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Pharmacological discrimination of calcitonin receptor - receptor activity modifying protein complexes

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Running title: Molecularly and pharmacologically distinct amylin receptors

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Abbreviations used: AM: Adrenomedullin; AMY: Amylin; BSA: bovine serum albumin; CGRP: calcitonin gene-related peptide; CL; calcitonin receptor-like receptor; $CT_{(a)}$: calcitonin receptor; DMEM: Dulbecco's modified Eagles medium; EDTA: ethylenediamine tetraacetic acid; FBS: fetal bovine serum; GPCR: G protein coupled receptor; HA: hemagglutinin; HEPES: 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; IMD: intermedin, IBMX: isobutylmethylxanthine; PBS: phosphate-buffered saline; RAMP: receptor activity modifying protein.

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Abstract

Calcitonin (CT) receptors dimerise with receptor activity modifying proteins (RAMPs) to create high affinity amylin (AMY) receptors, but there is no reliable means of pharmacologically distinguishing these receptors. We used agonists and antagonists to define their pharmacology, expressing the $CT_{(a)}$ receptor alone or with RAMPs in COS-7 cells and measuring cAMP accumulation. Intermedin (IMD) short (IMDS), otherwise known as adrenomedullin 2, mirrored the action of αCGRP, being a weak agonist at $CT_{(a)}$, $AMY_{2(a)}$ and $AMY_{3(a)}$ receptors but considerably more potent at $AMY_{1(a)}$ receptors. Similarly, the linear CGRP analogues, $(Cys(ACM)^{2,7})$hαCGRP and $(Cys(Et)^{2,7})$hαCGRP were only effective at $AMY_{1(a)}$ receptors, but were partial agonists. As previously observed in COS-7 cells, there was little induction of the $AMY_{2(a)}$ receptor phenotype, thus $AMY_{2(a)}$ was not examined further in this study. The antagonist peptide sCT_{8-32} did not discriminate strongly between CT and AMY receptors, however, AC187 was a more effective antagonist of AMY responses at AMY receptors, and AC413 additionally showed modest selectivity for $AMY_{1(a)}$ over $AMY_{3(a)}$ receptors. $CGRP_{8-37}$ also demonstrated receptor-dependent effects. $CGRP_{8-37}$ more effectively antagonised AMY at $AMY_{1(a)}$ than $AMY_{3(a)}$ receptors although it was only a weak antagonist of both, but did not inhibit responses at the $CT_{(a)}$ receptor. Low $CGRP_{8-37}$ affinity and agonism by linear CGRP analogues at $AMY_{1(a)}$ are the classical signature of a $CGRP_2$ receptor. Our data indicate that careful use of combinations of agonists and antagonists allow pharmacological discrimination of $CT_{(a)}$, $AMY_{1(a)}$ and $AMY_{3(a)}$ receptors providing a means to delineate the physiological significance of these receptors.

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Introduction

The peptides classically designated as calcitonin (CT) peptide family members include CT gene-related peptide (CGRP), amylin (AMY) and adrenomedullin (AM) (Poyner et al., 2002), although an assortment of related peptides have recently been identified including intermedin (IMD), also known as AM2 (Katafuchi et al., 2003; Roh et al., 2004; Takei et al., 2004). Whilst only weakly homologous in terms of amino acid sequence, several common features are shared, including an N-terminal ring structure that is the key to agonist activity. Nonetheless, the similarity in peptide structure leads to promiscuity for many of these peptides across their cognate receptors. Numerous biological activities have been attributed to these peptides. CT, for instance, is involved in bone homeostasis (Sexton et al., 1999). AMY is likely to be involved in nutrient intake and regulating blood glucose levels (Cooper, 1994). CGRP and AM are both potent vasodilators, with AM necessary for vascular integrity (Hinson et al., 2000; Shindo et al., 2001; Brain and Grant, 2004). As with many other peptides, significant advances in understanding the physiological, pathophysiological and clinical potential of CT family members are hampered by a lack of selective pharmacological agents that can be used to define function. Progress has been particularly slow for the CT peptide family as, until recently, the molecular nature of the cognate receptors for AMY, CGRP and adrenomedullin was unknown.

There is now some clarity regarding the nature of the receptor that likely mediates many of the effects of CGRP. It consists of a complex between a seven transmembrane protein belonging to the secretin family of G-protein coupled receptors (GPCRs), the CT receptor-like receptor (CL), with receptor activity modifying protein (RAMP) 1 (McLatchie et al., 1998). When these proteins are co-

expressed, classical $CGRP_1$-like pharmacology is observed (McLatchie et al., 1998; Hay et al., 2004). However, if CL is instead co-expressed with either of the two other RAMP family members, RAMP2 or RAMP3, adrenomedullin is recognized most effectively (McLatchie et al., 1998). Thus, RAMPs act as pharmacological switches. It was soon realized that the function of RAMPs may be much broader and there are now several examples of secretin family GPCRs with which these proteins are likely to interact (Christopoulos et al., 1999; Christopoulos et al., 2003).

