Identification and Pharmacological Characterization of Domains Involved in Binding of CGRP Receptor Antagonists to the Calcitonin-like Receptor

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ABSTRACT: The calcitonin-like receptor (CLR) and the calcitonin receptor (CTR) interact with receptor activity-modifying protein 1 (RAMP1) at the cell surface to form heterodimeric receptor complexes. CLR and CTR are members of the class II (family B) G-protein-coupled receptors (GPCR) and bind calcitonin gene-related peptide (CGRP) with similar affinities when coexpressed with RAMP1. The observation that various nonpeptide CGRP receptor antagonists display a higher affinity for the CLR/RAMP1 complex than for CTR/RAMP1 provided an opportunity to investigate the molecular determinants of the differential receptor affinities of these antagonists. A chimeric receptor approach was utilized to identify key domains within CLR responsible for conferring high-affinity antagonist binding. Initial chimera experiments implicated distinct regions within CLR as responsible for the affinities of structurally diverse CGRP receptor antagonists. Dissection of these key regions implicated amino acids 37−63 located in the amino terminus of CLR as responsible for the high-affinity interaction of one structural class, while transmembrane domain (TM) 7 was responsible for the interaction of a second class of antagonist. A unique binding interaction in the amino terminus of CLR is consistent with the observation that these compounds also interact with the extracellular region of RAMP1 and could suggest the formation of a binding pocket between the two proteins. Conversely, a compound which interacted with TM7 did not display a similar RAMP1 dependence, suggesting an allosteric mechanism of antagonism. Collectively, these data provide insight into two alternative mechanisms of antagonism for this unique heterodimeric receptor complex.

CGRP[1] is a 37 amino acid neuropeptide produced by tissue-specific alternative mRNA splicing of the calcitonin gene (*1*). CGRP and its receptors are widely expressed among peripheral tissues and the central and peripheral nervous systems (*2*). Research in the CGRP receptor field has been challenging due to the heterodimeric nature of the receptor. CGRP activity is mediated by the coexpression of a G-protein-coupled receptor (GPCR), calcitonin-like receptor (CLR), a single transmembrane-spanning protein designated receptor activity-modifying protein 1 (RAMP1) (*3*), and an intracellular protein, receptor component protein (RCP), required for G-protein signal transduction (*4*). RAMP1 has the ability to modulate the receptor phenotype of other GPCRs (*5*), in particular, the calcitonin receptor (CTR) (*6*). When coexpressed with RAMP1, the CTR displays high affinity for both amylin and CGRP. CLR and CTR are both members of the class II family of GPCRs which include receptors for secretin, glucagon, vasoactive intestinal peptide, corticotropin-releasing factor, and the parathyroid hormone in addition to the calcitonin family of peptides. CLR and CTR share approximately 57% identity on the amino acid level and are G_s-coupled receptors.

The pursuit of small molecule antagonists of the CGRP receptor, CLR-RAMP1, has intensified in recent years due to the mounting evidence that CGRP is involved in the pathophysiology of migraine headache (*7*). The first highly specific and potent CGRP receptor antagonist described was BIBN4096BS (*8*). Efficacy of BIBN4096BS in the acute treatment of migraine was reported recently (*9*). BIBN4096BS exhibits high affinity for the human CGRP receptor but >100-fold lower affinity for the rat CGRP receptor. Site-directed mutagenesis of RAMP1 identified amino acid 74 as responsible for the high-affinity binding of BIBN4096BS and its truncated analogues (*10*) to the human receptor. Although RAMP1 has been implicated in the high-affinity binding of this structural class, domains within CLR could not be ruled out as participating in antagonist binding to the heterodimeric receptor complex.

