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Determinants of 1-Piperidinecarboxamide, *N*-[2-[[5-Amino-*l*-[[4-(4-pyridinyl)-*l*-piperazinyl]carbonyl]pentyl]amino]-1-[(3,5-dibromo-4-hydroxyphenyl)methyl]-2-oxoethyl]-4-(1,4-dihydro-2-oxo-3(2*H*)-quinazolinyl) (BIBN4096BS) Affinity for Calcitonin Gene-Related Peptide and Amylin Receptors—The Role of Receptor Activity Modifying Protein 1

Debbie L. Hay, George Christopoulos, Arthur Christopoulos, and Patrick M. Sexton

School of Biological Sciences, University of Auckland, Symonds Street, Auckland, New Zealand (D.L.H.); and Drug Discovery Biology Laboratory, Department of Pharmacology, Monash University, Victoria, Australia (P.M.S., A.C., G.C.)

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ABSTRACT

1-Piperidinecarboxamide, *N*-[2-[[5-amino-*l*-[[4-(4-pyridinyl)-*l*-piperazinyl]carbonyl]pentyl]amino]-1-[(3,5-dibromo-4-hydroxyphenyl)methyl]-2-oxoethyl]-4-(1,4-dihydro-2-oxo-3(2*H*)-quinazolinyl) (BIBN4096BS), a calcitonin gene-related peptide (CGRP) receptor antagonist, can alleviate the symptoms of migraine and is highly selective for CGRP over adrenomedullin (AM) receptors. These receptors are heterodimers of the calcitonin receptor-like receptor (CL) and receptor activity modifying proteins (RAMPs), with the pharmacological properties determined by the RAMP subunit. BIBN4096BS-sensitive $CGRP_1$ receptors are CL/RAMP1, whereas BIBN4096BS-insensitive AM receptors are CL/RAMP2 or CL/RAMP3 (AM_1 and AM_2, respectively), implicating RAMP1 in conferring BIBN4096BS sensitivity. Because calcitonin receptors [$CT_{(a)}$] also interact with RAMP1 [$AMY_{1(a)}$ receptors], BIBN4096BS could also have affinity for these receptors. To test this, receptors were transfected into COS-7 cells and agonist-stimulated cAMP levels measured in the presence and absence of antagonists. We found that $AMY_{1(a)}$ receptors were ~150-fold less sensitive to BIBN4096BS antagonism than $CGRP_1$ receptors. In contrast, $AMY_{3(a)}$ [$CT_{(a)}$/RAMP3] or AM_2 receptors were not sensitive to BIBN4096BS antagonism. We investigated Trp74 in RAMP1, a residue implicated in the species selectivity of BIBN4096BS. BIBN4096BS affinity was reduced at $AMY_{1(a)}$ and $CGRP_1$ receptors when this residue was mutated to lysine or alanine. The equivalent residue in RAMP3, Glu74, when mutated to tryptophan (E74W), induced BIBN4096BS sensitivity at AM_2 and $AMY_{3(a)}$ receptors. It is interesting that a selective reduction in AM potency was observed at E74W AM_2 receptors, implicating this residue in AM interactions with this receptor. These data support the importance of Trp74 in RAMP1 in the interaction of BIBN4096BS with $CGRP_1$ and $AMY_{1(a)}$ receptors and identified Glu74 in RAMP3 as the first amino acid in RAMP important for agonist interactions with calcitonin-family receptors.

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D.L.H. and G.C. contributed equally to this work.

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Calcitonin (CT) gene-related peptide (CGRP), a 37-amino acid neuropeptide, has potent effects in the vasculature and has been implicated in migraine (Brain and Grant, 2004). CGRP-like immunoreactivity is elevated in a migraine attack, and triptans normalize these levels (Edvinsson, 2001). Furthermore, infusion of CGRP into subjects prone to migraine can trigger an attack (Lassen et al., 2002). However, the most compelling evidence for the in-

ABBREVIATIONS: CT, calcitonin; AM, adrenomedullin; AMY, amylin receptor phenotype; Amy, amylin; BIBN4096BS, 1-piperidinecarboxamide, *N*-[2[[5-amino-*l*-[[4-(4-pyridinyl)-*l*-piperazinyl]carbonyl]pentyl]amino]-1-[(3,5-dibromo-4-hydroxyphenyl)methyl]-2-oxoethyl]-4-(1,4-dihydro-2-oxo-3-(2*H*)-quinazolinyl; CGRP, calcitonin gene-related peptide; CL, calcitonin receptor-like receptor; $CT_{(a)}$, calcitonin receptor; HA, hemagglutinin; hCT, human calcitonin; RAMP, receptor activity modifying protein; rAmy, rat amylin; VPAC1, vasoactive intestinal polypeptide/pituitary adenylate cyclase-activating peptide receptor 1; WT, wild type; ANOVA, analysis of variance; VIP, vasoactive intestinal peptide.

volvement of the CGRP system in the pathogenesis of migraine comes from studies using the CGRP antagonist BIBN4096BS, which was able to normalize migraine pain (Olesen et al., 2004). Together, the data show the importance of CGRP in this condition and make the study of the mode of interaction of BIBN4096BS with CGRP receptors of particular interest.

In previous studies, it has been demonstrated that BIBN4096BS is highly selective for CGRP binding sites over those for the related peptide, adrenomedullin (AM) (Doods et al., 2000; Hay et al., 2002, 2003). Likewise, this compound has been shown to have particularly high species selectivity, having at least 100-fold greater affinity for primate over rodent CGRP receptors (Doods et al., 2000; Mallee et al., 2002). CGRP receptors are heterodimers of the CT receptor-like receptor (CL), a family B G protein-coupled receptor, and receptor activity modifying protein (RAMP) 1 (McLatchie et al., 1998). Only together are fully functional $CGRP_1$ receptors formed. On the other hand, AM receptors, for which BIBN4096BS has little affinity, are also heterodimers, this time of CL with RAMP2 or RAMP3 (AM_1 and AM_2, respectively; McLatchie et al., 1998; Fraser et al., 1999; Poyner et al., 2002). Given that CL is shared between these receptors but that BIBN4096BS only weakly interacts with AM receptors, RAMP1 is strongly implicated in conferring the high-affinity BIBN4096BS interaction with the $CGRP_1$ receptor. A predominant role for RAMP1 in BIBN4096BS affinity has been confirmed through studies of chimeras of rat and human RAMP1 that identified tryptophan at position 74 of RAMP1 as a key amino acid for the affinity differences across species (Fig. 1) (Mallee et al., 2002).