Notably, RAMPs have a strong interaction with the CT receptor, the closest relative to CL (Christopoulos et al., 1999). Together, RAMPs and the CT receptor generate receptors with high affinity for AMY, the precise nature of these receptors depending on the CT receptor splice variant and cellular background (Tilakaratne et al., 2000). To our knowledge, there have been no other reports of a distinct molecular entity capable of responding to AMY with such high affinity. It is noteworthy that early attempts to clone the AMY receptor usually produced the CT receptor, thus it is likely that CT receptor/RAMP complexes mediate at least some of the effects of AMY *in vivo* although this has yet to be directly tested. Crucially, there is no reliable means of distinguishing CT from AMY receptors or AMY receptor subtypes pharmacologically in functional systems. Although comprehensive binding and agonist-interaction analyses have been performed, there has been no critical analysis of the way that antagonists interact with these receptors. This type of information may allow the different biological effects of AMY and related peptides to be attributed to distinct receptor subtypes. It can also provide a basis for the rational design of more selective agents. This is important since an AMY analogue (Pramlintide) has now reached late-stage development for glycaemic control in diabetic patients, illustrating the clinical importance of this peptide.

Therefore, in this study we have sought to address this issue by transfecting the CT receptor ($CT_{(a)}$, Poyner et al., 2002) with, or without RAMPs into COS-7 cells that do not endogenously express phenotypically significant levels of RAMPs, CT receptors or CL. We have identified several key aspects of pharmacology that relate to the way that AMY and its related peptides have historically been reported to act in tissues.

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Materials and methods

Materials. Human adrenomedullin (AM), human adrenomedullin$_{22-52}$ (AM_{22-52}), rat AMY_{8-37}, human αCGRP, human $\alpha CGRP_{8-37}$, human βCGRP and acetyl-(Asn^{30},Tyr^{32})-calcitonin$_{8-32}$ (AC187) were purchased from Bachem (Bubendorf, Switzerland). Salmon calcitonin$_{8-32}$ (sCT_{8-32}) was from Peninsula (Belmont, CA, USA) and human Tyr^{0}αCGRP, (Cys(Et)^{2,7})-αCGRP, (Cys(Acm)^{2,7})-αCGRP and rat AMY (rAMY) were from Auspep (Parkville, Australia). AC413 was a generous gift from Dr. Andrew Young (Amylin Pharmaceuticals Inc., Lajolla, CA). Human CT was obtained from the American Peptide Company (Sunnyville, CA). IMD short (IMDS) was a generous gift from Dr. Teddy Hsu (Stanford University School of Medicine, Roh *et al.*, 2004). Peptide sequences are detailed in Figure 1. Bovine serum albumin (BSA) and isobutylmethylxanthine (IBMX) were from Sigma (St. Louis, MO, USA) and amplified luminescent proximity homogenous assay (ALPHA)-screen cAMP kits were purchased from Perkin Elmer (Boston, MA, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and HEPES were from Invitrogen (Carlsbad, CA, USA). Cell culture plasticware was manufactured by Nunc (Roskible, Denmark) and Metafectine was purchased from Scientifix (Cheltenham, VIC, Australia). ^{125}I-labeled goat anti-mouse IgG was obtained from Perkin Elmer (Boston, MA, USA). Na-^{125}I (100mCi/ml) was supplied by ICN Biochemicals (Irvine, CA, USA). ^{125}I-salmon CT (specific activity 700 Ci/mmol) was iodinated in-house as previously described (Findlay et al., 1980). N-succinimidyl 3-94-hydroxy,5,-[^{125}I] iodophenyl) propionate (Bolton-Hunter reagent; 2000 Ci/mmol) was from Amersham (Buckinghamshire, UK). ^{125}I-rat Amylin (specific activity, 2000 Ci/mmol) was iodinated by the Bolton-Hunter method and purified by reverse phase high-

performance liquid chromatography as previously described (Bhogal et al., 1992). All other reagents were of analytical grade.

Expression constructs.

Double hemagglutinin (HA) epitope tagged human $CT_{(a)}$ receptor was prepared as previously described (Pham et al, 2004). This receptor is the Leu^{447} polymorphic variant of the receptor (Kuestner et al, 1994). Human RAMP1, RAMP2 and RAMP3 and human CL receptor were a gift from Dr. Steven Foord (McLatchie et al, 1998).

Cell culture and transfection.

COS-7 cells were subcultured as described previously (Zumpe et al., 2000). One day prior to transfection, COS-7 cells were seeded into $25cm^2$ or $75cm^2$ cell culture flasks at high density in order to achieve 90 –100% confluency for transfection the next day. The cells were then transfected using Metafectine, as per the manufacturer's instructions, with the following amounts of DNA: For $25cm^2$ flasks, 1.25μg of receptor DNA ($CT_{(a)}$ or CL) and 1.9μg of RAMP or pcDNA3 DNA; for $75cm^2$ flasks, 3.8μg of receptor DNA and 5.7μg of RAMP or pcDNA3 DNA. The transfection mix was removed after 16 hours incubation and the cells recovered in complete media (DMEM with 5% FBS) for 8 hours. The cells were then serum-starved for a further 16 hours in order to minimize basal cAMP levels.

Measurement of cAMP production.

