 3.7 (t, 4H, J = 3.1, N(CH₂CH₂OH)₂); 3.9 (t, 4H, J = 3.1, N(CH₂CH₂OH)₂); 4.3 (q, 2H, J = 7.8, OCH₂CH₃); 6.8 (d, 2H, J = 10.1, Ph); 7.9 (d, 2H, J = 8.3, Ph) (Figure 33); GC-MS m/z (rel. intensity): 253 (10, M⁺), 222 (100), 208 (10), 150 (20); Anal. (C₁₃H₁₉O₄N); C, H, N (Table 8).

p-[N,N-bis-(2-chloroethyl)amino]benzoic acid (2). Conversion of 1 to p-[N,N-bis-(2-chloroethyl)-amino]benzoic acid (2) using, POCl₃ followed by hydrolysis in concentrated HCl, as previously reported, could not be reproduce in my hands. 2 was obtained from alternative published methods for the chlorination of the aliphatic alcohols of 1 by replacing POCl₃ with SOCl₂ at reflux in pyridine (Fleser et al., 1967), at reflux in benzene with a catalytic amount of ZnCl₂ (Squires et al., 1975) and neat SOCl₂ at reflux which gave 2 in yields of 20 %, 32 % and 39 %, respectively. Ultimately, 2 was obtained in highest yield using SOCl₂ at reflux in benzene (Ross et al., 1955) followed by acidic hydrolysis of the ester to provide an isolated yield of 41 %. The product from purification yielded 3.60 g (41 %) of cream colored needles. m.p. 172 – 173 °C (lit 172 – 173 °C (Elderfield and Liao, 1961)); Rf = 0.38 (chloroform); FTIR (KBr) 2962-2548, 1665 cm⁻¹ (Figure 34); 1H NMR (CDCl₃) δ: 3.7 (t, 4H, J = 4.9, N(CH₂CH₂Cl)₂); 3.9 (t, 4H, J = 4.9, N(CH₂CH₂Cl)₂); 6.7 (d, 2H, J = 6.2, Ph); 8.0 (d, 2H, J = 8.1, Ph); 11.3 (s, 1H, COOH) (Figure 35); GC-MS m/z (rel. intensity): 261 (10, M⁺⁺), 212 (100), 132 (10); Anal. (C₁₁H₁₃NO₂Cl₂); C, H, N, Cl (Table 8).
Conversion of 2 to
the acid chloride with SOCl₂ at reflux in benzene gave p-[N,N-bis(2-chloroethyl)-amino]benzoyl chloride (3) in an isolated yield of 94 %, although the reaction was complete only after 6 h, significantly longer than the 1 h originally reported (Elderfield and Liao, 1961). The product from purification yielded 101 mg (94 %) of white flakes. m.p. 94 – 96 °C (lit 94 – 96 °C (Elderfield and Liao, 1961)); Rᵣ = 0.73 (chloroform/ethyl acetate, 1:1); FTIR (KBr) 1665 cm⁻¹ (Figure 36); 1H NMR (CDCl₃) δ: 3.7 (t, 4H, J = 6.6, N(CH₂CH₂Cl)₂); 3.9 (t, 4H, J = 6.6, N(CH₂CH₂Cl)₂); 6.7 (d, 2H, J = 9.3, Ph); 8.0 (d, 2H, J = 9.3, Ph) (Figure 37); GC-MS m/z (rel. intensity): 279 (10, M⁺), 240 (100), 212 (15); Anal. (C₁₁H₁₂NOCI₃); C, H, N, Cl (Table 8).

Peptide Synthesis. Figure 38 depicts assembly of irreversible CGRP receptor antagonists 4 – 7 on the polystyrene resin. Briefly, the amino acid sequence of h-α-CGRP(8-37) was assembled on MBHA-resin using our previously published methods (Taylor et al., 2005) employing Boc-amino acid derivatives and in situ neutralization (Schnolzer et al., 1992). For analogues 4 and 6, after the N-terminal Boc group was removed the N-terminus was acylated with p-[N,N-bis-(2-chloroethyl)amino]benzoyl chloride or 4-(fluorosulfonyl)benzoyl chloride, respectively. For analogues 5 and 7, prior to removal of the N-terminal Boc group, the histidine imidazole side-chain, at position 10, of the resin bound peptide was benzylated at C4 using benzyl bromide (Smith et al., 2003), then the N-terminal Boc group was removed and the N-terminus was acylated with p-[N,N-bis-(2-chloroethyl)amino]benzoyl chloride or 4-(fluorosulfonyl)benzoyl
chloride, respectively. Peptides were cleaved from the resin and simultaneously freed of side-chain protecting groups using trifluoromethanesulfonic acid:trifluoroacetic acid (TFMSA:TFA) in the presence of thiol scavengers (Tam et al., 1986) and immediately purified by semi-preparative reversed-phase (RP) high performance liquid chromatography (HPLC). Peptide purity was assessed to be >98% by analytical RP-HPLC under isocratic elution conditions using three different RP-HPLC columns. Purified products had correct masses as determined by electrospray ionization-mass spectrometry (Table 9) and satisfactory amino acid composition (Table 10). No histidyl residues were found in the amino acid composition of analogues 5 and 7, which is consistent with irreversible benzylolation of these analogues in position 10 (Smith et al., 2003).

\[ \text{N-}\alpha-4-[\text{bis-(2-chloroethyl)amino]}\text{benzoyl-h-}\alpha\text{-CGRP(8-37)} \]  (4). The crude product from cleavage was loaded onto a \( C_{18} \) RP-HPLC column previously equilibrated with a mixture of buffer A (70%) and buffer B (30%). The product was eluted by raising the concentration of buffer B from 30% to 60% over 75 min. Fractions containing the desired product were identified by analytical RP-HPLC, pooled and loaded onto the same column previously equilibrated with a mixture of buffer C (78%) and buffer D (22%). The product was eluted by raising the concentration of buffer D from 22% to 52% over 75 min. Fractions were detected isocratically using a mixture of buffer C (67%) and buffer D (33%) by analytical RP-HPLC. Fractions containing only the desired product were pooled and lyophilized to yield 2.6 mg (4%) of analogue 4 as a fluffy white powder. The
The results of ESI-MS and amino acid analysis are shown in Table 9 and Table 10. The k' values are shown in Table 11.

N-α-4-[bis-(2-chloroethyl)amino]benzoyl-[His(4-benzyl)\textsuperscript{10}]-h-α-CGRP(8-37) (5). The crude product from cleavage was loaded onto a C\textsubscript{18} RP-HPLC column previously equilibrated with a mixture of buffer A (77 %) and buffer B (23 %). The product was eluted by raising the concentration of buffer B from 23 % to 68 % over 112 min. Fractions containing the desired product were identified by analytical RP-HPLC, pooled and loaded onto the same column previously equilibrated with a mixture of buffer C (83 %) and buffer D (17 %). The product was eluted by raising the concentration of buffer D from 17 % to 51 % over 150 min. Fractions containing only the desired product were pooled and lyophilized to yield 15 mg (22 %) of analogue 5 as a fluffy white powder. The results of ESI-MS and amino acid analysis are shown in Table 9 and Table 10. The k' values are shown in Table 11.

N-α-4-[fluorosulfon]benzoyl-h-α-CGRP(8-37) (6). The crude product from cleavage was loaded onto a C\textsubscript{18} RP-HPLC column previously equilibrated with a mixture of buffer C (82 %) and buffer D (18 %). The product was eluted by raising the concentration of buffer B from 18 % to 48 % over 112 min. Fractions containing the desired product were identified by analytical RP-HPLC, pooled and loaded onto a C\textsubscript{4} column previously equilibrated with a mixture of buffer C (82 %) in buffer D (18 %). The product was eluted by raising the concentration of buffer D from 18 % to 48 % over 112 min. Fractions were detected isocratically...
using a mixture of buffer C (69 %) and buffer D (31 %) by analytical RP-HPLC. Fractions containing only the desired product were pooled and lyophilized to yield 2.2 mg (3 %) of analogue 6 as a fluffy white powder. The results of ESI-MS and amino acid analysis are shown in Table 9 and Table 10. The k’ values are shown in Table 11.