In addition to heterodimerizing with CL, RAMPs functionally complex with the related CT receptor to form the AMY family of receptors, each having high affinity for the peptide hormone amylin (Amy) but a distinct specificity of interaction with the related peptides CT, CGRP, and AM (Christopoulos et al., 1999; Muff et al., 1999; Hay et al., 2006). In particular, the $AMY_{1(a)}$ receptor, the heterodimer of the $CT_{(a)}$ receptor and RAMP1, has high affinity for CGRP and thus may also be a target for BIBN4096BS with potential implication for the side-effect profile of the drug.

In this study, we explored the specificity of BIBN4096BS interaction at AMY receptors and, in particular, the contribution of Trp74 and the equivalent amino acid in other RAMPs to BIBN4096BS activity. We demonstrate that the RAMP1 containing $AMY_{1(a)}$ receptor has significant affinity for BIBN4096BS, but a high degree of selectivity is retained for the $CGRP_1$ receptor. Trp74 was a key residue for BIBN4096BS affinity for all receptor phenotypes, but the equivalent Glu74 in the AM_2 receptor also played an important role for AM potency at this receptor.

Materials and Methods

Materials. Human AM, human αCGRP, human $\alpha CGRP_{8-37}$, and human βCGRP were purchased from Bachem (Bubendorf, Switzerland). Rat Amy (rAmy) was from Auspep (Parkville, Australia). BIBN4096BS was kindly provided by Henri Doods (Boehringer Ingelheim GmbH, Ingelheim, Germany) or David Smith (AstraZeneca, Pharmaceuticals LP, Wilmington, DE) and was prepared as described previously (Hay et al., 2002); drugs from both sources had equivalent activity. Bovine serum albumin and isobutylmethylxanthine were from Sigma (St. Louis, MO) and amplified luminescent proximity homogenous assay-screen cAMP kits were purchased from Perkin Elmer (Boston, MA). Dulbecco's modified Eagle's medium, fetal bovine serum, and HEPES were from Invitrogen (Carlsbad, CA). Cell culture plastic ware was manufactured by Nunc (Roskible, Denmark), and Metafectine was purchased from Scientifix (Cheltenham, VIC, Australia). ^{125}I-labeled goat anti-mouse IgG (^{125}I-IgG) was obtained from Perkin Elmer. Na-^{125}I (100 mCi/ml) was supplied by ICN Biochemicals (Irvine, CA). *N*-Succinimidyl 3,4-hydroxy,5,-[^{125}I]iodophenyl)propionate (Bolton-Hunter reagent; 2000 Ci/mmol) was from Amersham (Little Chalfont, Buckinghamshire, UK). ^{125}I-rAmy (specific activity, 2000 Ci/mmol) was iodinated by the Bolton-Hunter method and purified by reverse-phase high-performance liquid chromatography as described previously (Bhogal et al., 1992). All other reagents were of analytical grade.

Expression Constructs and Site-Directed Mutagenesis. Double hemagglutinin (HA) epitope-tagged human $CT_{(a)}$ receptor was prepared as described previously (Pham et al., 2004). This receptor is the Leu447 polymorphic variant of the receptor (Kuestner et al., 1994). Human RAMP1, human N-terminally tagged mycRAMP1, RAMP3, and human CL receptor were gifts from Dr. Steven Foord (McLatchie et al., 1998). The vasoactive intestinal polypeptide/pituitary adenylate cyclase activating peptide receptor 1 (VPAC1 receptor) cDNA was a gift from Dr. Marc Laburthe (Couvineau et al., 1994). Single point mutations in the RAMPs were generated using the QuikChange method according to the manufacturer's instructions (Stratagene, La Jolla, CA).

Cell Culture and Transfection. In most experiments, COS-7 cells were subcultured and transfected as described previously (Zumpe et al., 2000; Hay et al., 2005). In experiments using the VPAC1 receptor, COS-7 cells were cultured in a similar manner, but cells were transfected with 0.25 μg of DNA per well (0.125 μg of VPAC1 with either 0.125 μg of pcDNA3 or 0.125 μg of mycRAMP1) in 96-well plates using polyethylenimine (Bailey and Hay, 2006).

Measurement of cAMP Production. Cells transfected with various receptor components were harvested approximately 40 h after transfection, and cAMP assays were performed as described previously (Hay et al., 2005). Agonists with or without antagonists were added to 384-well plates, and then transfected cell suspensions (20,000 cells/well) were added to this mixture for 30 min at 37°C before lysis and assay of cAMP content by amplified luminescent proximity homogenous assay screen (Hay et al., 2005). In experiments using the VPAC1 receptor, cAMP was measured using a radioreceptor assay as described previously (Bailey and Hay, 2006).

Radioligand Binding: ^{125}I-rAmy and ^{125}I-IgG. ^{125}I-rAmy binding was performed to confirm the interaction of mutant RAMPs with $CT_{(a)}$ at the cell surface and was undertaken as described previously

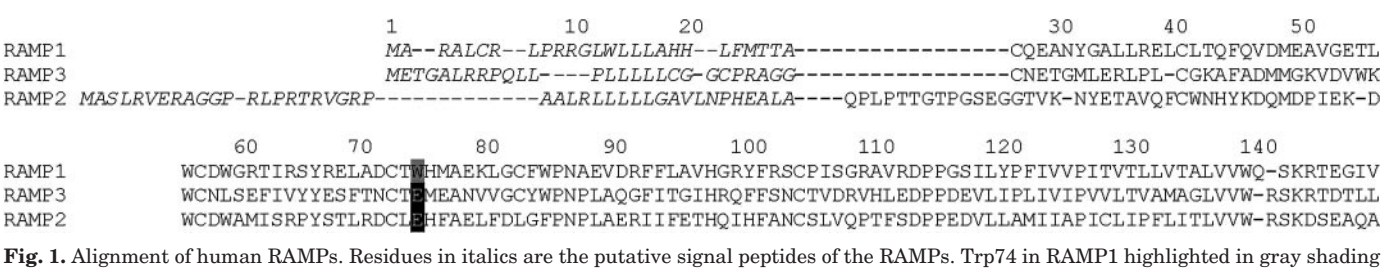

Fig. 1. Alignment of human RAMPs. Residues in italics are the putative signal peptides of the RAMPs. Trp74 in RAMP1 highlighted in gray shading and the equivalent residues (glutamic acid) in RAMP2 and -3 are shown in white text on black.