Cells transfected with $CT_{(a)}$ or CL plus pcDNA3, RAMP1, 2 or 3 were harvested approximately 40 hours after transfection. The cells were counted and

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diluted to 20,000 cells per 10µl and incubated, mixing, for at least 30 minutes in serum and phenol red free DMEM containing 0.1% (w/v) BSA and 1mM IBMX (stimulation buffer). Agonist and antagonist dilutions were prepared in stimulation buffer and added to white 384 well plates, either alone, or in combination to a total volume of 10µl. Following incubation of cells with stimulation buffer, 20,000 cells were added per well in a volume of 10µl. The plates were centrifuged very briefly to ensure thorough mixing of these small volumes. The plates were then incubated for 30 minutes at 37^{o}C. Drug-stimulated receptor activity was terminated by the addition of 20µl lysis buffer (0.3% v/v Tween 20, 5mM HEPES, 0.1% w/v BSA in water, pH7.4). Following addition of lysis buffer, the plates were again centrifuged briefly to ensure thorough mixing. The cAMP in the lysed cells was assayed in the same wells using ALPHA-screen assay kits. A cAMP standard curve was included in each assay. Briefly, cAMP was measured with acceptor and donor beads that were prepared in lysis buffer and added to the plates according to the manufacturer's instructions. Following over night incubation in the dark, the plates were read with an ALPHA-screen protocol on a Fusion plate reader PerkinElmer (Boston, MA, USA).

Radioligand binding.

When harvested for cAMP assay (above), the same transfected COS-7 cells were also seeded into 24-well culture plates at a density of approx. 250,000 cells per well. These cells were then assayed for receptor binding to either ^{125}I-rAMY or ^{125}I-sCT the next day (16 hours later). Cells were initially washed with 500µl phosphate buffered saline (PBS) and incubated for 30 mins at 37^{o}C in 500µl binding buffer (FBS free DMEM with 0.1% w/v BSA). Wells contained either 50pM ^{125}I-sCT or 100pM ^{125}I-rAMY. Nonspecific binding levels were determined by competing with 10^{-7} M

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sCT or 10^{-6} M rAMY respectively. Cells were then washed twice with 500µl PBS and were solubilised with 0.5ml 0.5M NaOH with the cell lysate counted for γ-radiation using a Packard gamma counter (75% efficiency).

For full curve, competition binding experiments, cells in 75cm^2 flasks were transfected for 5 hours using metafectene, with 3.7µg $CT_{(a)}$ and either 5.2µg[d1] pcDNA3, RAMP1 or RAMP3 DNA. The cells were allowed to recover for 16 hours and then harvested and seeded at around 80-90% confluence into 48 well plates. These were then allowed to adhere and recover for a further 16 hours. Competition binding was performed for 2 hours at room temperature. Each well contained 225µl DMEM +0.1% BSA, 200pM of ^{125}I-rAMY and 25µl of competing peptide (10^{-12} M – 10^{-7} M) or buffer control. Cells were washed once with PBS, lysed and counted as described above.

Measurement of cell surface expression by antibody binding.

As for binding assays, at the time of harvesting for cAMP assay, transfected COS-7 cells were plated into 24 well plates and later assayed for cell-surface expression of the HA-tagged receptor. Cells were rinsed twice with 0.5ml binding buffer (50mM Tris-HCl pH7.7, 100mM NaCl, 5mM KCl, 2mM $CaCl_2$ and 1% w/v BSA, adjusted to pH7.7 with HCl) followed by addition of 2µg per well HA-specific mouse antibody in 250µl binding buffer. Cells were incubated for 3 hours at 4^{o}C, with gentle agitation. Cells were then rinsed three times with binding buffer and ^{125}I-labeled goat anti-mouse IgG (diluted to give 200 pM/250µl per well) was added to the cells. The cells were incubated for a further 3 hours at 4^{o}C, then rinsed 3 times with binding buffer. Cells were solubilised with 0.5ml 0.5M NaOH and the cell lysate

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counted for γ-radiation. Non-specific binding was determined from the wells that received ^{125}I-labeled goat anti-mouse IgG but not the anti-HA primary antibody.

Data analysis and statistics.

Data were analyzed using Graphpad Prism 4.02. (San Diego, CA). In each assay, the quantity of cAMP generated was back-calculated from the raw data using a cAMP standard curve. For agonist responses, concentration-effect curves were fitted to a four parameter logistic equation (see Motulsky and Christopoulos, 2003).

For calculation of antagonist potency, agonist concentration-response curves in the absence and presence of antagonist were globally fitted to the following equation using Prism (see Motulsky and Christopoulos, 2003):

$$\text{Response}=E_{min}+\frac{(E_{max}-E_{min})[A]^{n_H}}{[A]^{n_H}+\left(10^{-pEC_{50}}\left[1+\left(\frac{[B]}{10^{-pA_2}}\right)^{s}\right]\right)^{n_H}}$$

where E_{max} represents the maximal asymptote of the concentration-response curves, E_{min} represents the lowest asymptote of the concentration-response curves, pEC_{50} represents the negative logarithm of the agonist EC_{50} in the absence of antagonist, [A] represents the concentration of the agonist, [B] represents the concentration of the antagonist, n_H represents the Hill slope of the agonist curve, s represents the Schild slope for the antagonist, and pA_2 represents the negative logarithm of the concentration of antagonist that shifts the agonist EC_{50} by a factor of 2. Parallelism of agonist concentration-response curves in the presence of antagonist relative to the

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absence of antagonist was assessed by F-test, which compared curve fits where the n_H parameter was shared across each family of curves to fits where each curve within a family was allowed its own Hill slope factor. The F-test was similarly used to determine whether the Schild slope was significantly different from unity within a given dataset. In the majority of instances, this was not the case, and thus all curves were re-fitted with the Schild slope constrained to a value of 1; under these conditions, the resulting estimate of pA_2 represents the pK_B.