N-α-4-[fluorosulfonyl]benzoyl-[His(4-benzy1)]10-h-α-CGRP(8-37) (7). The crude product from reaction was loaded onto a C18 RP-HPLC column previously equilibrated with a mixture of buffer C (82 %) and buffer D (18 %). The product was eluted by raising the concentration of buffer B from 18 % to 48 % over 112 min. Fractions containing the desired product were identified by analytical RP-HPLC, pooled and loaded onto a C4 column previously equilibrated with a mixture of buffer C (75 %) and buffer D (25 %). The product was eluted by raising the concentration of buffer D from 25 % to 45 % over 75 min. Fractions were detected isocratically using a mixture of buffer C (67 %) and buffer D (33 %) by analytical RP-HPLC. Fractions containing only the desired product were pooled and lyophilized to yield 5.1 mg (3 %) of analogue 7 as a fluffy white powder. The results of ESI-MS and amino acid analysis are shown in Table 9 and Table 10. The k’ values are shown in Table 11.

Functional Effects of N-Derivatized Analogues (4 – 7). CGRP plays an important role in the cardiovascular system as a neurotransmitter and neuromodulator. CGRP potently relaxes many blood vessels and is thought to regulate peripheral vascular tone and regional organ blood flow (Brain et al., 1985). The mouse thoracic aorta has been established as a sensitive system to
study the vasodilatory effects of CGRP (Pomerleau et al., 1997; Chan and Fiscus, 2001). Human SK-N-MC cells are an established model system for the study of CGRP receptors. CGRP receptors are positively coupled to the Gs protein, which stimulates adenylyl cyclase leading to increases in 3',5' cyclic adenosine monophosphate (cAMP) (Van Valen et al., 1990). Therefore, I used the mouse thoracic aorta and the human SK-N-MC cell line to examine the effect of the analogues 4 – 7 on CGRP-mediated vasodilation and cAMP production, respectively.

To determine if the analogues specifically interact with CGRP receptors I evaluated the effects of 4 – 7 on a receptor, other than CGRP receptors, coupled to vascular relaxation and cAMP production. Receptor specificity was established by their inability to antagonize ß-adrenergic receptor-mediated relaxation of pre-contracted mouse thoracic aorta and cAMP production in SK-N-MC cells. There was no significant difference in isoproterenol EC50 values after incubation with 4–7 (Table 12). The analogues also had no effect on ß-adrenergic receptor-mediated cAMP production in SK-N-MC cells. Sub-maximal isoproterenol-stimulated cAMP production was not blocked by incubation with analogues 4–7 (Figure 39).

4–7 had no effect on the basal level of contractile tone and did not relax pre-contracted thoracic aorta or stimulate cAMP production in SK-N-MC cells when used in concentrations up to 1 µM (Figure 39).

Analogues 4–7 were subsequently evaluated for their ability to irreversibly antagonize CGRP receptor-mediated responses at mouse and human CGRP
receptors in the thoracic aorta and SK-N-MC cell line, respectively. For these experiments, tissues or cells were incubated with analogues 4–7 for various time periods, followed by thorough washing to remove unbound antagonist. Antagonist incubation times were established by time course experiments (Figure 40). For functional assays using aortic rings, the rings were incubated for 15 minutes with analogues 4 and 5 or incubated for 45 minutes with analogues 6 and 7. SK-N-MC cells were incubated with analogues 4–7 for 15 minutes.

Analogues 4–7 displayed time and concentration-dependent irreversible antagonism. The data summarized in Table 13 showed that analogues containing the (bis-(2-chloroethyl)amino) (4, 5) and fluorosulfonyl (6, 7) alkylating moieties reduced the maximal efficacy of CGRP, which suggests that these compounds are antagonizing CGRP-mediated responses in an irreversible manner.

N-α-4-[bis-(2-chloroethyl)amino]benzoyl-h-α-CGRP(8-37) (4) and N-α-4-[bis-(2-chloroethyl)amino]benzoyl-[His(4-benzyl)10]-h-α-CGRP(8-37) (5). As described previously, the mouse thoracic aorta is a highly sensitive system to study the actions of CGRP. Previously it was shown that CGRP potently relaxes the pre-contracted mouse thoracic aorta with EC$_{50}$ values of 12.8 ± 1.4 nM (Pomerleau et al., 1997) and 8.1 ± 0.2 nM (Chan and Fiscus, 2001). In agreement with these studies, CGRP produced concentration-dependent and complete relaxation of the pre-contracted thoracic aorta with an EC$_{50}$ value of 10.8 ± 0.8 nM in my experiments (Table 13). In aortas, in the presence of 4 and 5, the potency of CGRP was reduced by 2-fold and 5-fold, respectively.
Furthermore, 4 and 5 demonstrated irreversible antagonist properties by inhibiting the ability of CGRP to completely relax the aortic rings back to the baseline level of tone at a concentration of 1.5 μM with a percent inhibition of 36.7 ± 6.4 % and 62.2 ± 1.8 %, respectively (Figure 41).

In SK-N-MC cells, CGRP caused a concentration-dependent stimulation of cAMP production with an EC\textsubscript{50} value of 8.7 ± 2.2 nM (Table 13). Incubation with both analogues for 15 min, followed by removal of the analogues by washing, caused a reduction in maximal CGRP efficacy. Treatment of SK-N-MC cells with 1.5 μM of 4 and 1 μM of 5 reduced the potency of CGRP by 2-fold and 5-fold, respectively. Furthermore, in the presence of 4 and 5 maximal CGRP-mediated cAMP production was reduced by 45.2 ± 3.1 % and 19.2 ± 5.4 %, respectively (Figure 42).

Incubation with analogues 4 and 5, followed by removal and thorough washing of the tissue or cells, caused a dose-dependent reduction in maximal CGRP-mediated relaxation. In particular, analogue 5, having a benzyl moiety on C4 of the imidazole side-chain of His\textsuperscript{10}, inhibited maximal CGRP-mediated relaxation the most.

N-α-4-[fluorosulfonyl]benzoyl-h-α-CGRP(8-37) (6) and N-α-4-[fluorosulfonyl]benzoyl-[His(4-benzyl)\textsuperscript{10}]-h-α-CGRP(8-37) (7). In aortas, in the presence of 6 and 7, the potency of CGRP was unaffected by 6 whereas 7 caused a 5-fold reduction in the potency of CGRP. Furthermore, 6 and 7 demonstrated irreversible antagonist properties by inhibiting the ability of CGRP to completely relax the aortic rings back to the baseline level of tone at a
concentration of 1.5 μM with a percent inhibition of 20.5 ± 1.4 % and 21.2 ± 1.8 %, respectively (Figure 41).

In SK-N-MC cells, CGRP caused a concentration-dependent stimulation of cAMP production with an EC$_{50}$ value of 8.7 ± 2.2 nM (Table 13). Incubation with both analogues for 15 min, followed by removal of the analogues by washing, caused a reduction in maximal CGRP efficacy. Treatment of SK-N-MC cells with 1.5 μM of 7 reduced the potency of CGRP by 5-fold whereas the potency of CGRP was unaffected by 1.5 μM of 6. Furthermore, in the presence of 6 and 7, maximal CGRP-mediated cAMP production was reduced by 29.3 ± 3.8 % and 39.0 ± 7.0 %, respectively (Figure 42).