(Hay et al., 2005). Likewise, cell surface expression of CL/RAMP complexes was assessed by detecting antibody bound to the N-terminal HA tag on CL (Hay et al., 2005). Mutant specific binding was expressed as a percentage of appropriate wild-type (WT)-specific binding (100%) for individual transfection days, and the individual values were combined to give mean data as shown in Fig. 5, A and 1B. For analysis of the dissociation kinetics of ^{125}I-Amy at the $AMY_{1(a)}$ receptor, COS-7 cells were seeded into 48-well plates at 90 to 100% confluence and transfected with 50 ng of $CT_{(a)}$ and 75 ng of hRAMP1 per well. Forty-eight hours after transfection, the cells were lysed, washed, and then preincubated for 30 min at 37°C in binding buffer (serum-free Dulbecco's modified Eagle's medium, 0.1% bovine serum albumin, and 0.45 M sucrose), with approximately 70,000 cpm of ^{125}I-rAmy dissociation. Immediately after this, each well was incubated with unlabeled rAmylin (10^{-6} M) alone or together with BIBN4096BS (10^{-6} M). Cells were then incubated at 37°C to determine the rate of ^{125}I-rAmy. At each time point wells were aspirated, and cells were washed once with ice-cold phosphate-buffered saline. The cells were then solubilized with 0.5 M NaOH. The level of radioactivity in the samples was determined on a Wallac γ-counter (PerkinElmer Wallac, Gaithersburg, MD).

Data Analysis and Statistics. GraphPad Prism 4.02 (GraphPad Software Inc., San Diego, CA) was used to analyze data. Agonist pEC_{50} (potency) and antagonist affinity values were calculated as described previously (Hay et al., 2005). Antagonist data were analyzed using Global Schild analysis (Motulsky and Christopoulos, 2004) to generate an estimate of the pA_2, which is the negative logarithm of the antagonist concentration that shifts the agonist EC_{50} value rightward by a factor of 2. Schild slopes were found not to differ significantly from unity and were therefore constrained to 1, and as such, antagonist affinity values are reported as pK_B values. For dissociation kinetics, experimental data were fit to a one-phase exponential decay curve to determine the dissociation rate *K*. Data shown are mean ± S.E.M. of multiple experiments performed in triplicate unless otherwise indicated. Comparisons between these values were performed using paired or unpaired *t* tests or one-way ANOVA as appropriate. Binding data were analyzed by ANOVA or paired *t* test as appropriate. Statistical differences between dissociation kinetics experiments was assessed by the *F* test within Prism 4. Significance was achieved at $p < 0.05$.

Results

Interaction of BIBN4096BS with CL, $CT_{(a)}$, and VPAC1-Based Receptors. Incubation of human $CT_{(a)}$ with

TABLE 1

Affinity (pK_B) of BIBN4096BS for antagonizing agonist-induced cAMP accumulation at WT human AMY, CT, $CGRP_1$, and AM_2 receptors

Data are presented as mean ± S.E.M. The values in parentheses represent the number of individual experiments performed.

	$AMY_{1(a)}$	$AMY_{3(a)}$	$CT_{(a)}$	$CGRP_1$	AM_2
hCT	<5 (4)	–[a]	<5 (6)		
rAmy	7.44 ± 0.2 (7)	≥5 (11)[b]	<5 (6)		
hβCGRP	7.67 ± 0.17 (12)			10.14 ± 0.44 (3)	6.29 ± 0.16 (4)
hαCGRP	7.49 ± 0.27 (10)			9.73 ± 0.24 (3)	
Tyr0- hαCGRP	8.74 ± 0.33 (4)*				
hAM					6.39 ± 0.25 (4)

* $p < 0.05$, Tyr0- hαCGRP versus rAmy, hβCGRP, and hαCGRP by one-way ANOVA followed by Tukey's test.
[a] BIBN4096BS pK_B values were only determined for peptides for which full dose-response curves at a specific receptor could be achieved.
[b] For 8 of 11 experiments, a pK_B value could not be determined, but on three occasions the pK_B was equal to 5.4.

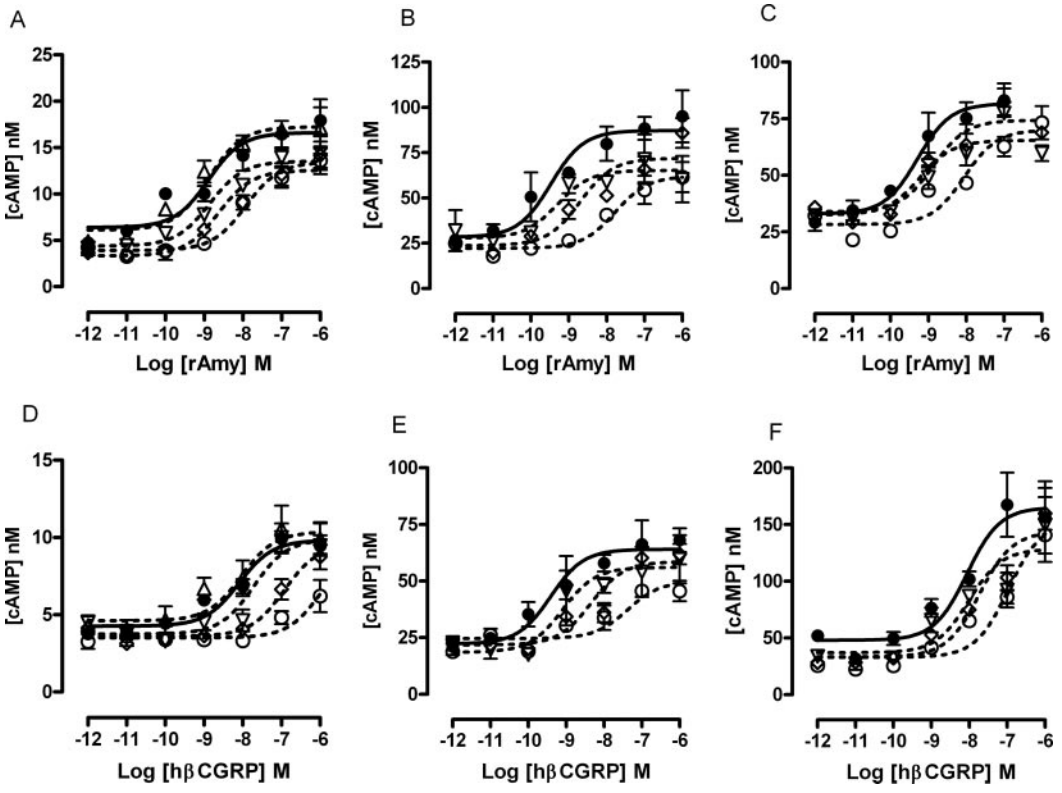

Fig. 2. BIBN4096BS antagonism of $AMY_{1(a)}$ receptors with WT RAMP1 (A and D), W74A RAMP1 (B and E), or W74K RAMP1 (C and F) using either rAmy (A–C) or βCGRP (D–F) as agonists. ●, control (agonist alone); △, $+10^{-8}$ M antagonist; ▽, $+10^{-7}$ M antagonist; ◇, $+10^{-6}$ M antagonist; ○, $+10^{-5}$ M antagonist.