In all cases, potency and affinity values were estimated as logarithms (Christopoulos, 1998). Data shown are the mean ± SEM. Comparisons between mean values were performed by unpaired *t* tests or one way ANOVA, as appropriate. Unless otherwise stated, values of $p < 0.05$ were taken as statistically significant.

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Results

COS-7 cells were chosen for transfection studies as they have been shown to lack phenotypically significant levels endogenous RAMPs, CT receptors and CL (Hay et al., 2003). Without significant background expression of such receptor components, defined receptor subtypes can be accurately compared.

Agonist pharmacology

The approach taken to generate a detailed pharmacological analysis of the molecularly defined AMY receptors was to compare the effects of all available antagonists against the major agonists that were capable of eliciting reliable receptor activation. Therefore, we initially examined agonist-induced cAMP responses in cells transfected with $CT_{(a)}$ alone, or in combination with individual RAMPs to assess the relative agonist activation profiles of the receptors defined as $CT_{(a)}$, $AMY_{1(a)}$, $AMY_{2(a)}$ and $AMY_{3(a)}$, respectively. In most experiments, cell surface expression of the $CT_{(a)}$ was confirmed by binding of an anti-HA antibody to the epitope tag incorporated into the N-terminus of the receptor (Figure 2). In addition, in some experiments ^{125}I-sCT binding was also performed and confirmed that similar levels of the receptor protein were expressed at the cell surface (not shown). Expression of the AMY receptor phenotype was confirmed by concomitant ^{125}I-rAMY binding (data not shown).

As shown in table 1 and in accordance with previous results, hCT displayed equivalent high potency in cells transfected with $CT_{(a)}$ or $AMY_{1(a)}$ receptors but had ~10-fold lower potency at $AMY_{3(a)}$ receptors ($p<0.05$, n=6). In contrast, rAMY and the CGRPs had low potency at the $CT_{(a)}$ receptor and exhibited ~100-fold increased potency at the $AMY_{1(a)}$ receptor. As seen previously in this cellular background, preliminary analysis of radioligand binding and cAMP response indicated very little induction of $AMY_{2(a)}$ phenotype with pEC_{50} values for rAMY at this receptor

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equivalent to that seen with $CT_{(a)}$ alone (data not shown, Christopoulos et al, 1999; Tilakaratne et al., 2000). rAMY had high potency at the $AMY_{3(a)}$ receptor but the CGRPs showed only modest increases in potency (<10-fold) at this receptor. At all receptor phenotypes Tyr^0-hαCGRP was weaker than unmodified hαCGRP, but exhibited similar modulation of potency to α- and β-CGRP at $AMY_{1(a)}$ receptors.

IMD displays efficacy at CL/RAMP-based receptors (Roh et al., 2004; Takei et al., 2004). We examined the interaction of the short form of this peptide, IMDS, with CT and AMY receptors and compared it to the behavior of the peptide at CGRP and AM receptors. IMDS had low potency at $CT_{(a)}$ and $AMY_{2(a)}$ receptors and displayed a similar increase in potency at $AMY_{1(a)}$ (~40 fold) and $AMY_{3(a)}$ (<10 fold) receptors, as seen for the CGRPs (Figure 3; Table 2). This contrasts with the interaction of IMDS at CGRP and AM receptors assayed in the same cellular background where IMDS displayed similar high efficacy at all three receptors but differed from the activity of hαCGRP at these receptors, which only had high potency at the $CGRP_1$ receptor (Figure 3; Table 2).

The linear CGRP analogues $(Cys(Et)^{2,7})$-αCGRP and $(Cys(Acm)^{2,7})$-αCGRP have been used to sub-classify CGRP receptors into $CGRP_1$ and $CGRP_2$ receptors (Dennis et al., 1990; 1991; Poyner et al, 2002). As AMY receptors can also function as high affinity CGRP receptors, it was of interest to assess the potency of the linear CGRP analogues at CT and AMY receptors. Both analogues had very low potency and efficacy at $CT_{(a)}$, $AMY_{2(a)}$ and $AMY_{3(a)}$ receptors, but displayed moderate potency at the $AMY_{1(a)}$ receptor (Table 1; Figure 4A). However, both analogues were only partial agonists at the latter receptor exhibiting $\%E_{max}$ responses of 47.9 ± 5.4 and 22.8 ± 6.0, respectively, for $(Cys(Et)^{2,7})$-αCGRP and $(Cys(Acm)^{2,7})$-αCGRP. At the $CGRP_1$ receptor, both analogues displayed high potency, pEC_{50} 9.4 ± 0.12 (n=5) and

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9.08 ± 0.63 (n=4) for (Cys(Et)^{2,7})-αCGRP and (Cys(Acm)^{2,7})-αCGRP, respectively), similar to unmodified hαCGRP (9.51 ± 0.14 (n=5)), but were again partial agonists. However, (Cys(Et)^{2,7})-αCGRP was considerably more efficacious than (Cys(Acm)^{2,7})-αCGRP with %E_{max} values of 83.5 ± 7.2 and 8.1 ± 2.1, respectively (Figure 4B).