To further define the binding sites of CGRP receptor antagonists, we capitalized on the observation that although both the CLR and the related CTR interact with RAMP1 to form complexes that bind CGRP with similar high affinities, various nonpeptide CGRP receptor antagonists display higher affinities for CLR/RAMP1 than for the CTR/RAMP1 complex. Chimeric receptors therefore were generated by

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[1] Abbreviations: CLR, calcitonin-like receptor; CGRP, calcitonin gene-related peptide; CTR, calcitonin receptor; GPCR, G-protein-coupled receptor; hCGRP, human CGRP; RAMP, receptor activity-modifying protein; RCP, receptor component protein; TM, transmembrane domain.

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exchanging regions of CLR with the corresponding regions of CTR followed by coexpression with RAMP1 and determination of antagonist affinities. By using this approach, the binding domains of structurally diverse CGRP receptor antagonists within CLR have been delineated. The results of this study may provide insight into the development of selective and high-affinity nonpeptide antagonists of this unique heterodimeric receptor.

EXPERIMENTAL PROCEDURES

Human Calcitonin Receptor cDNA Cloning. The insert-negative human calcitonin receptor was isolated from human substantia nigra using RT-PCR. PCR primers were based upon accession number X69920. The forward primer (5′-GGCTAGCACCATGAGGTTCACATTTAC) contained a *Nhe*I restriction site followed by a Kozak sequence, and the reverse primer contained a *Bam*HI site (5′-GGGATCCT-CAAGCAGATGACTCTTGC). The 1.4 kb product was sequenced bidirectionally with 100% coverage in each direction and confirmed to be identical to accession number X69920 (polymorphic variant Leu 447).

Expression Constructs and Chimeras. Human cDNA for CLR was provided by Dr. Douglas MacNeil (Merck Research Laboratories, Rahway, NJ) and subcloned as a 5′*Nhe*I−3′*Not*I fragment into pcDNA3.1/Zeo(+). Human CLR was also subcloned as a 5′*Nhe*I−3′*Pme*I fragment into the pIREShyg2 which had been digested with *Nhe*I and *Eco*RV. Human RAMP1 and RAMP2 cDNAs were provided by Dr. Bruce Daugherty (Merck Research Laboratories, Rahway, NJ) and subcloned as a 5′*Hin*dIII−3′*Not*I fragment into pcDNA3.1/Hygro(+) for RAMP1 and as a 5′*Nhe*I−3′*Not*I fragment into pIRESpuro2 for RAMP2. Human CTR was subcloned as a 5′*Nhe*I−3′*Bam*HI fragment into pcDNA3.1/Hygro(−). Chimeric receptors NH_2(1−63), NH_2-(1−145), EC1−CO_2H, EC2−CO_2H, EC3−CO_2H, EC3-(357−363), EC3(357−371), TM7−CO_2H, and CO_2H were generated by overlap extension PCR. Chimeric receptors NH_2(24−35), NH_2(37−63), and NH_2(24−63) were generated by annealing overlapping oligonucleotides and filled-in using DNA polymerase I large fragment (Klenow) followed by overlap extension PCR. Chimeric receptors were subcloned as 5′*Nhe*I−3′*Not*I fragments into pcDNA3.1/Hygro(−). The TMpred program was used to predict membrane-spanning regions (*11*). Chimeras NH_2(24−35), NH_2(37−63), and NH_2-(24−63) were constructed so as to retain the CLR signal peptide which has a putative cleavage site between amino acids 22 and 23 (*12*).

Cell Culture and DNA Transfection. 293 EBNA and HEK293 cells were cultured in DMEM with 4.5 g/L glucose, 1 mM sodium pyruvate, and 2 mM glutamine supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin and maintained at 37 °C, 5% CO_2, and 95% humidity. Cells were subcultured by treatment with 0.25% trypsin with 0.1% EDTA in HBSS.

For transient transfections, 24 h prior to transfection the cells were seeded at 2.0×10^7/dish in 500 cm^2 dishes. Transfections were performed by combining 60 μg/dish DNA with 180 μg/dish Lipofectamine 2000 (Invitrogen). Expression vector constructs for the appropriate wild-type or chimeric receptor and RAMP1 were cotransfected in equal amounts. The transfection cocktail was added directly to the medium, and this mixture was replaced with fresh medium 24 h later. The cells were harvested for membranes 48 h post-transfection.