Incubation of aortic rings and SK-N-MC cells with 6 and 7, followed by removal and thorough washing of the tissue, caused a decrease in maximal CGRP-mediated responses. These analogues were less potent than 4 and 5 and the decrease in maximal CGRP-mediated response was less in both assays. Addition of the benzyl moiety to C4 of the imidazole side-chain of His$^{10}$ (7) did not produce a statistically significant increase in inhibition of maximal CGRP-mediated relaxation as compared to the unmodified His$^{10}$ analogue (6).
Figure 30. Structures of high affinity and irreversible antagonists, analogues 4, 5, 6 and 7 respectively.
Figure 31. Synthesis of \( p-[N,N\text{-bis-(2-chloroethyl)amino}]\text{benzoyl chloride (3).} \)
Figure 32. FTIR spectrum of ethyl $p$-[N,N-bis(2-hydroxyethyl)amino]benzoate (1).
Figure 33. 1H-NMR spectrum of ethyl $p$-[N,N-bis(2-hydroxyethyl)amino]benzoate (1).
Figure 34. FTIR spectrum of \(p\)-[N,N-bis-(2-chloroethyl)amino]benzoic acid (2).
Figure 35. 1H-NMR spectrum of \( p-[N,N\text{-bis-(2-chloroethyl)amino}]\text{benzoic acid (2).} \)
Figure 36. FTIR spectrum of $p$-[N,N-bis-(2-chloroethyl)amino]benzoyl chloride (3).
Figure 37. 1H-NMR spectrum of p-[N,N-bis-(2-chloroethyl)amino]benzoyl chloride (3).
Table 8. Elemental analysis of ethyl $p$-[N,N-bis-(2-hydroxyethyl)amino]benzoate (1), $p$-[N,N-bis-(2-chloroethyl)amino]benzoic acid (2) and $p$-[N,N-bis-(2-chloroethyl)amino]benzoyl chloride (3).

<table>
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<tr>
<th>Compound #</th>
<th>Compound</th>
<th>C</th>
<th>H</th>
<th>N</th>
<th>Cl</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$p$-[N,N-bis-(2-hydroxyethyl)amino]benzoate</td>
<td>61.8 (61.7)</td>
<td>7.6 (7.6)</td>
<td>5.6 (5.5)</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>$p$-[N,N-bis-(2-chloroethyl)amino]benzoic acid</td>
<td>50.6 (50.6)</td>
<td>5.0 (5.0)</td>
<td>5.4 (5.4)</td>
<td>27.0 (27.2)</td>
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<tr>
<td>3</td>
<td>$p$-[N,N-bis-(2-chloroethyl)amino]benzoyl chloride</td>
<td>47.2 (47.1)</td>
<td>4.4 (4.3)</td>
<td>4.8 (5.0)</td>
<td>36.2 (37.9)</td>
</tr>
</tbody>
</table>

*Theoretical values are shown in parentheses.*
Figure 38. Synthesis of irreversible CGRP receptor antagonists 4 – 7. Rapid SPPS protocol employing in situ neutralization for the synthesis of CGRP(8-37), benzylation of the imidazole side-chain of His at position 10 and coupling of [bis-(2-chloroethyl)amino]benzoyl chloride and 4-(fluorosulfonfyl)benzoyl chloride to the N-terminus of resin-bound side-chain protected sequences of CGRP(8-37) and [His(4-benzyl)-10]-CGRP(8-37).
Table 9. Observed masses of N-terminally and His^{10} modified analogues of h-\(\alpha\)-CGRP(8-37).

<table>
<thead>
<tr>
<th>Analogue #</th>
<th>Analogue</th>
<th>ESI-MS (amu)</th>
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<th>Calculated</th>
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<td>4</td>
<td>N-(\alpha)-4-[bis-(2-chloroethyl)amino]benzoyl-h-(\alpha)-CGRP(8-37)</td>
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<td>3369.7</td>
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<td>5</td>
<td>N-(\alpha)-4-[bis-(2-chloroethyl)amino]benzoyl-[His(4-benzyl)^{10}]-h-(\alpha)-CGRP(8-37)</td>
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<td>3459.8</td>
</tr>
<tr>
<td>6</td>
<td>N-(\alpha)-4-[fluorosulfonyl]benzoyl-h-(\alpha)-CGRP(8-37)</td>
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<td>3311.7</td>
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<tr>
<td>7</td>
<td>N-(\alpha)-4-[fluorosulfonyl]benzoyl-[His(4-benzyl)^{10}]-h-(\alpha)-CGRP(8-37)</td>
<td></td>
<td>3401.7</td>
<td>3401.9</td>
</tr>
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Table 10. Amino acid composition of N-terminally and His^{10} modified analogues of h-α-CGRP(8-37)^{a}.

<table>
<thead>
<tr>
<th>Analogue #</th>
<th>Asp</th>
<th>Ser</th>
<th>Gly</th>
<th>His^{b}</th>
<th>Arg</th>
<th>Thr</th>
<th>Ala</th>
<th>Pro</th>
<th>Val^{c}</th>
<th>Lys</th>
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<tr>
<td>4</td>
<td>3.17(3)</td>
<td>3.09(3)</td>
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<td>4.13(5)</td>
<td>1.98(2)</td>
<td>3.03(3)</td>
<td>2.05(2)</td>
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<td>2.91(3)</td>
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<td>3.85(4)</td>
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<td>1.93(2)</td>
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<td>2.05(2)</td>
<td>1.16(1)</td>
<td>3.63(5)</td>
<td>2.06(2)</td>
<td>2.95(3)</td>
<td>2.08(2)</td>
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<tr>
<td>6</td>
<td>3.12(3)</td>
<td>2.93(3)</td>
<td>4.09(4)</td>
<td>1.07(1)</td>
<td>1.86(2)</td>
<td>1.89(2)</td>
<td>1.93(2)</td>
<td>1.11(1)</td>
<td>4.31(5)</td>
<td>1.90(2)</td>
<td>3.02(3)</td>
<td>1.97(2)</td>
</tr>
<tr>
<td>7</td>
<td>3.19(3)</td>
<td>2.89(3)</td>
<td>4.17(4)</td>
<td>0.03(1)</td>
<td>1.83(2)</td>
<td>2.04(2)</td>
<td>2.20(2)</td>
<td>1.18(1)</td>
<td>4.00(5)</td>
<td>2.05(2)</td>
<td>3.20(3)</td>
<td>2.18(2)</td>
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</tbody>
</table>

^{a} Unnatural amino acids were not determined. Theoretical values shown in parentheses.

^{b} Benzylation of His^{10} results in loss from amino acid composition.

^{c} N-benzoyl-Val^{8} peptide bond is resistant to hydrolysis.
Table 11. k' values for N-terminally and His$^{10}$ modified analogues of h-α-CGRP(8-37)$^a$.

<table>
<thead>
<tr>
<th>#</th>
<th>Analogue</th>
<th>System 1</th>
<th>System 2</th>
<th>System 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>N-α-4-[bis-(2-chloroethyl)amino]benzoyl-h-α-CGRP(8-37)</td>
<td>3.3</td>
<td>5.5</td>
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<td>5</td>
<td>N-α-4-[bis-(2-chloroethyl)amino]benzoyl-[His(4-benzyl)$^{10}$]-h-α-CGRP(8-37)</td>
<td>9.4</td>
<td>5.3</td>
<td>7.4</td>
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<tr>
<td>6</td>
<td>N-α-4-[fluorosulfonyl]benzoyl-h-α-CGRP(8-37)</td>
<td>1.5</td>
<td>1.8</td>
<td>2.9</td>
</tr>
<tr>
<td>7</td>
<td>N-α-4-[fluorosulfonyl]benzoyl-[His(4-benzyl)$^{10}$]-h-α-CGRP(8-37)</td>
<td>2.8</td>
<td>5.5</td>
<td>3.0</td>
</tr>
</tbody>
</table>

$^a$ Columns used were System 1, Vydac C$_{18}$ monomeric 238TP54 column (250 x 4.6 mm); System 2, Waters Symmetry C$_{18}$ 300Å column (250 x 4.6 mm); System 3, Kromasil C$_{8}$ column (250 x 4.6 mm).

$^b$ Peptides were eluted under isocratic conditions using 0.1 % TFA in water/acetonitrile 69/31 (v/v) at a flow rate of 1 mL/min and detected at 214nm.
Table 12. Specificity of N-terminally and His\textsuperscript{10} modified analogues of h-\(\alpha\)-CGRP(8-37). Effect of antagonist on isoproterenol-mediated relaxation of the mouse thoracic aorta.