Antagonist pharmacology

N-terminally truncated analogues of CT and related peptides have traditionally been used as "specific" antagonists of the primary receptors at which they interact. However, the specificity of interaction across the range of CT and AMY receptor phenotypes has not been systematically addressed. We have therefore assessed the relative effectiveness of these peptide antagonists and a number of chimeras of sCT_{8-32} and rAMY (Figure 1) as antagonists of $CT_{(a)}$, $AMY_{1(a)}$ and $AMY_{3(a)}$ receptors. Antagonist studies were not performed at the $AMY_{2(a)}$ receptor because of the weak AMY phenotype we observe in COS-7 cells.

Of the peptides examined, sCT_{8-32} was the most effective antagonist with a pK_B of ~8 across all receptors examined. It did not display significant selectivity, with a similar pK_B observed for $CT_{(a)}$, $AMY_{1(a)}$ and $AMY_{3(a)}$ receptors, for each of the agonists (Table 3; Figure 5A,E; Figure 6A,E; Figure 7A,E), although there was a weak trend for lower affinity at $AMY_{1(a)}$ receptors with either rAMY or the CGRPs as agonists (Figure 8A).

In contrast, the $CGRP_1$ receptor antagonist, $CGRP_{8-37}$ was selective for AMY receptors over CT receptors (Figure 8B), with no antagonism of agonist responses at CT receptors with concentrations of antagonist up to 10^{-5} M (Table 3; Figure 5B,F). However, $CGRP_{8-37}$ was only a weak antagonist at $AMY_{1(a)}$ and $AMY_{3(a)}$ receptors with pK_B values of < 7 (Table 3; Figure 6B,F; Figure 7B,F). With AMY as agonist,

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$CGRP_{8-37}$ exhibited weak selectivity for $AMY_{1(a)}$ over $AMY_{3(a)}$ receptors, although this did not reach statistical significance (t-test; p=0.11) in the current study. Intriguingly, there was an apparent agonist-dependent component to antagonism by $CGRP_{8-37}$ with no effect seen at any of the receptors when hCT was used as the agonist (Table 3; Figure 5B; Figure 6B; Figure 7B).

In support of the weak effect of AM at these receptors (Table 1), AM_{22-52}, an antagonist of AM receptors, had no effect at either CT or AMY receptors (Table 3). Confirmation of the integrity of AM_{22-52} was obtained in experiments with AM_2 receptors, where this peptide is known to be an antagonist (data not shown, Hay et al., 2003). $rAMY_{8-37}$ was almost without activity, exhibiting only very weak antagonist activity at $AMY_{1(a)}$ receptors, and only when rAMY was the agonist (Table 3).

The peptide chimeras of rAMY and sCT_{8-32}, AC187 and AC413, each had affinity for $CT_{(a)}$, $AMY_{1(a)}$ and $AMY_{3(a)}$ receptors, but displayed selectivity between receptor phenotypes (Table 3; Figure 8C,D). AC187 was ~10-fold more potent an antagonist of $AMY_{1(a)}$ receptors compared with $CT_{(a)}$ receptors when rAMY was used as the agonist (Table 3; Figure 5G; Figure 6G; Figure 8C). Similarly, AC187 was more potent at $AMY_{3(a)}$ receptors over $CT_{(a)}$ receptors when rAMY was the agonist (Table 3; Figure 5G; Figure 7G; Figure 8C), but no significant difference was seen between $AMY_{1(a)}$ and $AMY_{3(a)}$ receptors (Figure 8C). As seen with $CGRP_{8-37}$, there was an apparent agonist-dependent effect observed with the antagonist potency of AC187 when hCT was the agonist, as no significant change in AC187 potency was seen across the 3 receptor types (Table 3; Figure 8C). Equivalent antagonist behavior was observed for AC413 when hCT was the agonist, with no difference in antagonist potency between $CT_{(a)}$, $AMY_{1(a)}$ and $AMY_{3(a)}$ receptors (Table 3; Figure 5D; Figure 6D; Figure 7D; Figure 8D). However, additional receptor-dependent and agonist-

dependent behavior was seen for AC413. For each of the receptors, AC413 was more potent when rAMY was the agonist vs when hCT was the agonist (Table 3; Figures 5, 6, 7, panels H vs D; Figure 8D), although this was not significant at the $AMY_{3(a)}$ receptor. AC413 also appeared to discriminate between $AMY_{1(a)}$ vs $AMY_{3(a)}$ receptors when rAMY was used as the agonist, being more effective at $AMY_{1(a)}$ (Figure 8D).

In competition for ^{125}I-rAMY binding, sCT_{8-32}, AC187 and AC413 each displayed high affinity at both $AMY_{1(a)}$ and $AMY_{3(a)}$ receptors, while $CGRP_{8-37}$ had lower affinity for both receptors (Table 4). However, consistent with their lack of antagonist potency at AMY receptors, $rAMY_{8-37}$ and hAM_{22-52} both exhibited very low affinity (Table 4).

Discussion

Many factors alter the potency of agonists at GPCRs; affinity and intrinsic efficacy are receptor-dependent, while receptor density and G protein-coupling efficiency are system dependent (Armour et al, 1999; Kenakin, 1997). In this study we examined the effect of agonists and antagonists on CT and AMY receptors expressed at similar levels in the same cellular background to reduce system-dependent variables and to allow comparison of relative affinity and intrinsic efficacy of the agents used (Armour et al, 1999).