For stable transfections, 24 h prior to transfection cells were seeded at 3×10^6/T75 flask. Transfections were performed by combining 5 μg of hCLR and 5 μg of hRAMP2 with 30 μg of Lipofectamine 2000 (Invitrogen). The transfection cocktail was added directly to the medium, 24 h later the cells were passaged into fresh medium, and growth medium plus 300 μg/mL hygromycin and 1.0 μg/mL puromycin was added the following day. Clonal cell populations were generated via single cell sorting and maintained in growth medium containing 150 μg/mL hygromycin and 0.5 μg/mL puromycin.

Membrane Preparation and Radioligand Binding Studies. Transiently transfected 293 EBNA or stably transfected HEK293 cells were washed once with PBS and harvested in harvest buffer containing 50 mM HEPES, 1 mM EDTA, and Complete protease inhibitors (Roche). The cell suspension was disrupted with a laboratory homogenizer and centrifuged at 48000*g* to isolate membranes. The pellets were resuspended in harvest buffer plus 250 mM sucrose. Membranes were stored at −70 °C as aliquots. Membranes from rat brains were prepared similarly.

Binding assays were assembled by combining antagonist or 500 nM hCGRP for nonspecific binding and 10 pM ^{125}I-hCGRP (GE Healthcare), followed by 0.5−75 μg of membranes from transiently transfected cells (dependent upon receptor expression levels) or 100 μg of rat brain membranes, and incubated for 3 h at room temperature in binding buffer (10 mM HEPES, 5 mM $MgCl_2$, 0.2% BSA) in a total volume of 1 mL. Adrenomedullin binding assays were set up as above except with 7.5 μg of CLR/RAMP2 membranes, 10 pM ^{125}I-rat adrenomedullin (GE Healthcare) as the radioligand, and 50 nM adrenomedullin for nonspecific binding. Incubations were terminated by filtration through GF/B 96-well filter plates that had been blocked with 0.5% polyethylenimine. Saturation binding experiments were carried out on wild-type CLR and the receptor spanning chimeras after coexpression with RAMP1 and the dissociation constant (K_d) and the receptor density (B_{max}) determined from either a one-site or two-site binding model. K_d and B_{max} values for each membrane preparation are summarized in the Supporting Information. Dose response curves were plotted and IC_{50} values determined from a four-parameter fit as defined by the equation $y = ((a - d)/[1 + (x/c)^b] + d$, where $y =$ response, $x =$ dose, $a =$ maximum response, $d =$ minimum response, $c =$ inflection point, and $b =$ slope. K_i values were determined from the high-affinity binding site by using the equation $K_i = IC_{50}/1 + ([\text{ligand}]/K_d)$.

Compounds **1**, **3**, and **4** were synthesized in the Medicinal Chemistry Department at Merck Research Laboratories.

RESULTS

Rationale and Construction of CLR/CTR Chimeric Receptors. To identify critical regions within CLR responsible for high-affinity binding of antagonists, we chose to replace CLR domains with the corresponding domains from the related CTR. The relative locations of the seven transmembrane domains were identified using the TMpred program (*11*), and extracellular or intracellular termini and loops were

Table 1: Identification of General Antagonist Binding Domains via Competitive Binding Experiments Using Membranes from Transiently Transfected Cells[a]

	K_i						
	CLR	NH_2(1-63)	NH_2(1-145)	EC1-CO_2H	EC2-CO_2H	EC3-CO_2H	CTR
	nM	*nM*	*nM*	*nM*	*nM*	*nM*	*nM*
Compound 1	33 ± 3.08	450 ± 14	190 ± 3.0	52 ± 1.4	28 ± 4.2	43 ± 5.1	490 ± 33
Compound 3	0.019 ± 0.0015	0.27 ± 0.0085	0.56 ± 0.039	0.016 ± 0.0025	0.010 ± 0.00057	0.018 ± 0.0022	1.2 ± 0.26
Compound 4	3,500 ± 510	5100 ± 760	2900 ± 430	>100,000	>100,000	>100,000	>100,000

[a] 293 EBNA cells were transiently transfected with CLR, CLR/CTR chimera, or CTR with RAMP1. Membranes were prepared 48 h after transfection, and K_i values were determined by competition of 10 pM ^{125}I-hCGRP by each antagonist. Data represent the mean ± SEM from at least three independent experiments. GPCRs are represented by simplified structures where black regions are derived from CLR and gray regions are derived from CTR.