<table>
<thead>
<tr>
<th>#</th>
<th>Analogue</th>
<th>Thoracic Aorta EC\textsubscript{50} ((\mu)M)\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>N-(\alpha)-4-[bis-(2-chloroethyl)amino]benzoyl-h-(\alpha)-CGRP(8-37)</td>
<td>21.7 ± 12.2</td>
</tr>
<tr>
<td>5</td>
<td>N-(\alpha)-4-[bis-(2-chloroethyl)amino]benzoyl-[His(4-benzyl)\textsuperscript{10}]-h-(\alpha)-CGRP(8-37)</td>
<td>32.6 ± 8.1</td>
</tr>
<tr>
<td>6</td>
<td>N-(\alpha)-4-[fluorosulfonyl]benzoyl-h-(\alpha)-CGRP(8-37)</td>
<td>21.0 ± 4.6</td>
</tr>
<tr>
<td>7</td>
<td>N-(\alpha)-4-[fluorosulfonyl]benzoyl-[His(4-benzyl)\textsuperscript{10}]-h-(\alpha)-CGRP(8-37)</td>
<td>48.8 ± 13.3</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Each value is the mean ± S.E.M. of four experiments using individual aortas, each taken from different animals.
Figure 39. Effect of analogues on basal, forskolin-stimulated (300 nM) and isoproterenol-stimulated (3 nM) cAMP production in SK-N-MC cells.
Figure 40. Time course experiments to establish incubation times for analogues 4 – 7. CGRP concentration-response curves for relaxation of mouse thoracic aorta before and after treatment with N-terminal and His\textsuperscript{10} modified irreversible analogues of h-\textalpha-CGRP(8-37) for various times. Aortic rings were pre-contracted with norepinephrine and CGRP concentration-response curves were obtained. The rings were then incubated with the irreversible analogues for 15, 30 45 or 180 min followed by removal of unbound analogue by washing and CGRP concentration-response curves were repeated. Points are the mean ± S.E.M. of responses of one to six experiments using individual thoracic aortas, each taken from different animals. \textbf{Panel A)} 0.5 \textmu M 4; \textbf{Panel B)} 0.5 \textmu M 5; \textbf{Panel C)} 1.5 \textmu M 6; \textbf{Panel D)} 1.5 \textmu M 7.
Figure 41. CGRP concentration-response curves for relaxation of mouse thoracic aorta before and after treatment with N-terminal and His\textsuperscript{10} modified irreversible analogues of h-\textalpha-CGRP(8-37). Aortic rings were pre-contracted with norepinephrine and CGRP concentration-response curves were obtained. The rings were then incubated with irreversible analogues 4 or 5 for 15 min or 6 or 7 for 45 min, followed by removal of unbound analogue by washing and CGRP concentration-response curves were repeated. Points are the mean ± SEM of responses of four to six experiments using individual thoracic aortas, each taken from different animals.
Figure 42. CGRP concentration-response curves for CGRP-mediated cAMP production in SK-N-MC cells before and after treatment with N-terminal and His\textsuperscript{10} modified irreversible analogues of h-\textalpha-CGRP(8-37). Untreated control cells were stimulated with CGRP and cAMP production was measured. Treated cells were incubated with an irreversible analogue for 15 minutes followed by removal of unbound analogue by washing. Cells were stimulated with CGRP and cAMP production was measured. Points are the mean ± S.E.M. of responses of three individual experiments performed in duplicate, with each experiment using cells grown in different culture plates on different days.
Table 13. Irreversible antagonism of CGRP-mediated functional responses by analogues 4 – 7.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CGRP-Mediated Relaxation(^a)</th>
<th>CGRP-Mediated cAMP Production(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC(_{50}) (nM)</td>
<td>Inhibition (% Control)</td>
</tr>
<tr>
<td>Control</td>
<td>10.8 ± 0.8</td>
<td>−</td>
</tr>
<tr>
<td>4 N-α-4-[bis-2-chloroethyl]amino]benzoyl-h-α-CGRP(8-37)</td>
<td>97.9 ± 23.4</td>
<td>36.7 ± 6.4</td>
</tr>
<tr>
<td>5 N-α-4-[bis-(2-chloroethyl]amino]benzoyl-[His(4-benzyl)(^{10})]-h-α-CGRP(8-37)</td>
<td>22.8 ± 3.1</td>
<td>62.2 ± 1.8</td>
</tr>
<tr>
<td>6 N-α-4-[fluorosulfanyl]benzoyl-h-α-CGRP(8-37)</td>
<td>8.4 ± 1.0</td>
<td>20.5 ± 1.4</td>
</tr>
<tr>
<td>7 N-α-4-[fluorosulfanyl]benzoyl-[His(4-benzyl)(^{10})]-h-α-CGRP(8-37)</td>
<td>11.5 ± 3.1</td>
<td>21.2 ± 1.8</td>
</tr>
</tbody>
</table>

\(^{a}\)n = 4 – 6.

\(^{b}\)n = 3, performed in duplicate. Data are expressed as the mean ± S.E.M.
DISCUSSION

Previous structure activity studies have identified the amino terminus of CGRP(8-37) as a principal site for derivatization to significantly increase antagonist affinity for CGRP receptors and produce significantly more potent competitive antagonists. Derivatization of the amino terminus of CGRP(8-37) with acetyl and benzyl groups resulted in 5- and 10-fold increases in potency, respectively, while the most favorable modification was a benzoyl group which increased the potency 53-fold (Smith et al., 2003). We also determined that the imidazole side-chain of His at position 10 is intimately involved in CGRP(8-37) binding to the CGRP receptor. Benzylating the N-terminus and His$^{10}$ resulted in a significant increase in affinity and potency over N-terminal benzylation alone. These studies have identified the bulky hydrophobic benzoyl group on the N-terminus as well as the benzyl group on the His$^{10}$ side-chain as favorable structures for increased binding affinity and potency.

Irreversible receptor antagonists are important tools that permit the study of receptor structure and functions, including ligand binding site mapping, receptor occupancy response relationships and physiological functions.

N-α-benzoyl-h-α-CGRP(8-37) was used as a lead structure to synthesize novel analogues containing electrophilic moieties incorporated at the 4-position of the N-terminal benzoyl group. Incorporation of such electrophilic moieties at the N-terminus of N-α-benzoyl-h-α-CGRP(8-37), with and without the imidazole side-chain of His$^{10}$ benzylated, resulted in irreversible antagonism. Preincubation of isolated mouse thoracic aorta and SK-N-MC cells with micromolar
concentrations of 4, 5, 6 and 7 followed by extensive washing produced inhibition of maximal CGRP-mediated relaxation (Figure 41) and CGRP-mediated cAMP production (Figure 42). The extended incubation time required for 6 and 7 suggests that the fluorosulfonyl group is less effective at forming covalent bonds with nucleophilic groups within CGRP receptors than the BAM group present in 4 and 5. These studies suggest that CGRP receptors are covalently modified by the analogues and CGRP is unable to illicit a maximal response due to a decrease in the number of functional CGRP receptors.

In agreement with our previous studies using high affinity competitive antagonists, where benzylation of the His\textsuperscript{10} side-chain increased the affinity of N-benzyl-CGRP(8-37), these studies provide further evidence for the role of His\textsuperscript{10} side-chain benzylation in providing a favorable interaction with the active site of the receptor. The observed increase in potency and increase in the reduction of maximal CGRP efficacy by addition of the bulky hydrophobic benzyl group to C4 of the His\textsuperscript{10} imidazole side-chain of the analogue possessing a bis-(2-chloroethyl)amino group but not observed with the analogue possessing a fluorosulfonyl group is consistent with these analogues interacting with the receptor in a dissimilar manner. The structure activity studies of position 10 suggests that analogues containing the bis-(2-chloroethyl)amino group adopt a conformation comparable with our previously characterized high affinity competitive antagonists for binding to the active site of CGRP receptors. The potency and reduction in maximal CGRP efficacy was not affected by His\textsuperscript{10} benzylation of analogues bearing the fluorosulfonyl group, which suggests that
these analogues either adopt a different conformation than the high affinity competitive antagonists for binding to the active site or bind to CGRP receptors at a different location, distinct from the active site.