As seen previously (Christopoulos et al, 1999, Muff et al, 1999), co-expression of $CT_{(a)}$/RAMP1 led to receptors that were potently stimulated by rAMY and CGRP, while $CT_{(a)}$/RAMP3 expression generated receptors potently stimulated by rAMY but only moderately by CGRP. In contrast, $CT_{(a)}$ expressed alone responded weakly to peptides aside from hCT. hCT potently stimulated cAMP production in COS-7 cells co-expressing $CT_{(a)}$/RAMP1 but was right-shifted (10-fold) in cells expressing $CT_{(a)}$/RAMP3. In all cases antagonist pK_B values were equivalent across receptors when hCT was used as the agonist, suggesting that hCT stimulation of cAMP is via the same receptor ($CT_{(a)}$), regardless of co-transfected RAMPs. This implies that hCT has only very low affinity for AMY receptors. This was consistent with competition binding studies where hCT had low affinity at both $AMY_{1(a)}$ and $AMY_{3(a)}$ receptors (Table 4; Christopoulos et al., 1999). Unlike CL, $CT_{(a)}$ expresses at the cell surface in a RAMP-independent manner (Lin et al., 1991; Kuestner et al, 1994) so co-transfection with RAMP leads to mixed populations of “free” and heterodimerised receptor. The reduced hCT potency at $AMY_{3(a)}$ is consistent with a marked decrease in the level of “free” $CT_{(a)}$, contrasting with the lack of modulation of hCT efficacy seen with RAMP1 co-transfection. This implies that $CT_{(a)}$ has a stronger interaction

with RAMP3 than RAMP1, and is supported by the consistent reduction in CT potency with RAMP3 that is not seen with RAMP1 (Christopoulos et al, 1999, Tilakaratne et al, 2000; Muff et al, 1999, Armour et al, 1999, Kuwasako et al, 2004) and also that only RAMP3 is able to induce an AMY receptor phenotype in melanophores (Armour et al, 1999). However, it is also possible that hCT has lower efficacy at $AMY_{3(a)}$ versus $AMY_{1(a)}$ receptors.

Initial studies with IMDS indicated that it could interact, with similar potency, with CGRP and AM receptors (Roh et al, 2004). We have confirmed this observation. Its efficacy was equivalent to that of hαCGRP but there were marked differences in the relative potency of these two peptides for individual CL/RAMP combinations. However, at $CT_{(a)}$-based receptors, the activity of IMDS tracked that of hαCGRP. This suggests that the IMDS binding interface at $CT_{(a)}$-based receptors is similar to that of the CGRPs and contrasts to its mode of interaction with CL-RAMP receptors. In our COS-7 cell background, the overall potency of IMDS was weaker at CT-based receptors than at CL/RAMP receptors, suggesting that the physiological target of IMDS is more likely to be the latter receptor family. During the preparation of this manuscript, a study examining the effect of IMD at $CT_{(a)}$-based receptors in COS-7 cells was published, with similar findings to ours (Takei et al., 2004).

Unlike agonist behavior, antagonist potency is viewed as a receptor-dependent variable and so antagonists are the preferred tool for defining receptor subtypes (Christopoulos and El-Fakahany, 1999). We have delineated the pharmacology of $CT_{(a)}$-based receptors through functional analysis of the effects of N-terminally truncated analogues of CT and related peptides, including chimeras between rAMY and sCT_{8-32}.

sCT_{8-32} had high affinity for all three receptor subtypes but discriminated little between them. However, the small, non-significant, decrease in affinity against AMY versus CT receptors was similar to sCT_{8-32} behavior at $CT_{(a)}$ and $AMY_{3(a)}$ receptors in melanophores where higher affinity at $CT_{(a)}$ receptors was observed (Armour et al, 1999).

$CGRP_{8-37}$ was highly selective for AMY receptors over CT receptors and was weakly selective for $AMY_{1(a)}$ over $AMY_{3(a)}$ receptors, mirroring the effects of αCGRP at these receptors. However, its potency against AMY receptors was much lower than against $CGRP_1$ (CL/RAMP1) receptors expressed in the same system (pK_B 9.34±0.38 (n=5), Hay D. L., manuscript in preparation). As such it is a useful research tool for investigation of receptor subtypes, but only in combination with a range of other antagonists that can distinguish between CGRP-responsive receptors.

AC187 had high affinity for AMY receptors and was ~10-fold selective for these receptors over CT receptors. AC187 has only low affinity for $CGRP_1$ receptors (Howitt and Poyner, 1997; Hay D. L., manuscript in preparation) and therefore is useful for discriminating between CL- and CT-based receptors. However, low selectivity between AMY versus CT receptors limits its usefulness.

AC413 provided the first evidence for selectivity between $AMY_{1(a)}$ and $AMY_{3(a)}$ receptors with pK_Bs of 7.92 and 7.10, respectively, against rAMY. Whilst the difference is small, the peptide may guide the design of more specific antagonists. The different pK_B values of AC413 for rAMY versus hCT at $CT_{(a)}$ receptors are difficult to reconcile with simple competitive antagonism, where the nature of the agonist should not alter the pK_B. There may be differences in the mode of binding of rAMY and hCT at this receptor. It is possible that, although partially overlapping, the binding sites of hCT and rAMY at the $CT_{(a)}$ receptor are significantly different

allowing an allosteric interaction; such interactions are often characterized by apparent agonist-dependent antagonist pK_B values (Christopoulos and Kenakin, 1992). Unlike AC187, which has only 2 amino acids of rAMY substituted into the sCT_{8-32} backbone, AC413 is also homologous to rAMY over residues 8-18 (Figure 1) and so may interact with higher affinity at the site occupied by rAMY versus that occupied by hCT. Alternatively, each of the agonists may provide a unique receptor conformation, leading to alteration in system dependent activity of the receptor that is manifest as differential antagonist affinity. However, we believe this is less likely, as such changes could be expected to alter affinity of other antagonists.