BIBN4096BS (up to 10 μM) in the presence of hCT or rAmy did not significantly attenuate the agonist response (Table 1). However, when human $CT_{(a)}$ was cotransfected with human RAMP1 to give $AMY_{1(a)}$ receptors, rAmy but not hCT could be antagonized (Table 1 and Fig. 2A). Other agonists of the $AMY_{1(a)}$ receptor; hαCGRP and hβCGRP (Fig. 2D), were also antagonized by BIBN4096BS with a pK_B value similar to that of rAmy (Table 1). It is interesting that when Tyr0-hαCGRP was used as the agonist of this receptor, the pK_B value was approximately 10-fold higher (Table 1). BIBN4096BS was not an effective antagonist of rAmy responses at $AMY_{3(a)}$ (Table 1 and Fig. 3A) and was not able to effectively shift the concentration-effect curve to rAmy even at a concentration of 10 μM.

We compared the effect of BIBN4096BS at $CT_{(a)}$-based receptors with those at CL-based receptors. As expected, BIBN4096BS was potent at inhibiting CGRP responses at $CGRP_1$ receptors (Table 1) but was a very weak antagonist of AM and βCGRP responses at AM_2 receptors (Table 1). The difference in antagonist potency (BIBN4096BS versus hαCGRP) between $CGRP_1$ and $AMY_{1(a)}$ receptors was approximately 150-fold.

Given that the VPAC1 receptor also interacts with RAMP1 (Christopoulos et al., 2003), we investigated whether BIBN4096BS could also inhibit vasoactive intestinal peptide (VIP)-stimulated cAMP responses in cells transfected with both the VPAC1 receptor and RAMP1. However, unlike with CL and $CT_{(a)}$ receptors, RAMP1 did not confer BIBN4096BS

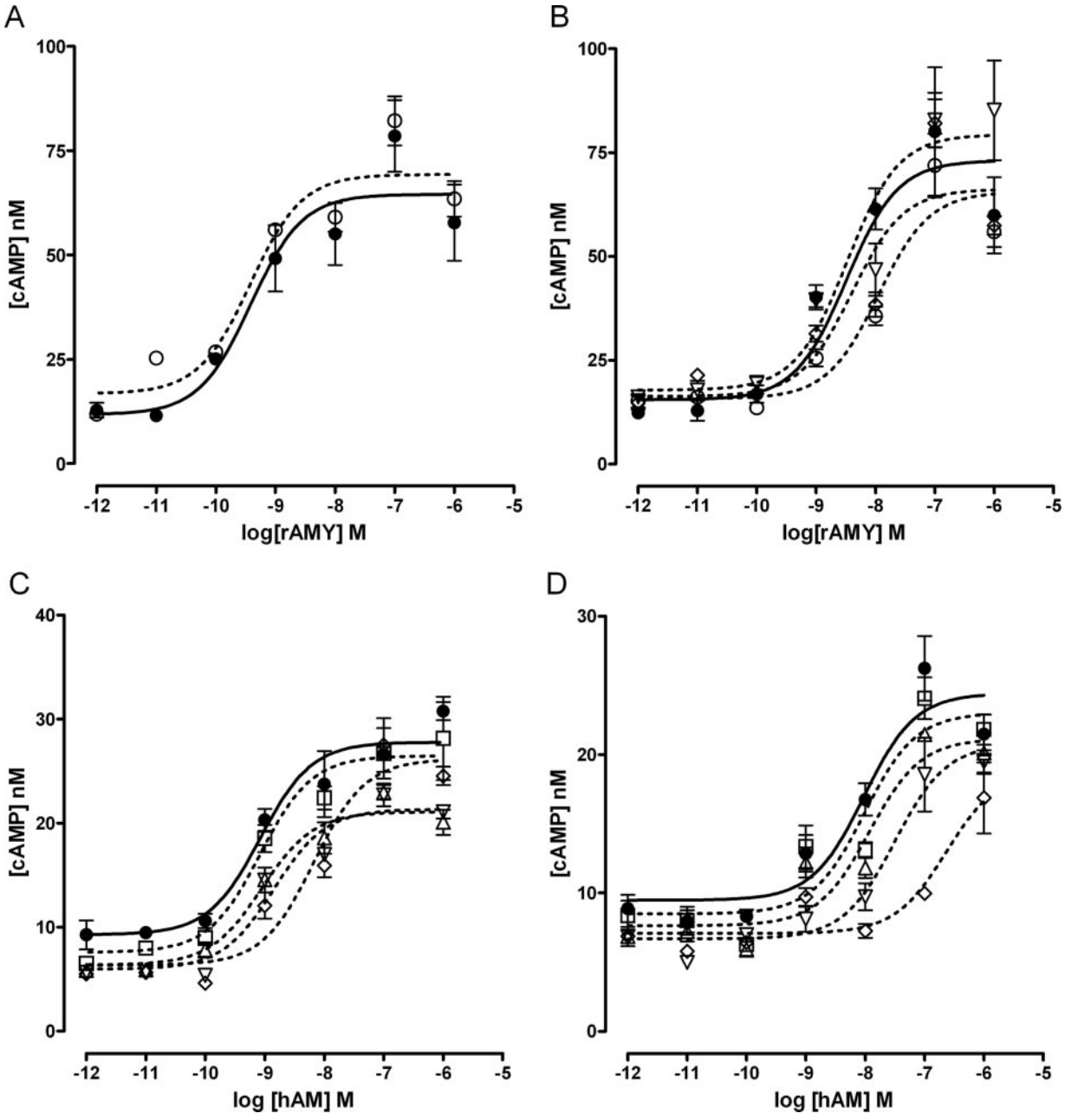

Fig. 3. BIBN4096BS antagonism of $AMY_{3(a)}$ receptors with WT RAMP3 (A) or E74W RAMP3 (B) using rAmy as the agonist or AM_2 receptors with WT RAMP3 (C) or E74W RAMP3 (D) using hAM. ●, control (agonist alone); □, $+10^{-9}$ M antagonist; △, $+10^{-8}$ M antagonist; ▽, $+10^{-7}$ M antagonist; ◇, $+10^{-6}$ M antagonist; and ○, $+10^{-5}$ M antagonist.

affinity upon the VPAC1 receptor. In VPAC1/vector (pcDNA3)-transfected cells, VIP stimulated cAMP production with a pEC_{50} value of 10.35 ± 0.13 (n = 3). In the presence of 10 μM BIBN4096BS there was no shift in the VIP concentration-effect curve (pEC_{50} = 10.36 ± 0.15, n = 3). Likewise, in VPAC1/mycRAMP1-transfected cells, VIP stimulated cAMP production with pEC_{50} values of 10.28 ± 0.08 (n = 3) without BIBN4096BS and 10.38 ± 0.12 (n = 3) in the presence of 10 μM BIBN4096BS.