CGRP1 receptors are comprised of two proteins, the calcitonin-like receptor protein (CL) and an accessory protein termed receptor activity-modifying protein 1 (RAMP1). Formation of a 1:1 heterodimer between CL and RAMP1 is required to form a functional CGRP receptor as well as for receptor expression (McLatchie et al., 1998). The exact mechanism of how these proteins heterodimerize and form the binding pocket for CGRP remains largely unknown. Cross-linking studies have demonstrated that $^{125}$I-CGRP cross-links the CL-RAMP1 complex suggesting both proteins may participate in forming the binding pocket or RAMP1 allosterically modifies the conformation of CL, enabling different ligand-binding states (Hilairet et al., 2001). Portions of the N-terminal domains of both CL and RAMP1 are directly involved in conferring high affinity CGRP binding and in maintaining CGRP potency and efficacy (Koller et al., 2002; Fitzsimmons et al., 2003; Kuwasako et al., 2003; Chauhan et al., 2005; Ittner et al., 2005). Based on the nucleophile selectivity between the bis-(2-chloroethyl)amino and fluorosulfonyl alkylating groups, analogues 4 and 5 bind to a nitrogen nucleophile while analogues 6 and 7 may bind to an NH₂, SH or OH nucleophile within the active site, formed by the association of the N-terminii of CL and RAMP1. There are multiple Arg, Ser, Thr and Lys residues in the N-terminii of CL and RAMP1 to which these irreversible antagonists could covalently bind. Alternatively, if analogues 6 and 7 are alkylating CL at an
allosteric site, there are multiple Ser or Thr residues in transmembrane exoloops 1, 2, 4 and 5, as well as the N-terminii of CL or RAMP1 that are possible alkylation sites.
SUMMARY AND CONCLUSIONS

SUMMARY

The overall goal of my research was to design, synthesize and pharmacologically characterize novel CGRP receptor antagonists. I was interested in the development of irreversible antagonists because they permit the investigation of receptor structure and function such as ligand binding site mapping, receptor occupancy response relationships and physiological functions. Thus, the development of irreversible CGRP receptor antagonists would provide novel pharmacological tools to characterize the interaction of CGRP with its receptors \textit{in vitro} and investigate the physiological roles of CGRP \textit{in vivo}. Also, I was interested in the development of competitive antagonists that are selective for human receptors because they would provide novel tools to investigate CGRP receptors in human tissues and the consequence of structural disparity in the binding pocket of the CGRP receptor of different species.

This was accomplished using organic synthesis, solid phase peptide synthesis, multiple chromatography methods for the purification of reaction intermediates and peptide analogues, multiple techniques to structurally characterize reaction intermediates and peptide analogues and functional assays to pharmacologically characterize the effects of the novel antagonists on CGRP.
receptor-mediated responses in the mouse thoracic aorta and human SK-N-MC cell line.
CONCLUSIONS

In Chapter 2, I investigated solvent effects during the synthesis of CGRP(8-37), which revealed a correlation between solvation of the resin-bound side-chain protected peptide, solvent properties and coupling yields. Solvation of (para-methylbenzhydrylamine)copoly(styrene-1 % DVB) (resin) and resin covalently bound to the fully protected amino acid sequence of CGRP(8-37) (peptide-resin) was correlated to solvent Hildebrand solubility (δ) and hydrogen-bonding (δ_h) parameters. Contour solvation plots of δ_h versus δ revealed maximum solvation regions of resin and peptide-resin. Maximum resin solvation occurred with NMP, NMP:DMSO (8:2) and DMSO. Inefficient solvation of the peptide-resin occurred with these solvents and resulted in poor syntheses with unacceptable average coupling yields. Superior peptide-resin solvation was obtained using DMA and DMF, which resulted in significantly higher average coupling yields. The region of maximum peptide-resin solvation shifted to solvents with higher δ_h values. DMF provided the most effective peptide-resin solvation and was the only solvent from which CGRP(8-37) was obtained as a single major product in the crude cleaved material.

In Chapter 3, I synthesized p-[N,N-bis-(2-chloroethyl)amino]benzoyl chloride and synthesized and structurally characterized four analogues containing bis-(2-chloroethyl)amino or fluorosulfonyl moiety incorporated at the para-position of the phenyl ring of N-α-benzoyl-h-α-CGRP(8-37) and N-α-benzoyl-[His(4-benzyl)^10]-h-α-CGRP(8-37). Subsequently, I used functional
assays to pharmacologically characterize the analogues and determine if they acted in an irreversible manner.

*p-[N,N-bis-(2-chloroethyl)amino]benzoyl chloride was synthesized in a three-step reaction, purifying and structurally characterizing reaction intermediates and the final product. Reaction of ethyl p-aminobenzoate with ethylene oxide, as described by Elderfield and Liao, gave *p-[N,N-bis(2-hydroxyethyl)-amino]benzoate (1) and a previously unreported impurity. The impurity was removed by flash chromatography on silica and 1 was obtained in a significantly improved overall yield of 81 %. Contrary to the published method, conversion of 1 to *p-[N,N-bis-(2-chloroethyl)amino]benzoic acid (2) using POCI$_3$ followed by hydrolysis in concentrated HCl could not be reproduced. Therefore, 2 was obtained from alternate published methods for the chlorination of the aliphatic alcohols by replacing POCI$_3$ with SOCl$_2$ at reflux in pyridine, at reflux in benzene with catalytic amount of ZnCl$_2$ and neat SOCl$_2$ at reflux which gave 2 in low yields of 20, 32 and 39 %, respectively. Ultimately, 2 was obtained in highest yield, using SOCl$_2$ at reflux in benzene followed by acid hydrolysis of the ester in an isolated yield of 41 %. Also, contrary to the published method, conversion of 2 to *p-[N,N-bis(2-chloroethyl)amino]benzoyl chloride (3) using SOCl$_2$ at reflux in benzene was incomplete after 1 h and gave 3 in poor yield. The reaction was monitored by TLC and determined to be complete after 6 h and 3 was isolated in an improved yield of 94 %.

Functional studies confirmed that derivatization of 1-α-benzoyl-h-α-CGRP(8-37) and 1-α-benzoyl-[His(4-benzyl)$_{10}$]-h-α-CGRP(8-37) at the para-
position of the phenyl ring with bis-(2-chloroethyl)amino or fluorosulfonyl moieties produced potent irreversible antagonism of CGRP receptors. The bis-(2-chloroethyl)amino-modified analogues were more potent irreversible antagonists than fluorosulfonyl-modified analogues. Benzylation of the His\textsuperscript{10} side-chain increased the potency of the bis-(2-chloroethyl)amino-modified irreversible antagonists but had no effect on the potency of fluorosulfonyl-modified irreversible antagonists. The most potent irreversible antagonist was, N-\textalpha-4-[bis-(2-chloroethyl)amino]benzoyl-[His(4-benzyl)\textsuperscript{10}]-\textalpha-CGRP(8-37) (5).

These structure activity studies describe the addition of electrophilic moieties to high affinity CGRP antagonists which resulted in the first irreversible antagonists of CGRP receptors. Future studies may determine amino acid side-chains to which these irreversible antagonists are forming a covalent bond in the active site or allosteric sites on the CGRP receptor. These selective irreversible antagonists provide novel tools to identify structural requirements of antagonists for CGRP receptor interaction and may prove invaluable for fully characterizing the actions of CGRP in cells organs and whole animals.

The mouse CL shares 90 % amino acid residues with human CL while mouse RAMP1 shares only 71 % amino acid residues with human RAMP1. Most of the amino acid sequence dissimilarity resides in the extracellular domain of RAMP1 which has been shown to modulate the affinity of non-peptide antagonists. In Chapter 4, based on the amino acid composition and analysis of hydropathy plots of human and mouse CL and RAMP1, I hypothesized that
position of the phenyl ring with bis-(2-chloroethyl)amino or fluorosulfonyl moieties produced potent irreversible antagonism of CGRP receptors. The bis-(2-chloroethyl)amino-modified analogues were more potent irreversible antagonists than fluorosulfonyl-modified analogues. Benzylation of the His\(^{10}\) side-chain increased the potency of the bis-(2-chloroethyl)amino-modified irreversible antagonists but had no effect on the potency of fluorosulfonyl-modified irreversible antagonists. The most potent irreversible antagonist was, N-α-4-[bis-(2-chloroethyl)amino]benzoyl-[His(4-benzyl)]\(^{10}\)-h-α-CGRP(8-37) (5).