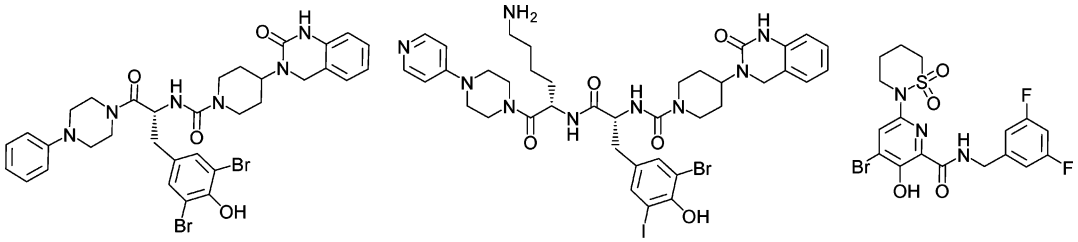

FIGURE 1: Structures of CGRP receptor antagonists compound **1**, compound **3**, and compound **4**.

assigned accordingly. Five receptor spanning chimeras (Table 1) were generated initially to localize general binding domains within CLR responsible for antagonist binding. Two amino-terminal chimeras were constructed, one replacing the first 63 amino acids [chimera NH_2(1−63)] of CLR with the corresponding 70 residues of CTR and the second exchanging the entire amino terminus of CLR [chimera NH_2(1−145)] with the corresponding region of CTR. Three chimeras spanning the remainder of the receptor were generated by exchanging the first (chimera EC1−CO_2H), second (chimera EC2−CO_2H), or third (chimera EC3−CO_2H) extracellular loop through the carboxy terminus. The K_d of the radioligand, ^{125}I-hCGRP, for the chimeric receptors (4−28 pM) was similar to the wild-type CGRP receptor (10 pM). All CLR/CTR chimeras, including CTR itself, when coexpressed with RAMP1 displayed a high- and low-affinity binding site with the exception of chimeras containing the third extracellular loop of CTR (EC1−CO_2H, EC2−CO_2H, EC3−CO_2H, EC3-(357−363), EC3(357−371). No specific binding of ^{125}I-hCGRP was observed in nontransfected 293 EBNA cell membranes as previously shown in Mallee et al. (*10*). The antagonists employed in this study include the previously reported compound **1**, compound **3**, which is an analogue of BIBN4096BS, and the novel antagonist compound **4** (Figure 1).

Pharmacological Profile and Selectivity of Antagonists. We have previously shown that the high-affinity binding and marked species selectivity of BIBN4096BS and its truncated analogue compound **1** are governed by RAMP1 (*10*). Compound **3**, a closely related analogue to BIBN4096BS, also displayed marked species selectivity for the CGRP receptor with K_i values of 0.019 nM on CLR coexpressed with RAMP1 and a K_i of 1.4 nM on rat brain membranes. Compounds **1** and **3** also exhibited a great deal of selectivity against the related adrenomedullin receptor (CLR/RAMP2) with K_i values of >100000 and 9200 nM, respectively. A structurally unrelated compound, compound **4**, did not exhibit a marked species selectivity with K_i values of 3500 and 6800 nM on the human and rat CGRP receptors, respectively. Additionally, compound **4** displayed a similar affinity for the adrenomedullin receptor (CLR/RAMP2) with a K_i of 7500 nM.