Modulation of ^{125}I-Amy Dissociation from $AMY_{1(a)}$ Receptors by BIBN4096BS. The agonist-dependent pK_B values of BIBN4096BS at the $AMY_{1(a)}$ receptor suggested that the antagonist may be acting allosterically at the receptor. To empirically test this, we determined the rate of ^{125}I-Amy dissociation in the presence and absence of BIBN4096BS. BIBN4096BS caused an acceleration of ^{125}I-Amy dissociation kinetics from a $t_{1/2}$ of 12.32 to 8.85 min (K = 0.056 ± 0.005 versus K = 0.078 ± 0.007, respectively; $p < 0.05$, F test) (Fig. 4). Because competitive orthosteric ligands cannot alter ligand dissociation (Christopoulos and Kenakin, 2002), these data indicate that BIBN4096BS is acting allosterically at the $AMY_{1(a)}$ receptor.

Interaction of BIBN4096BS with $CGRP_1$ and $AMY_{1(a)}$ Receptors: W74K RAMP1 Mutation. In binding studies, Trp74 in human RAMP1 was shown previously to be a vital component of the species selectivity (human > rat) that BIBN4096BS displays (Mallee et al., 2002). Therefore we investigated the role of this residue in determining BIBN4096BS affinity for $AMY_{1(a)}$ and $CGRP_1$ receptors. When tryptophan was mutated to lysine (W74K), the antagonistic potency of BIBN4096BS was significantly decreased; against rAmy and βCGRP at W74K $AMY_{1(a)}$ receptors (Table 2 and Fig. 2, C and F) and against α- or βCGRP at W74K $CGRP_1$ receptors compared with WT $AMY_{1(a)}$ or $CGRP_1$ receptors (Table 2). Of note, the BIBN4096BS potency at the $CGRP_1$ receptor mutant was agonist-dependent, with greater impact on βCGRP- versus αCGRP-mediated responses. In contrast, sCT_{8-32} and $CGRP_{8-37}$, other antagonists of the $AMY_{1(a)}$ receptor (Hay et al., 2005), were not affected (Table 2), suggesting a selective perturbation of BIBN4096BS interaction. Likewise, antagonism by $CGRP_{8-37}$ at $CGRP_1$ receptors was not different with WT or W74K RAMP1 (Table 2). This mutation did not perturb agonist responses or expression of the receptors (Table 3 and Fig. 5, ■).

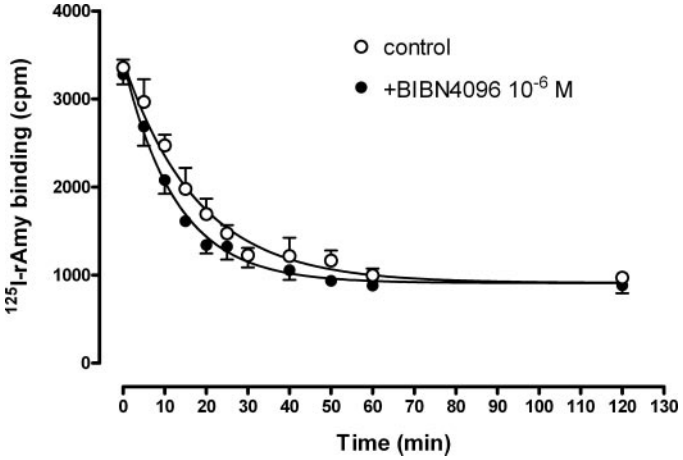

Fig. 4. Dissociation of ^{125}I-Amy binding to $AMY_{1(a)}$ receptors in the presence (●) or absence (○) of BIBN4096BS 10^{-6} M. Lysed cells expressing the $AMY_{1(a)}$ receptor were prebound for 30 min with ^{125}I-Amy. Lysates were washed briefly, and dissociation was assessed through further incubation, at 37°C, in the presence of excess (10^{-6} M) unlabeled rat amylin to prevent rebinding of the radioligand.

Interaction of BIBN4096BS with $CGRP_1$ and $AMY_{1(a)}$ Receptors: W74A RAMP1 Mutation. To delve further into the role of Trp74 in governing antagonism of $CGRP_1$ and $AMY_{1(a)}$ receptors by BIBN4096BS, the human RAMP1 tryptophan to alanine (W74A) mutant was generated. This mutant had similar properties to W74K in that BIBN4096BS pK_B values were lower against both receptors generated using this mutant than against wild-type receptors (Table 2 and Fig. 2B), although the effect was not as pronounced as W74K against rAmy at $AMY_{1(a)}$. The W74A mutation also led to reduced BIBN4096BS antagonism compared with WT with hβCGRP as the agonist at $AMY_{1(a)}$ receptors (Table 2 and Fig. 2E) or with hα- or βCGRP at $CGRP_1$ receptors (Table 2). In accordance with the data for W74K, agonists (Table 3) other antagonists (Table 2), and receptor expression (Fig. 5, A and B, ■) were not affected by the W74A mutation at either receptor.