In contrast to the N-terminally truncated peptides already described, $rAMY_{8-37}$ was essentially without antagonist activity at any of the receptors, consistent with its low affinity in competition binding studies (Table 4; Aiyar et al., 1995). Nonetheless, this peptide can antagonize some AMY-induced responses (Wang et al, 1993; Ye et al, 2001).

Subdivision of CGRP receptors was first proposed by Quirion and colleagues (Dennis et al., 1990; 1991), based primarily on the observation that $CGRP_{8-37}$ exhibits high affinity antagonism for only $CGRP_1$ receptors. Conversely, linear analogues of hαCGRP (most commonly $(Cys(Acm)^{2,7})$-αCGRP) have higher potency at $CGRP_2$ receptors. However, the range of reported values for these peptides is extremely broad (Poyner et al., 2002; Hay et al., 2004) and differences seen in functional assays are not apparent in competition binding assays (Rorabaugh et al., 2001). While it is now generally accepted that CL/RAMP1 represents the $CGRP_1$-receptor phenotype (Poyner et al., 2002), the molecular identity of the receptor(s) giving rise to $CGRP_2$ pharmacology is obscure. Recent work with $(Cys(Acm)^{2,7})$-αCGRP and $(Cys(Et)^{2,7})$-αCGRP has provided some evidence that AMY receptors may contribute to $CGRP_2$

pharmacology (Kuwasako et al, 2004). Taken with this latter work, the current study identifies a spectrum of agonist and antagonist behavior at AMY receptors that provides a potential explanation for $CGRP_2$ receptor pharmacology. The $AMY_{1(a)}$ receptor is potently activated by CGRP and its analogues and antagonized weakly by $CGRP_{8-37}$, fitting in with the classical definition of the $CGRP_2$ receptor (Dennis et al., 1990, 1991). The $AMY_{3(a)}$ receptor also has reasonable affinity for CGRP and is weakly antagonized by $CGRP_{8-37}$ but shows little stimulation by linear CGRP analogues. Nonetheless, since these latter analogues are rarely used, it may also contribute to reports of $CGRP_2$ receptors in the literature.

The actions of CGRP-derived agonists call for comment. Here, $(Cys(Acm)^{2,7})$-αCGRP and $(Cys(Et)^{2,7})$-αCGRP were partial agonists, in contrast to the data of Kuwasako et al (2004). It is highly likely that this discrepancy may be explained by the HEK293 cells used by Kuwasako and colleagues having more efficient receptor coupling to G-proteins, masking partial agonist behavior. In support of this, αCGRP was also much more potent in their study. It is also significant that Kuwasako et al showed that there was relatively little difference in the dissociation constants for CGRP and the two cys-modified analogues as measured in binding studies; a consistent theme in the literature has been the failure to observe a $CGRP_1$/$CGRP_2$ difference using radioligand binding (e.g Dennis et al., 1990). In the porcine aorta, $(Cys(Acm)^{2,7})$-αCGRP was a partial agonist (Waugh et al., 1999).

In summary, despite the complicated pharmacology of CT/RAMP complexes there are several useful tools in defining these receptors including agonists (rAMY, hCT) that are specific for CT and AMY receptor subtypes and antagonists (sCT_{8-32}, AC187, $CGRP_{8-37}$) that used in conjunction can help define these receptor classes. Individual receptor subtypes, such as $AMY_{1(a)}$ and $AMY_{3(a)}$ receptors, can also be

discriminated with careful use of additional agonists such as the CGRPs. However, system-dependent factors such as coupling efficiency must also be considered. Finally, it is likely that most $CGRP_2$ receptor behavior can be attributed to existing CT/RAMP and CL/RAMP based receptors.

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Footnotes

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Figure 1

Peptide sequences and alignment. Sequences were aligned according to the Clustal V methods (PAM250) using the MegAlign program from DNAstar (Madison, WI, USA). For agonist peptides, residues that match the consensus CGRP sequence are boxed (upper panel). For antagonist peptides, residues that match the overall consensus are boxed (lower panel). The location of the disulphide-linked cysteines in agonist peptides is also indicated. The exception to this are the analogues Cys(Et)^{2,7}-αCGRP and Cys(Acm)^{2,7}-αCGRP where the disulphide linkage has been blocked. Modification to these cysteines is indicated by bold boxes.

Figure 2

Cell surface expression of $CT_{(a)}$-protein, in COS-7 cells transiently transfected with $CT_{(a)}$ alone or $CT_{(a)}$ in the presence of either RAMP1 ($AMY_{1(a)}$), RAMP2 ($AMY_{2(a)}$) or RAMP3 ($AMY_{3(a)}$), measured by binding of anti-HA antibody to the 2xHA epitope incorporated at the N-terminus of the receptor. Primary antibody binding is detected by incubation of a ^{125}I-labelled goat anti-mouse IgG antibody as described in Methods. In untransfected or mock transfected cells the level of binding was < 15% of binding seen in $CT_{(a)}$ transfected cells. Data are expressed as a percentage of the binding of ^{125}I-antibody to cells expressing the $CT_{(a)}$ protein in the absence of RAMP co-transfection. Data are from 10 independent experiments with duplicate repeats.