Identification of General Binding Domains Responsible for Two Structural Classes of Antagonist. Compound **3** and its truncated analogue compound **1** both displayed high affinity for CLR when coexpressed with RAMP1 as measured by their ability to compete with ^{125}I-hCGRP binding with K_i values of 0.019 and 33 nM, respectively. As expected, compounds **1** and **3** displayed reduced affinity for CTR when coexpressed with RAMP1 with K_i values of 490 and 1.2 nM, respectively. Substitution of the first, second, or third extracellular loop through the carboxy terminus of CLR with the corresponding region of CTR had essentially no effect on the affinity of compounds **1** and **3** when compared to the affinities for the wild-type receptor (Table 1). However, coexpression of either amino-terminal chimera, NH_2(1−63) or NH_2(1−145), with RAMP1 caused a decrease in binding affinity for compound **1** of 6−14-fold (K_i = 450 and 190 nM, respectively) and of 14−29-fold for compound **3** (K_i = 0.27 and 0.56 nM, respectively), implicating the first 63 amino acids as important for binding of this class of antagonist (Table 1).

Compound **4** displayed moderate affinity for CLR when coexpressed with RAMP1 as measured by its ability to

Table 2: Identification of Amino-Terminal Binding Domains via Competitive Binding Experiments Using Membranes from Transiently Transfected Cells[a]

	K_i				
	CLR	NH_2(24-35)	NH_2(24-63)	NH_2(37-63)	CTR
	nM	*nM*	*nM*	*nM*	*nM*
Compound 1	33 ± 3.1	29 ± 2.2	480 ± 32	220 ± 16	490 ± 33
Compound 3	0.019 ± 0.0015	0.019 ± 0.0029	0.28 ± 0.052	0.13 ± 0.0040	2.3 ± 0.50

[a] 293 EBNA cells were transiently transfected with CLR, CLR/CTR chimera, or CTR with RAMP1. Membranes were prepared 48 h after transfection, and K_i values were determined by competition of 10 pM ^{125}I-hCGRP by each antagonist. Data represent the mean ± SEM from at least three independent experiments. GPCRs are represented by simplified structures where black regions are derived from CLR and gray regions are derived from CTR.

compete with ^{125}I-hCGRP binding with an K_i value of 3500 nM. Compound **4** was inactive (0% inhibition at 100 μM) on the CTR/RAMP1 complex. Substitution of the amino terminus of CLR with the comparable region of CTR had no effect on its affinity when compared to the wild-type receptor (Table 1). Substitution of the first, second, or third extracellular loop through the carboxy terminus resulted in the complete loss of affinity (0% inhibition at 100 μM) for compound **4** (Table 1), implicating the region of CLR between extracellular loop 3 and the carboxy terminus as critical for binding of this antagonist.

*Identification of the Amino-Terminal Binding Domain for Compounds **1** and **3**.* Three chimeras (Table 2) were constructed to identify the region in the amino terminus responsible for the high-affinity binding of compounds **1** and **3**. The putative signal peptide sequence was retained from CLR and either amino acids 24−35 [chimera NH_2(24−35)], amino acids 37−63 [chimera NH_2(37−63)], or amino acids 24−63 [chimera NH_2(24−63)] of CLR were replaced with the corresponding region of CTR. Substitution of amino acids 24−35 had no effect on the affinity of either compound **1** (K_i = 29 nM) or compound **3** (K_i = 0.019 nM), but the substitution of amino acids 24−63 decreased the affinity of both compounds approximately 15-fold (K_i = 480 and 0.28 nM, respectively; Table 2). Additionally, the substitution of amino acids 37−63 also decreased the affinity of compounds **1** and **3** approximately 7-fold (K_i = 220 and 0.13 nM, respectively; Table 2), thus identifying amino acids 37−63 as critical for receptor recognition by this class of CGRP antagonist (Figure 2A,B).