E74W RAMP3 Mutation. Given the apparent importance of tryptophan at position 74 in human RAMP1 in determining BIBN4096BS interactions with CGRP and AMY receptors, we hypothesized that by introducing this residue into a BIBN4096BS-insensitive RAMP we might be able to achieve BIBN4096BS affinity for the receptor. In RAMP3, Trp74 is Glu74 (Fig. 1), and this RAMP affords only very weak antagonism by BIBN4096BS (Table 1 and Fig. 3, A and C; Hay et al., 2002). Here, we mutated Glu74 to tryptophan (E74W) in human RAMP3 and evaluated the ability of BIBN4096BS to antagonize rAmy responses at mutant or WT $AMY_{3(a)}$ receptors and hAM or hβCGRP responses at AM_2 receptors. Consistent with our predictions, we were able to induce increased BIBN4096BS affinity (albeit weakly) for the E74W $AMY_{3(a)}$ receptor (Table 2 and Fig. 3B) and gained an approximate 10-fold increase in affinity for the E74W AM_2 receptor (Table 2 and Fig. 3D). It is surprising that this point mutation led to a significant and selective decrease in AM potency at AM_2 receptors (Table 3 and Fig. 6); two other agonists of this receptor (hα- and βCGRP) were not affected (Table 3). This reduction in agonist potency does not seem to be the result of a loss of cell surface expression of the mutant receptor (Fig. 5A, □). Maximal cAMP stimulation in response to AM was not significantly different between WT (17.7 ± 2.6 nM, n = 4) and E74W AM_2 receptors (13.0 ± 1.5 nM, n = 4). At $AMY_{3(a)}$, a very small decrease in rAmy potency was observed (Table 3); this was accompanied by an apparent decrease in the total amount of Amy binding, but this did not reach significance (Fig. 5B, ■).

The role of amino acid 74 in RAMP3 for AM potency was further interrogated by mutation of glutamic acid to either glutamine (E74Q), aspartic acid (E74D), or lysine (E74K). For the E74D, mutant no significant change in AM potency was observed, whereas removal of the charge in the E74Q mutant led to a small decrease in AM potency (Table 3). Of the three mutants, E74K led to the greatest decrease in AM potency (Table 3), albeit to a lesser extent than seen for the E74W mutant.

Discussion

BIBN4096BS, a high-affinity CGRP receptor antagonist has shown proof of concept in the clinic for this class of agent in the treatment of migraine (Olesen et al., 2004). This antagonist is approximately 100-fold more selective for primate than rodent CGRP receptors (Doods et al., 2000), and it has been documented that amino acid position 74 in RAMP1 is the major determinant of this species selectivity (Mallee et al., 2002). Human RAMP1 contains tryptophan at this position, whereas in rat RAMP1, this is lysine (Fig. 1). In addition to species selectivity, BIBN4096BS also has considerable selectivity for CGRP over AM receptors (at least 1000-fold; Hay et al., 2002, 2003; Table 1). This is interesting given that these receptors share the same G protein-coupled receptor subunit, CL, and therefore, RAMP1 itself is implicated in determining the affinity of the $CGRP_1$ receptor for BIBN4096BS.

In the current study, we demonstrate that BIBN4096BS does not have significant affinity for $CT_{(a)}$ receptors, consistent with the inability of BIBN4096BS to displace CT from its binding sites (Doods et al., 2000). In contrast, when human $CT_{(a)}$ was cotransfected with human RAMP1 to give $AMY_{1(a)}$ receptors, rAmy but not hCT responses could be antagonized. The lack of CT antagonism is consistent with CT mediating its agonistic actions through a population of $CT_{(a)}$ receptors that are not associated with RAMP1 (Hay et al., 2005). Other agonists of the $AMY_{1(a)}$ receptor, hαCGRP and hβCGRP, were similarly antagonized by BIBN4096BS, although when Tyr0-hαCGRP was used as the agonist of this receptor, the pK_B value was approximately 10-fold higher. Although the mechanistic basis for this is unclear, there are at least two potential explanations for the observation: 1) Tyr0-hαCGRP may differentially, to other agonists, modulate the receptor (e.g., via phosphorylation or recruitment of

TABLE 2

pK_B values for antagonists at mutant or WT receptors

Data are presented as mean ± S.E.M. The values in parentheses represent the number of individual experiments performed.

Receptor	Agonist	Antagonist		
		sCT_{8-32}	$CGRP_{8-37}$	BIBN4096BS
WT $AMY_{1(a)}$	rAmy	7.78 ± 0.13 (11)[e]	6.62 ± 0.13 (11)[e]	7.44 ± 0.2 (7)
W74A $AMY_{1(a)}$	rAmy	7.48 ± 0.33 (3)	6.39 ± 0.48 (3)	6.50 ± 0.16 (3)*
W74K $AMY_{1(a)}$	rAmy	7.47 ± 0.24 (5)	6.15 ± 0.15 (5)	5.67 ± 0.18 (5)**
WT $AMY_{1(a)}$	hβCGRP	7.68 ± 0.18 (12)[e]	6.78 ± 0.13 (14)[e]	7.67 ± 0.17 (12)
W74A $AMY_{1(a)}$	hβCGRP	7.28 ± 0.29 (4)	6.25 ± 0.26 (4)	6.24 ± 0.32 (5)††
W74K $AMY_{1(a)}$	hβCGRP	7.86 ± 0.25 (3)	6.23 ± 0.56 (3)	6.25 ± 0.22 (3)††
WT $AMY_{3(a)}$	rAmy	7.92 ± 0.19 (6)[e]	6.17 ± 0.26 (7)[e]	≥ 5[f] (11)
E74W $AMY_{3(a)}$	rAmy	8.28 ± 0.16 (4)	5.85 ± 0.15 (4)	5.6 ± 0.12 (4)
WT $CGRP_1$	hαCGRP		7.60 ± 0.44 (4)	10.14 ± 0.44 (3)
W74A $CGRP_1$	hαCGRP		7.66 ± 0.30 (4)	8.59 ± 0.18 (4)[a]
W74K $CGRP_1$	hαCGRP		8.02 ± 0.25 (4)	8.41 ± 0.24 (4)[aa]
WT $CGRP_1$	hβCGRP		7.00 ± 0.43 (3)	9.73 ± 0.24 (3)
W74A $CGRP_1$	hβCGRP		7.26 ± 0.35 (4)	8.45 ± 0.31 (4)[bb]
W74K $CGRP_1$	hβCGRP		7.25 ± 0.25 (4)	7.49 ± 0.12 (4)[bb]
WT AM_2	hAM		6.51 ± 0.06 (4)	6.39 ± 0.25 (4)
E74W AM_2	hAM		6.14 ± 0.13 (3)	7.56 ± 0.11 (4)[cc]
WT AM_2	hβCGRP		6.02 ± 0.38 (3)	6.29 ± 0.16 (4)
E74W AM_2	hβCGRP		5.96 ± 0.23 (3)	7.35 ± 0.19 (3)[d]

* $p < 0.05$, ** $p < 0.01$ versus WT $AMY_{1(a)}$ for rAmy with BIBN4096BS.
†† $p < 0.01$ versus WT $AMY_{1(a)}$ for βCGRP with BIBN4096BS.
[a] $p < 0.05$, [aa] $p < 0.01$ verus WT $CGRP_1$ for hαCGRP with BIBN4096BS.
[bb] $p < 0.01$ versus WT $CGRP_1$ for hβCGRP with BIBN4096BS.
[cc] $p < 0.01$ versus WT AM_2 for hAM with BIBN4096BS.
[d] $p < 0.05$ versus WT AM_2 for hβCGRP with BIBN4096BS, one-way ANOVA followed by Dunnett's multiple comparison test.
[e] Data from Hay et al. (2005); experiments were performed in parallel with this study.
[f] For 8 of 11 experiments, a pK_B value could not be determined, but on three occasions, the pK_B was equal to 5.4.