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The mouse CL shares 90 % amino acid residues with human CL while mouse RAMP1 shares only 71 % amino acid residues with human RAMP1. Most of the amino acid sequence dissimilarity resides in the extracellular domain of RAMP1 which has been shown to modulate the affinity of non-peptide antagonists. In Chapter 4, based on the amino acid composition and analysis of hydropathy plots of human and mouse CL and RAMP1, I hypothesized that
RAMP1 participates in forming a hydrophobic binding pocket with CL and synthesize a novel competitive antagonist, [N-α-benzoyl-His\textsuperscript{10}-benzyl]α-CGRP(8-37). This antagonist was designed to favor interaction with human RAMP1 by hydrophobic modifications I predicted would confer species selectivity due to the hydrophobic amino acid composition of human RAMP1.

I synthesized and structurally characterized the prototypical CGRP receptor antagonist CGRP(8-37), a previously reported high affinity competitive antagonist [N-α-benzoyl]α-CGRP(8-37) (bzl-CGRP(8-37)) and a novel antagonist [N-α-benzoyl-His\textsuperscript{10}-benzyl]α-CGRP(8-37) (bzl-bn-CGRP(8-37)). Subsequently, I pharmacologically characterized these antagonists at mouse CGRP receptors in the mouse aorta and at human CGRP receptors in the human SK-N-MC cells. I compared the antagonist affinity of CGRP(8-37), bzl-CGRP(8-37) and bzl-bn-CGRP(8-37) in mouse aorta and in human SK-N-MC cells as well as between mouse and human CGRP receptors and determined that vascular relaxation in the mouse aorta is mediated by CGRP1 receptors. bzl-bn-CGRP(8-37) is a potent species selective competitive antagonist discriminating human CGRP receptors and antagonist hydrophobicity correlated with human selectivity. Taken together these studies suggest that the human CGRP receptor binding pocket for peptide antagonists is more hydrophobic than in the mouse.

I think that my research will have a broad impact on the CGRP research area. There are significant discoveries which could be made utilizing these novel antagonists. I anticipate the use of these antagonists \textit{in vivo} and/or \textit{in vitro} will be
to further understand the physiological functions of CGRP, to ascertain information about the structure of CGRP receptor binding pocket using mass spectrometry and perhaps differentiation of CGRP receptors by determining the pharmacological properties of these antagonists in various tissues and cell lines.
APPENDIX A

CONCENTRATION-DEPENDENT RECEPTOR ALKYLATION, SUBSEQUENT DETERMINATION OF CGRP AFFINITY USING IRREVERSIBLE ANTAGONISTS

INTRODUCTION

Furchgott’s method of irreversible receptor inactivation has been extensively used to determine receptor reserve and calculate agonist equilibrium dissociation constants (K_a) for numerous G protein-coupled receptors, including α and β adrenergic (Besse and Furchgott, 1976; Ruffolo et al., 1979a; Ruffolo et al., 1979b; Ruffolo et al., 1979c; Ruffolo et al., 1980a; Ruffolo et al., 1980b), muscarinic (Furchgott and Bursztyn, 1967; Sastry and Cheng, 1972; Takeyasu et al., 1979; Ringdahl and Jenden, 1983), adenosine (Morey et al., 1998) and serotonin (Meller et al., 1990) receptors. Until recently, this method has not been valid to determine CGRP receptor reserve or determination of K_A values for CGRP due to the lack of appropriate antagonists. In Chapter 3, I described the synthesis and characterization of the first irreversible CGRP receptor antagonists that serve as valuable pharmacological tools to study CGRP receptor reserve in various organs and tissues, selective organ and tissue responses to various agonists, differentiation of receptors and determination of K_A values for CGRP in various organs and tissues. In isolated mouse thoracic aorta, I investigated
receptor reserve in isolated aorta using CGRP and analogues 4 – 7 and determined $K_A$ values for CGRP, by the method of Furchgott (Furchgott, 1966).
EXPERIMENTAL PROCEDURES

Thoracic Aorta Relaxation.

Identical experimental procedures were used for the dissection, mounting equilibration, pre-contraction of aortic rings and measurement of CGRP-induced relaxation as those stated in Chapter 3, Experimental Procedures, Thoracic Aorta Relaxation. Rings were incubated with different concentrations of 4 or 5 for 15 min or with different concentrations of 6 or 7 for 45 min and then the ring segments were thoroughly washed for 45 min. Cumulative concentration-response curves for h-α-CGRP were then repeated.

Data Analysis.

Functional data were analyzed by nonlinear regression curve fitting using Graphpad Prism 4.0 (San Diego, CA). Cumulative concentration-response curves were constructed and EC50 values and % maximal responses were calculated using all points on the concentration-response curve. Data are expressed as the mean ± SEM.

The agonist equilibrium dissociation constant, K_A, of CGRP was determined by the method of Furchgott (Furchgott, 1966). Equieffective concentrations of CGRP before [A] and after [A'] treatment with several different concentrations of 4, 5, 6 or 7 were determined at ten levels of response between 15 and 80 % of the maximum effect by interpolation from concentration-response curves. A Lineweaver-Burk (Double Reciprocal) plot was constructed from the data. 1/[A] was plotted vs. 1/[A'] and a straight line was fitted to the data by linear regression. From the slope and the intercept on the ordinate axis, the K_A value of
the agonist-receptor complex and the fraction, \( q_{\text{functional}} \), of functional receptors remaining were calculated.

\[
P_{AR} = \frac{N_{AR}}{N}
\]

\[
N_{AR} = N \frac{[A]}{K_A + [A]}
\]

\[
N_{AR} = qN \frac{[A']}{K_A + [A']}
\]

\[
N \frac{[A]}{K_A + [A]} = qN \frac{[A']}{K_A + [A']}
\]

\[
\frac{1}{A} = \frac{1}{q} \left[ A' \right] + \frac{(1 - q)}{qK_A}
\]

A plot of \( 1/[A] \) versus \( 1/[A'] \) should give a straight line with:

\[
slope = \frac{1}{q} \quad y - \text{intercept} = \frac{(1 - q)}{qK_A}
\]

\[
K_A = \frac{(slope - 1)}{y - \text{intercept}}
\]

Differences of % inhibition between different concentrations of irreversible antagonists were determined using analysis of variance followed by Newman-Keuls post test with a \( p < 0.05 \) level of probability accepted as a significant difference.

\( p_{AR} \) is the proportion of binding sites occupied by \( A \).
\( N_{AR} \) is the number of receptors \( A \) occupies.
\( N \) is the total number of receptors.

After irreversible antagonist \( N \) is reduced to \( qN \). A greater concentration of agonist \( [A'] \) must be applied in order to achieve the same value of \( N_{AR} \).

The same tissue response before and after irreversible antagonist corresponds to corresponds to the same receptor occupancy by agonist.
RESULTS

Concentration-Dependent Receptor Alkylation. In aortas, 4 and 5 demonstrated irreversible antagonist properties at concentrations of 0.1, 0.4, 1.5 and 2.5 μM (Figure 43) and 0.025, 0.1, 0.4 and 1.5 μM (Figure 44), respectively, with percent inhibition of 10.4 ± 0.2, 17.5 ± 3.9, 35.1 ± 2.8 and 55.5 ± 4.7 %, and 18.6 ± 1.6, 29.7 ± 4.6, 52.3 ± 4.9 and 62.1 ± 1.8 %, respectively, for each individual concentration of irreversible antagonist used (Table 14). Incubation with increasing concentrations of analogue 4 or 5, followed by removal and thorough washing of the tissue, caused dose-dependent reductions in maximal CGRP-mediated relaxation that were significantly different. In particular, analogue 5, having a benzyl moiety on C4 of the imidazole side-chain of His^{10}, inhibited maximal CGRP-mediated relaxation the most.

In aortas, 6 and 7 demonstrated irreversible antagonist properties at concentrations of 1.5, 2.5 and 5 μM (Figure 45, Figure 46), with percent inhibition of 20.5 ± 1.4, 28.2 ± 1.9 and 33.9 ± 1.7 %, and 21.2 ± 1.8, 27.4 ± 2.7 and 33.9 ± 1.5 %, respectively, for each individual concentration of irreversible antagonist used (Table 14). Incubation of aortic rings with increasing concentration of 6 and 7, followed by removal and thorough washing of the tissue, caused dose-dependent reductions in maximal CGRP-mediated relaxation that were significantly different. These analogues were less potent than 4 and 5 and the decrease in maximal CGRP-mediated response was less in both assays. Addition of the benzyl moiety to C4 of the imidazole side-chain of
His$^{10}$ (7) did not produce a statistically significant increase in inhibition of maximal CGRP-mediated relaxation as compared to the unmodified His$^{10}$ analogue (6).