*Identification of the CLR Binding Domain for Compound **4**.* Initial chimera data identified a fairly large region of CLR as potentially responsible for the interaction of compound **4**. In total, four chimeras (Table 3) were generated to explore the region encompassing the third extracellular loop through the carboxy terminus. Two chimeric receptors explored the role of the third extracellular loop in binding of this antagonist. One of these chimeras substituted the entire third extracellular loop of CLR including the putative first amino acid in transmembrane domain 7 [amino acids 357−371; chimera EC3(357−371)] with the corresponding region of CTR. The other third extracellular loop chimera substituted a region, amino acids 357−363 [chimera EC3(357−363)], based on the observation that it exhibited limited homology

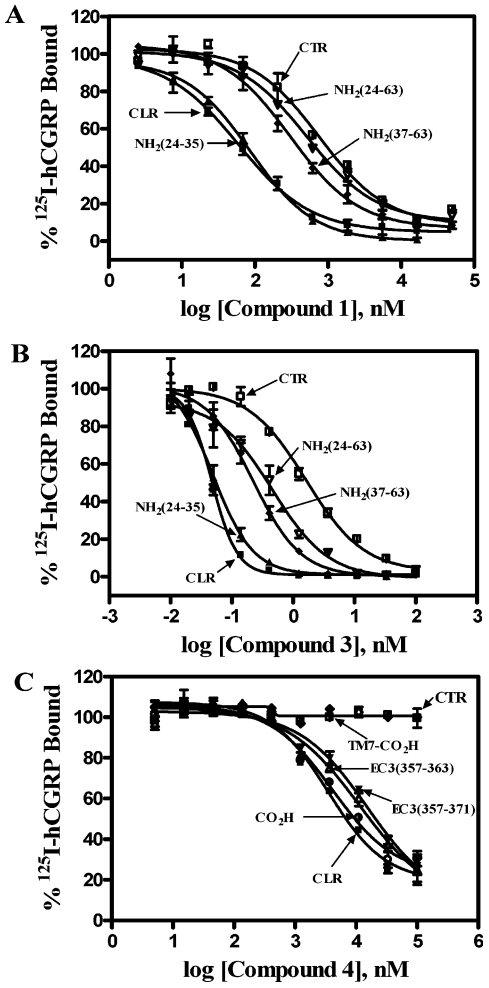

FIGURE 2: Competition binding analysis of CLR/CTR chimeric receptors coexpressed with hRAMP1. Affinities of compound **1** (A), compound **3** (B), and compound **4** (C). Symbols: (■) CLR, (▲) NH_2(24−35), (▽) NH_2(24−63), (◆) NH_2(37−63), (△) EC3(357−363), (▼) EC3(357−371), (◇) TM7−CO_2H, (○) CO_2H, and (□) CTR.

between CLR and CTR. Coexpression of either third extracellular loop chimera with RAMP1 had little effect on the affinity of compound **4** (Table 3). Exploration of TM7 and the carboxy terminus was accomplished by generating two additional chimeras. One chimera substituted TM7 through the carboxy terminus (chimera TM7−CO_2H) and

Table 3: Identification of the Transmembrane 7 Binding Domain via Competitive Binding Experiments Using Membranes from Transiently Transfected Cells[a]

	K_i					
	CLR	EC3(357-363)	EC3(357-371)	TM7- CO_2H	CO_2H	CTR
						
	nM	*nM*	*nM*	*nM*	*nM*	*nM*
Compound 4	3,500 ± 510	6,900 ± 1,500	6,000 ± 680	>100,000	2800 ± 410	>100,000

[a] 293 EBNA cells were transiently transfected with CLR, CLR/CTR chimera, or CTR with RAMP1. Membranes were prepared 48 h after transfection, and K_i values were determined by competition of 10 pM ^{125}I-hCGRP by each antagonist. Data represent the mean ± SEM from at least three independent experiments. GPCRs are represented by simplified structures where black regions are derived from CLR and gray regions are derived from CTR.

the other just the carboxy terminus (chimera CO_2H) of CLR with the corresponding region of CTR. Substitution of the carboxy terminus alone had little effect on the affinity of compound 4 (K_i = 2800 nM), whereas substitution of TM7 along with the carboxy terminus significantly reduced the affinity of compound **4** (0% inhibition at 100 μM), thus implicating TM7 as critical for binding of this antagonist (Table 3; Figure 2C).