TABLE 3

Agonist potencies (pEC_{50}) for stimulation of cAMP production by mutant or WT AMY, CGRP, or AM receptors.

Data are presented as mean ± S.E.M. The values in parentheses represent the number of individual experiments performed.

	rAmy	hβCGRP	hαCGRP	hAM
WT $AMY_{1(a)}$	8.98 ± 0.19 (5)	9.17 ± 0.41 (3)		
W74A $AMY_{1(a)}$	9.05 ± 0.37 (3)	8.84 ± 0.28 (4)		
W74K $AMY_{1(a)}$	9.27 ± 0.12 (5)	9.44 ± 0.09 (3)		
WT $AMY_{3(a)}$	9.17 ± 0.05 (4)			
E74W $AMY_{3(a)}$	8.93 ± 0.06 (4)*			
WT $CGRP_1$		9.76 ± 0.14 (11)	9.52 ± 0.08 (12)	
W74A $CGRP_1$		9.97 ± 0.1 (4)	9.7 ± 0.06 (4)	
W74K $CGRP_1$		9.85 ± 0.07 (4)	9.73 ± 0.09 (4)	
WT AM_2		7.36 ± 0.14 (5)	6.67 ± 0.2 (4)	9.21 ± 0.08 (4)
E74W AM_2		7.34 ± 0.11 (3)	7.01 ± 0.16 (3)	7.91 ± 0.19 (4)†††
E74Q AM_2				8.88 ± 0.07 (4)*
E74D AM_2				8.94 ± 0.10 (4)
E74K AM_2				8.72 ± 0.10 (4)*

* $p < 0.05$, WT $AMY_{3(a)}$ rAmy versus E74W $AMY_{3(a)}$ or WT AM_2 versus E74Q or E74K AM_2 by t test.
††† $p < 0.001$, WT AM_2 hAM versus E74W AM_2 by t test.

interacting proteins), leading to a population of receptors with altered affinity for BIBN4096BS; 2) BIBN4096BS, which is structurally distinct from peptide ligands, may be an allosteric modulator of the $AMY_{1(a)}$ receptor; it is well-established that the magnitude of interaction between allosteric antagonists and orthosteric agonists is dependent on the type of agonist that is used (May et al., 2004). Consistent with the latter hypothesis, BIBN4096BS caused acceleration of the dissociation of ^{125}I-rAmy, indicating that it does, indeed, act allosterically. Overall, BIBN4096BS had approximately 150-fold selectivity for $CGRP_1$ over $AMY_{1(a)}$ receptors in this study. At $AMY_{3(a)}$, BIBN4096BS was not an effective antagonist of rAmy responses, consistent with RAMP1 being a key determinant of BIBN4096BS affinity. Doods et al. (2000) reported previously that BIBN4096BS did not have significant affinity for Amy binding sites; however, they provided few details, and thus, it is difficult to make comparisons between studies. It is possible that the preparation they used contained RAMP2- or -3-enriched AMY receptors, explaining the lack of competitive binding by BIBN4096BS, although, given that BIBN4096BS can act allosterically to modulate the $AMY_{1(a)}$ receptor, this may not be evident in competitive binding assays. Given that VPAC1 receptors are able to interact with RAMP1 (Christopoulos et al., 2003) and thus could be potential targets for BIBN4096BS, we investigated VIP antagonism by this compound in the presence and absence of RAMP1. In neither circumstance did BIBN4096BS show significant affinity for this receptor. Therefore it seems that whereas RAMP1 is a critical determinant of BIBN4096BS affinity for $CGRP_1$ and $AMY_{1(a)}$ receptors, CL and $CT_{(a)}$ are also important, the data supporting the interaction of BIBN4096BS at the interface between these proteins. Salvatore et al. (2006) came to similar conclusions in their recent investigation of the CL receptor domains involved in the binding of the BIBN4096BS analog, compound 1, in which the region delimited by amino acids 37 to 63 was implicated.

Recently, we reported that the $AMY_{1(a)}$ receptor may represent one form of the "$CGRP_2$" receptor (Hay et al., 2005). Our data suggest that, used carefully, BIBN4096BS may be a useful tool for the discrimination of potential CGRP receptor subtypes.

We went on to investigate the role of Trp74 in RAMP1 in determining the affinity of BIBN4096BS for $CGRP_1$ and $AMY_{1(a)}$ receptors. As predicted from previous observations with rat CL and K74W rat RAMP1 (Mallee et al., 2002), mutation of Trp74 to lysine selectively reduced BIBN4096BS potency at both receptors. The W74A mutant essentially had similar properties to W74K, confirming the requirement for tryptophan at this position. It is interesting that there were agonist-dependent effects of these mutations. For example, the inhibition of rAmy responses by BIBN4096BS at $AMY_{1(a)}$