**Determination of CGRP Affinity after Irreversible Receptor Blockade.**

Double reciprocal plots were constructed from each concentration of analogue 4 – 7 (Figure 47, Figure 48, Figure 49 and Figure 50). The $K_A$ values for CGRP was determined, from the $y$-intercept of the linear regression, for each concentration of analogue 4 – 7 used and are listed in Table 14. There was no significant difference among $K_A$ values determined from different concentrations of analogue 4 – 7.

The % of functional receptors remaining ($q_{\text{functional}}$) was determined from the slope of the linear regression for each concentration of analogue 4 – 7 used and is listed in Table 14. The amount of $q_{\text{functional}}$ decreased with increasing concentration of analogue 4 – 7.
Figure 43. CGRP concentration-response curves for relaxation of mouse thoracic aorta before and after treatment with different concentrations of N-α-4-[bis-(2-chloroethyl)amino]benzoyl-h-α-CGRP(8-37). Aortic rings were pre-contracted with norepinephrine and CGRP concentration-response curves were obtained. The rings were then incubated with the irreversible analogues for 15 min followed by removal of unbound analogue by washing and CGRP concentration-response curves were repeated. Points are the mean ± SEM of responses of four to six experiments using individual thoracic aortas, each taken from different animals.
Figure 44. CGRP concentration-response curves for relaxation of mouse thoracic aorta before and after treatment with different concentrations of N-α-4-[bis-(2-chloroethyl)amino]benzoyl-[His(4-benzyl)¹⁰]-h-α-CGRP(8-37). Aortic rings were pre-contracted with norepinephrine and CGRP concentration-response curves were obtained. The rings were then incubated with the irreversible analogues for 15 min followed by removal of unbound analogue by washing and CGRP concentration-response curves were repeated. Points are the mean ± SEM of responses of four to six experiments using individual thoracic aortas, each taken from different animals.
Figure 45. CGRP concentration-response curves for relaxation of mouse thoracic aorta before and after treatment with different concentrations of N-α-4-[fluorosulfonyl]benzoyl]-h-α-CGRP(8-37). Aortic rings were pre-contracted with norepinephrine and CGRP concentration-response curves were obtained. The rings were then incubated with the irreversible analogues for 45 min followed by removal of unbound analogue by washing and CGRP concentration-response curves were repeated. Points are the mean ± SEM of responses of four to six experiments using individual thoracic aortas, each taken from different animals.
Figure 46. CGRP concentration-response curves for relaxation of mouse thoracic aorta before and after treatment with different concentrations of N-α-4-[fluorosulfonyl]benzoyl-[His(4-benzyl)10]-h-α-CGRP(8-37). Aortic rings were pre-contracted with norepinephrine and CGRP concentration-response curves were obtained. The rings were then incubated with the irreversible analogues for 45 min followed by removal of unbound analogue by washing and CGRP concentration-response curves were repeated. Points are the mean ± SEM of responses of four to six experiments using individual thoracic aortas, each taken from different animals.
Figure 47. Determination of $K_A$ value of CGRP and $q_{\text{functional}}$ from irreversible blockade of CGRP receptors with different concentrations of N-\(\alpha\)-4-[bis-(2-chloroethyl)amino]benzoyl-\(\alpha\)-CGRP(8-37). A) Double reciprocal plot of $1/[A]$ vs. $1/[A']$. Values for [A] and [A'] were obtained from control concentration response curves and after incubation, with different concentrations of irreversible antagonist. B) Calculated amount of functional receptors remaining after incubation with different concentrations of irreversible antagonist.
Figure 48. Determination of $K_A$ value of CGRP and $q_{\text{functional}}$ from irreversible blockade of CGRP receptors with different concentrations of N-α-[bis-(2-chloroethyl)amino]benzoyl-[His(4-benzyl)$_{10}$]-h-α-CGRP(8-37). **A)** Double reciprocal plot of $1/[A]$ vs. $1/[A']$. Values for $[A]$ and $[A']$ were obtained from control concentration response curves and after incubation, with different concentrations of irreversible antagonist. **B)** Calculated amount of functional receptors remaining after incubation with different concentrations of irreversible antagonist.
Figure 49. Determination of $K_A$ value of CGRP and $q_{\text{functional}}$ from irreversible blockade of CGRP receptors with different concentrations of N-$\alpha$-4-[fluorosulfonyl]benzoyl-h-$\alpha$-CGRP(8-37). A) Double reciprocal plot of $1/[A]$ vs. $1/[A']$. Values for $[A]$ and $[A']$ were obtained from control concentration response curves and after incubation, with different concentrations of irreversible antagonist. B) Calculated amount of functional receptors remaining after incubation with different concentrations of irreversible antagonist.
Figure 50. Determination of $K_A$ value of CGRP and $q_{\text{(functional)}}$ from irreversible blockade of CGRP receptors with different concentrations of $\text{N-}\alpha-4$-[fluorosulfonyl]benzoyl-[His(4-benzyl)$^{10}$]-h-$\alpha$-CGRP(8-37). A) Double reciprocal plot of $1/[A]$ vs. $1/[A']$. Values for $[A]$ and $[A']$ were obtained from control concentration response curves and after incubation, with different concentrations of irreversible antagonist. B) Calculated amount of functional receptors remaining after incubation with different concentrations of irreversible antagonist.
Table 14. Concentration dependent irreversible antagonism of CGRP-mediated functional responses in mouse aorta by analogues 4 – 7, functional receptors remaining and $K_A$ of CGRP.

<table>
<thead>
<tr>
<th>Analogue 4</th>
<th>Concentration</th>
<th>Inhibition (%) Control</th>
<th>$Q_{(functional)}$ (% Receptors Remaining)</th>
<th>$K_A$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 0.1 μM</td>
<td>10.4 ± 0.2</td>
<td>91.6 ± 1.4</td>
<td>520</td>
<td></td>
</tr>
<tr>
<td>B 0.4 μM</td>
<td>17.5 ± 5.9</td>
<td>87.4 ± 3.1</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td>C 1.5 μM</td>
<td>35.1 ± 2.8ab</td>
<td>78.2 ± 4.4</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>D 2.5 μM</td>
<td>55.5 ± 4.7ab, c</td>
<td>77.0 ± 1.0</td>
<td>130</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean $K_A$</td>
<td>179 ± 23</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Analogue 5</th>
<th>Concentration</th>
<th>Inhibition (%) Control</th>
<th>$Q_{(functional)}$ (% Receptors Remaining)</th>
<th>$K_A$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 0.025 μM</td>
<td>18.6 ± 1.6</td>
<td>98.4 ± 0.1</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>B 0.1 μM</td>
<td>29.7 ± 4.6a</td>
<td>98.0 ± 0.3</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>C 0.4 μM</td>
<td>52.3 ± 4.9ab</td>
<td>95.9 ± 0.5</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>D 1.5 μM</td>
<td>62.1 ± 1.8ab, c</td>
<td>89.1 ± 1.8</td>
<td>172</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean $K_A$</td>
<td>91 ± 36</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Analogue 6</th>
<th>Concentration</th>
<th>Inhibition (%) Control</th>
<th>$Q_{(functional)}$ (% Receptors Remaining)</th>
<th>$K_A$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 1.5 μM</td>
<td>20.5 ± 1.4</td>
<td>98.5 ± 0.1</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>B 2.5 μM</td>
<td>28.2 ± 1.9a</td>
<td>97.8 ± 0.1</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>C 5.0 μM</td>
<td>33.9 ± 1.7ab</td>
<td>97.0 ± 0.1</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean $K_A$</td>
<td>33 ± 8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Analogue 7</th>
<th>Concentration</th>
<th>Inhibition (%) Control</th>
<th>$Q_{(functional)}$ (% Receptors Remaining)</th>
<th>$K_A$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 1.5 μM</td>
<td>21.2 ± 1.8</td>
<td>97.9 ± 0.1</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>B 2.5 μM</td>
<td>27.4 ± 2.7</td>
<td>96.9 ± 0.1</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>C 5.0 μM</td>
<td>33.9 ± 1.5ab</td>
<td>95.6 ± 0.1</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean $K_A$</td>
<td>42 ± 20</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$a$ Significantly different from the value in row A ($p < 0.05$).
$b$ Significantly different from the value in row B ($p < 0.05$).
$c$ Significantly different from the value in row C ($p < 0.05$).
APPENDIX B