DISCUSSION

The CGRP receptor requires dimerization of CLR, a classical seven transmembrane receptor, with a single transmembrane accessory protein RAMP1. RAMP1 is a type I transmembrane protein that modulates the receptor phenotype of multiple receptors, in particular CLR and CTR. Coexpression of RAMP1 with CLR results in a receptor that binds CGRP with high affinity (*3*) whereas coexpression with CTR results in amylin receptor pharmacology (*6*). CLR and CTR are members of the class II family of GPCRs which recognize various regulatory peptides. It is well documented that the large amino terminus of the class II GPCRs plays a critical role in agonist binding; similarly, the amino terminus of CLR has been shown to participate in binding of CGRP (*13*). In the case of the CGRP receptor, RAMP1 also contains a large extracellular domain which has been implicated in the formation or modulation of the agonist binding pocket (*14*).

The heterodimeric nature of the CGRP receptor poses additional challenges in the discovery and development of small molecule antagonists. The observation that CGRP could be cross-linked to both CLR and RAMP1 (*15*) suggests that RAMP1 is in close proximity to the CGRP binding site or that the peptide interacts directly with both CLR and RAMP1. This close proximity of CLR and RAMP1 might suggest that both proteins are involved in antagonist binding through the formation of a binding pocket. It has recently been shown that amino acids 23−60 of CLR are required and sufficient for the transport of RAMP1 to the cell surface (*16*), supporting the hypothesis that the amino terminus of CLR and RAMP1 are in close proximity. Interestingly, chimeras NH_2(24−63), NH_2(37−63), and NH_2(1−63) had the lowest receptor density (data in Supporting Information), as determined by B_{max} from saturation binding experiments, potentially a result of diminished RAMP1 and CLR/CTR chimera cotransport to the plasma membrane. Also of interest was the observation that chimeric receptors containing the third extracellular loop of CTR, but not CTR itself, displayed single-site high-affinity binding whereas the remainder of the chimeras (and CLR) all exhibited both a high- and low-affinity site. This could suggest that the N-terminus of CLR and third extracellular loop of CTR form a unique high-affinity CGRP binding site which is unable to isomerize to the low-affinity site.

We have previously demonstrated that tryptophan 74 in the amino terminus of RAMP1 is responsible for the high-affinity binding of the BIBN4096BS class of antagonist (*10*). Although RAMP1 could be solely responsible for the high-affinity binding of certain classes of antagonist by a direct interaction, the involvement of other binding domains could not be ruled out. It could be envisioned that tryptophan 74 of RAMP1 is involved in the formation of a binding pocket with additional amino acids located within the CLR−RAMP1 heterodimer. Compound **3** and its truncated analogue compound **1** are closely related to BIBN4096BS (*8*), demonstrate profound species-selective pharmacology, and share the requirement of a tryptophan at position 74 of RAMP1 for high-affinity binding. Furthermore, the RAMP1 dependency of binding for this class of antagonist is illustrated by the fact that BIBN4096BS exhibits no significant affinity for the related adrenomedullin receptor (*8*), comprised of CLR and RAMP2. In contrast, the structurally unrelated compound **4** exhibits similar affinity for human and rat CGRP receptors, as well as similar binding to CLR/RAMP2, suggesting that receptor recognition by this antagonist is RAMP-independent. The observation that some structural classes of antagonist demonstrate a RAMP1-dependent phenotype (compounds **1** and **3**) while others exhibit a RAMP-independent phenotype (compound **4**) led us to explore the role of CLR in the binding of nonpeptide CGRP antagonists.