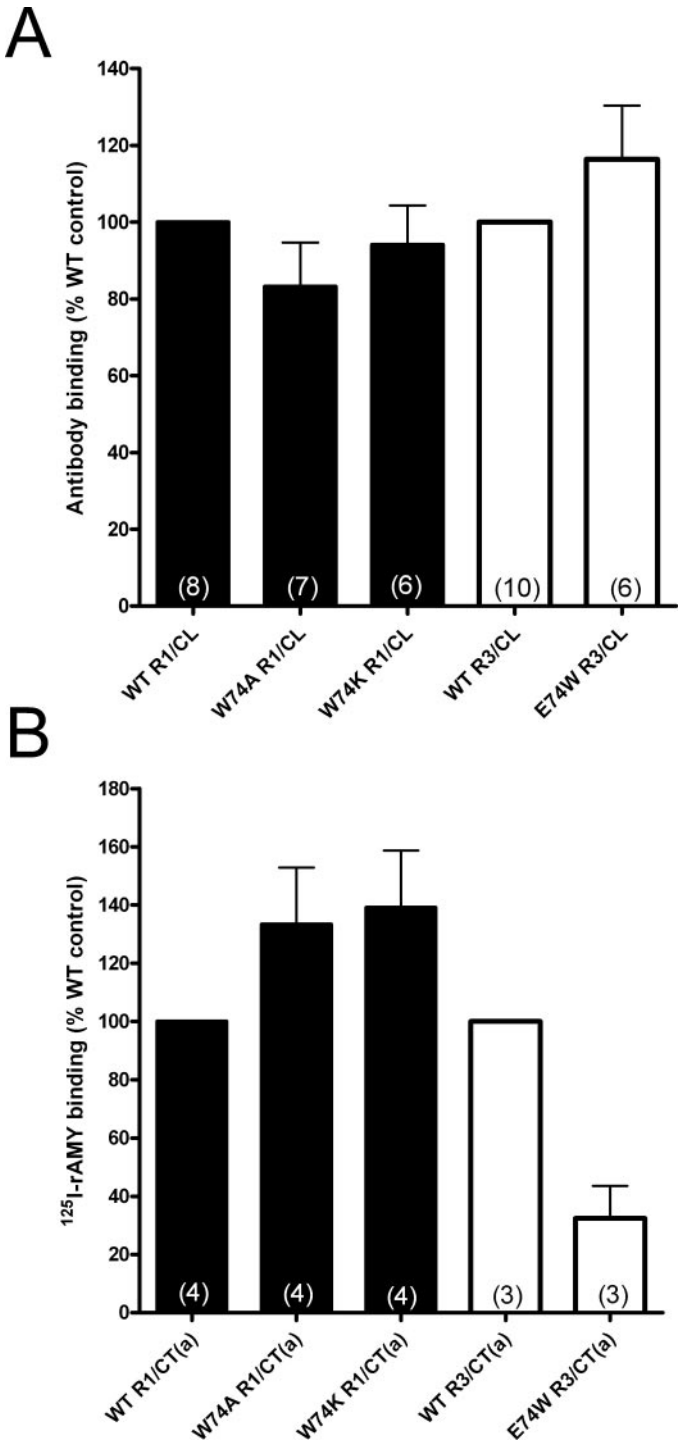

Fig. 5. A, cell surface expression of HA-tagged CL in complexes with various RAMPs as measured by antibody binding. Data are expressed as a percentage of specific binding in WT receptor-expressing cells. B, specific ^{125}I-Amy binding at mutant and WT $AMY_{1(a)}$ and $AMY_{3(a)}$ receptors. Data are expressed as a percentage of specific binding in WT receptor-expressing cells. In both parts A and B, data shown are mean ± S.E.M. of 3 to 10 experiments performed in duplicate. For each data set, *n* is shown in parentheses. There were no significant differences.

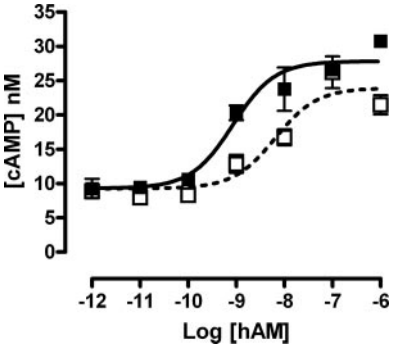

Fig. 6. Stimulation of cAMP by hAM at WT AM_2 receptors (■) or E74W RAMP3 AM_2 receptors (□).

receptors were perturbed to a greater extent with W74K than with W74A, whereas with βCGRP, both mutations diminished the interaction to a similar extent, providing additional evidence that BIBN4096BS may be acting allosterically. Likewise, at the W74K $CGRP_1$ receptor mutant, agonist-dependent differences in BIBN4096BS pK_B values were observed for α- versus βCGRP. Although, based on competitive binding studies, it has been suggested that BIBN4096BS is a competitive antagonist of $CGRP_1$ receptors, there are marked differences in the level of dissociated [^{3}H]BIBN4096BS in the presence of an equivalent molar excess of unlabeled BIBN4096BS or CGRP (Schindler and Doods, 2002). This behavior is, in fact, consistent with an allosteric interaction rather than a competitive interaction. It should be noted that strongly negatively cooperative ligands seem to behave as competitive inhibitors of binding (Christopoulos and Kenakin, 2002), which may explain the apparent discrepancy in the different binding studies and the apparent Schild slope of 1 seen in the current antagonist studies.

Trp74 in BIBN4096BS-sensitive human RAMP1 is Glu74 in BIBN4096BS-insensitive human RAMP3. We made human RAMP3 RAMP1-like by mutating Glu74 to tryptophan. This substitution engendered increases in BIBN4096BS potency at both $AMY_{3(a)}$ and AM_2 receptors. There was a slight reduction in rAmy potency at E74W $AMY_{3(a)}$ receptors, which may have been due to reduced expression of this mutant relative to the WT receptor complex (Fig. 5B). Of most interest was the selective decrease in AM potency observed at E74W AM_2 receptors (hα- and βCGRP were not affected) in the face of equal expression to WT AM_2 receptors. Interrogation of the nature of the interaction through additional mutation to aspartic acid, glutamine, or lysine indicated that, although the negative charge of Glu74 had a small overall contribution to AM potency, the introduction of a positive charge was detrimental to peptide activity. Nonetheless, the greatest effect was observed with the tryptophan substitution, suggesting that the bulky aromatic ring of the tryptophan may modulate AM potency through steric hindrance. The data suggest that Glu74 in RAMP3 could be important for hAM interactions with AM_2 receptors; this is the first single-amino acid residue in RAMP identified as important for agonist interactions with any receptor from the CT family. It is noteworthy that this residue is conserved in RAMP2 and could also be important for AM interactions with this receptor. We are currently investigating this possibility.

In summary, we have characterized the role of Trp74 in human RAMP1 in determining BIBN4096BS affinity for $CGRP_1$ and $AMY_{1(a)}$ receptors and have shown that RAMP3-containing AM and AMY receptors can be made BIBN4096BS-sensitive by introducing this residue, suggesting that there is a high degree of conservation in the tertiary structure of RAMPs 1 and 3. In the course of this study, we also identified the importance of Glu74 in human RAMP3 for AM interactions with AM_2 receptors. Together, the data support the hypothesis that RAMP is not a passive bystander but actively contributes to the binding of peptidic and nonpeptidic ligands of CT family receptors.

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Address correspondence to: Dr. Patrick M. Sexton, Drug Discovery Biology Laboratory, Department of Pharmacology, Monash University, Victoria 3800, Australia. E-mail: patrick.sexton@med.monash.edu.au