AGONIST EFFECTS OF ANALOGUE 7

RESULTS

Agonist Effects of N-α-4-[fluorosulfonyl]benzoyl-[His(4-benzyl)10]-h-α-CGRP(8-37) (7). During the characterization of analogues 4 – 7, specifically while investigating non-specific effects in the mouse thoracic aorta and SK-N-MC cell line, I observed agonist effects with analogue 7. In aorta, upon addition to the organ bath, analogue 7 caused contraction which was sustained for the entire time period of incubation. The amount of contraction increased in a concentration-dependent manner (Figure 51), incubation with 1.5, 2.5 and 5 μM caused 13 ± 12, 64 ± 8 and 107 ± 7 % of norepinephrine-mediated (0.1 μM) contraction. In SK-N-MC cells, incubation with 1 μM of analogue 7 caused no stimulation of cAMP production while increasing the concentration to 5 μM caused stimulation of cAMP production (Figure 52). The amount of cAMP produced by 5 μM of analogue 7 was 12 ± 3 pmol.

In principle, an irreversible antagonist expressing agonism could compromise its use to estimate agonist equilibrium dissociation constants (K_A) due to desensitization of the receptor occurring in addition to receptor inactivation.

It is unclear how or why analogue 7 expresses agonism in both of these functional assays. The use of a competitive antagonist of CGRP receptors in the
functional assays and testing to see if the agonism by 7 is shifted to the right would determine if agonism is CGRP receptor-mediated.

In aorta, when analogue 7 binds to the CGRP receptor, the CL/RAMP1 complex may adopt a conformation where G protein switching occurs and the receptor preferentially couples to Gq, instead of Gs, to causes contraction through activation of PLC. Also, alteration of the CL/RAMP1 complex may lead to preferential activation of calcium channels.

Another explanation may be release of Cl from the fluorosulfonating moiety and a contaminating amount of AlF3 present in the buffers. Cl− + AlF3 → AlClF3− and AlClF3− directly activates adenylate cyclase and calcium channels.

It is possible that the purified product contains an impurity which is mediating the agonist effects, although no impurity is evident in the RP-HPLC chromatograms during purification or peptide homogeneity analyses. There is still the possibility that an impurity could co-elute with analogue 7 which would not be observed in the chromatograms.

Future studies, using CGRP receptor antagonists, calcium channel blockers, a PLC inhibitor or a Gs protein inhibitor may determine the mechanism of action of contraction and cAMP production and could elucidate structural information on how this antagonist interacts with the receptor.
Figure 51. Agomist effects of N-Fs-bzl-His(bn)\textsuperscript{10}-CGRP\textsubscript{(8-37)} (7) in mouse aorta. Aortic rings were incubated with increasing concentrations of (7) for 45 min. Points are the mean ± SEM of responses of three to four experiments using individual thoracic aortas, each taken from different animals.
Figure 52. Agomist effects of N-Fs-bzl-His(bn)\textsuperscript{10}-CGRP(8-37) (7) in SK-N-MC cells. SK-N-MC cells were incubated with increasing concentrations of (7) for 30 min. Points are the mean ± S.E.M. of responses of three individual experiments performed in duplicate, with each experiment using cells grown in different culture plates on different days.
BRADYKININ-RELATED PEPTIDES AND TRYPTOPHILLINS IN THE SKIN SECRETIONS OF THE MOST PRIMITIVE EXTANT FROG, *Ascaphus truei*

**ABSTRACT**

The tailed frog *Ascaphus truei* occupies a unique position in phylogeny as the most primitive extant anuran and is regarded as the sister taxon to the clade of all other living frogs. A previous study led to the isolation of 8 antimicrobial peptides, termed ascaphins, from norepinephrine-stimulated skin secretions. Peptidomic analysis (HPLC separation followed by MALDI mass spectrometry and Edman degradation) of these secretions has led to the identification and structural characterization of 14 additional peptides present in relatively high concentration. In addition to bradykinin (BK; RPPGFSPFR), a C-terminally extended bradykinin (peptide RD-11; RPPGFSPFRVD), a bradykinin-like peptide (peptide AR-10; APVPGLSPFR) and a C-terminally extended form of this peptide (peptide AV-12; APVPGLSPFRVV) were obtained in pure form. These peptides produced concentration-dependent relaxation of precontracted mouse tracheal rings with a rank order of potency of BK > RD-11 > AR-10 > AV-12 but only RD-11 caused the same maximal relaxation as BK.
EXPERIMENTAL PROCEDURES

Isolated Tracheae Relaxation.

Male albino CF1 mice (30 g) were euthanized using CO₂ and the tracheae were isolated as described previously (Garssen et al., 1990). Tracheal rings (3 mm long) were mounted in an organ bath by passing two stainless steel pins through the tracheal lumen. One pin was attached to a Grass FT.03 isometric force transducer (Grass Instruments, Quincy, MA) for measurement of isometric tension while another pin was held in a fixed position. The trachea was bathed with Krebs solution (composition in mmol/l): NaCl, 126; KCl, 5.5; CaCl₂, 2.5; NaH₂PO₄, 1.2; MgCl₂, 1.2; NaHCO₃, 25; dextrose, 11.1; Na₂Ca EDTA, 0.029; pH 7.4) maintained at 37 °C and gassed with 95 % O₂/5 % CO₂. Rings were equilibrated in Krebs solution for 45 min at a resting tension of 300 mg and then precontracted with 0.3 μM methacholine. After the contraction reached a plateau, a single concentration of BK, RD-11, AR-10 or AV-12 was added to the bath and tracheal relaxation recorded. After the tissues had been washed for 30 min with Krebs solution and precontracted again with 0.3 μM methacholine, a single relaxation-response to a higher concentration of BK, RD-11, AR-10 or AV-12 was obtained. This non-cumulative concentration-response procedure was necessary to avoid development of tachyphylaxis which has been reported with cumulative addition of BK and related peptides (Li et al., 1998). Concentration-response curves for each peptide were obtained and potencies were calculated using Graphpad Prism 4.0. For each peptide the EC₄₀ was determined to be the
concentration required to produce 40 % of the maximal BK-mediated relaxation. Data points are the mean ± SEM of 4-5 experiments.

RESULTS

Myotropic Activities of BK, RD-11, AR-10 and AV-12. As shown in Figure 53, BK, RD-11, AR-10 and AV-12 produced concentration-dependent relaxation of mouse trachea that had been precontracted with methacholine. The rank order of potency was BK > RD-11 > AR-10 > AV-12. The EC₄₀ values were BK: 53.0 ± 3.3 nM, RD-11: 0.4 ± 0.3 µM, and AR-10: 3.3 ± 1.0 µM. Due to the weak potency of AV-12 an EC₄₀ value could not be determined. At the highest concentration tested (10 µM), BK caused maximal relaxation of the trachea. RD-11 caused the same maximal relaxation compared to BK while AR-10 and AV-12 were less effective. The maximal relaxations at 10 µM relative to the BK-induced maximal relaxation were RD-11: 96 ± 22 %, AR-10 47 ± 9 %, and AV-12: 15 ± 7 %.
Figure 53. Myotropic activities of BK, RD-11, AR-10 and AV-12. Non-cumulative concentration-response curves to (■) BK, (●) RD-11, (▲) AR-10 and (▼) AV-12 (0.01 – 10 μM) in the isolated mouse trachea after methacholine (0.3 μM) precontraction. Each point is plotted as a % of the BK-mediated maximal relaxation and represents mean ± SEM (n = 4 – 5).
REFERENCES