Compounds **1** and **3** interact with the amino terminus of CLR, specifically between amino acids 37−63. While it cannot be completely ruled out that steric alterations in the chimeric receptor are at least partially responsible for the decrease in antagonist affinity, there are a number of observations that indicate a direct binding interaction. The extracellular amino-terminal interaction with CLR is consistent with the critical requirement of tryptophan 74 in the extracellular domain of RAMP1 for the affinity of these compounds. The close proximity of residues 37−63 of CLR to one of the putative CGRP binding domains (*13*) could at

least partially explain the ability of these compounds to act as antagonists. One could envision the amino termini of CLR and RAMP1 positioned in such a way that residue 74 of RAMP1 is in close proximity to residues 37−63 of CLR. The aromatic side chain of tryptophan 74 in RAMP1 could directly interact with compounds **1** and **3**, thereby positioning these compounds to interact with important residues in CLR. The amino-terminal extracellular domain has been shown to be the site of contact between the receptor and peptide ligand for numerous class II GPCRs. Photoaffinity labeling studies with the vasoactive intestinal peptide (VIP) receptor (*17*) and the calcitonin receptor (*18*) have both indicated a close physical proximity of the amino-terminal extracellular domain of the receptor and ligand. Additionally, the N-terminal extracellular domain of the mouse corticotropin-releasing factor (CRF) has been shown to be the major ligand recognition domain through chemical shift perturbation experiments (*19*). Although residues 37−63 of CLR have been identified as important for antagonist binding, other regions within CLR cannot be excluded as participating in the binding interaction. The observation that compound **3** is not completely shifted to a CTR pharmacological profile supports the idea that other residues within CLR may play a role in binding of this structural class of antagonists. Perhaps transmembrane domains 1 and 2, which were not explored and therefore cannot be ruled out, play a role in the binding interaction of compound **3**. It should be noted that competition binding experiments with compound **3** on CLR and NH_2(24−35) coexpressed with RAMP1 exhibit a Hill slope which is steeper than unity (Figure 2B). This binding deviation from homogeneity could indicate that compound **3** has multiple binding sites, lending support to the hypothesis of a transmembrane domain 1 or 2 interaction. Perhaps residues 37−63 are the key determinants to a very high affinity binding domain in CLR, and once these residues have been replaced with the corresponding region of CTR, a single site law of mass action is followed.

Compound **4** binds to the CGRP receptor through interactions with TM7 of CLR. This antagonist was designated RAMP-independent because it exhibits similar affinity for both the human and rodent CGRP receptors as well as similar binding to the adrenomedullin receptor. These observations led us to hypothesize that this antagonist may have a unique binding site within CLR as compared to compounds **1** and **3**. A TM7 interaction should not be surprising due to the numerous reports in the literature that nonpeptide GPCR antagonists often bind in TM regions (*20*−*22*). The ability of this class of compounds to prevent CGRP binding could be the result of an indirect mechanism. One possible scenario for this indirect mechanism could involve compound **4** binding in the upper region of transmembrane domain 7, resulting in a modulation in the conformation of the binding pocket of CGRP. It could also be possible that CGRP interacts in the upper region of TM7 and compound **4** could be disrupting the agonist binding site.

In conclusion, two distinct domains of CLR were identified as critical to antagonist binding. RAMP1-dependent compounds were shown to interact in the amino terminus, specifically with residues 37−63, whereas a RAMP-independent compound was shown to interact in TM7. Our results provide the first evidence that nonpeptide antagonists of the CGRP receptor interact directly with CLR. While key regions were identified within CLR, the heterodimeric nature of this receptor presents us with other potential interaction sites, perhaps transmembrane domains 1 or 2, within this receptor complex. Although defining the exact nature of the antagonist binding site will require elucidating a three-dimensional structure of the CLR−RAMP1 heterodimer, the present study has provided insight into the mode of interaction of small molecule antagonists with the CGRP receptor.

SUPPORTING INFORMATION AVAILABLE

One table listing K_d and B_{max} values for each membrane preparation. This material is available free of charge via the Internet at http://pubs.acs.org.

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