Figure 3

Induction of cAMP accumulation by IMDS in COS-7 cells transiently transfected with $CT_{(a)}$-based receptor phenotypes (A) and CL-based receptor phenotypes (B).

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For CGRP and AM receptors, the response across receptors likely represents different levels of receptor expression. The E_{max} for IMDS and hαCGRP was equivalent for all. The graph is of a representative experiment, with triplicate repeats, of at least 6 independent experiments.

Figure 4

Induction of cAMP accumulation at $AMY_{1(a)}$ (A) or $CGRP_1$ (B) receptors by linear CGRP analogues. Closed squares (hαCGRP); closed circles ((Cys(Et)^{2,7})-αCGRP); open circles ((Cys(Acm)^{2,7})-αCGRP). pEC_{50} and E_{max} values, respectively, at the $CGRP_1$ receptor were: hαCGRP, 9.51 ± 0.14, 100% (n=5); (Cys(Et)^{2,7})-αCGRP, 9.40 ± 0.12, 83.54 ± 7.19% (n=5); (Cys(Acm)^{2,7})-αCGRP, 9.08 ± 0.63, 8.08 ± 2.09%. The graph is of a representative experiment, with triplicate repeats, of at least 4 independent experiments. pEC_{50} and E_{max} values for peptides at the $AMY_{1(a)}$ receptor are detailed in Table 1.

Figure 5

Representative antagonist curves at $CT_{(a)}$ receptors; sCT_{8-32} (A, E), $CGRP_{8-37}$ (B, F), AC187 (C, G) and AC413 (D, H) with hCT (A - D) or rAMY (E – H) as agonist.
Closed circles – control (agonist alone); open triangles (+ 10^{-8}M antagonist); open inverted triangles (+ 10^{-7}M antagonist); open diamonds (+ 10^{-6}M antagonist); open circles (+ 10^{-5}M antagonist).

Figure 6

Representative antagonist curves at $AMY_{1(a)}$ receptors; sCT_{8-32} (A, E), $CGRP_{8-37}$ (B, F), AC187 (C, G) and AC413 (D, H) with hCT (A - D) or rAMY (E – H) as agonist.

Closed circles – control (agonist alone); open triangles (+ 10^{-8}M antagonist); open inverted triangles (+ 10^{-7}M antagonist); open diamonds (+ 10^{-6}M antagonist); open circles (+ 10^{-5}M antagonist).

Figure 7

Representative antagonist curves at $AMY_{3(a)}$ receptors; sCT_{8-32} (A, E), $CGRP_{8-37}$ (B, F), AC187 (C, G) and AC413 (D, H) with hCT (A - D) or rAMY (E – H) as agonist. Closed circles – control (agonist alone); open triangles (+ 10^{-8}M antagonist); open inverted triangles (+ 10^{-7}M antagonist); open diamonds (+ 10^{-6}M antagonist); open circles (+ 10^{-5}M antagonist).

Figure 8

Distribution of pK_B values for the antagonists sCT_{8-32} (A), $CGRP_{8-37}$ (B), AC187 (C) and AC413 (D). Comparisons were performed using an unpaired t-Test. The number of individual experiments used to calculate the pK_B values are indicated in parentheses. The boxed values for pK_B of AC187 for the agonists rAMY, αCGRP and βCGRP at the $AMY_{1(a)}$ receptor were all significantly different from the indicated comparator.

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Table 1. Agonist potencies (pEC_{50} values) for stimulation of cAMP accumulation at human CT and AMY receptors. Data are presented as mean ± standard error of the mean. Values in parentheses represent the number of individual experiments analysed.

	$CT_{(a)}$	**$AMY_{1(a)}$**	**$AMY_{3(a)}$**
hCT	8.99 ± 0.1 (8)	8.93 ± 0.09 (7)	8.02 ± 0.22 (7)
rAMY	6.95 ± 0.18 (8)	9.12 ± 0.16 (10)	8.63 ± 0.09 (7)
hαCGRP	6.80 ± 0.05 (5)	8.70 ± 0.17 (6)	7.60 ± 0.17 (6)
Tyr^0-hαCGRP	<6 (2)	7.55 ± 0.17 (7)	<6 (3)
hβCGRP	7.18 ± 0.22 (2)	9.16 ± 0.18 (9)	7.67 ± 0.23 (6)
$(Cys(Et)^{2,7})$hαCGRP	<6 (3)	7.79 ± 0.14 (5)[a]	<6 (6)
$(Cys(ACM)^{2,7})$hαCGRP	<6 (3)	7.46 ± 0.06 (4)[a]	<6 (6)
hAM	6.73 ± 0.45 (3)	6.48 ± 0.28 (4)	6.89 ± 0.51 (3)
IMDS	6.53 ± 0.09 (6)	8.07 ± 0.19 (6)[b]	7.12 ± 0.19 (6)

[a]Note that these CGRP analogues were weak partial agonists at this receptor with E_{max} values of 47.9 ± 5.4% and 22.8 ± 6% for $(Cys(Et)^{2,7})$hαCGRP and $(Cys(ACM)^{2,7})$hαCGRP, respectively. These values were generated by comparing the curve maximum asymptotes of the hαCGRP analogues with that for hαCGRP itself (set at 100%), which was used as the reference full agonist for these experiments.

[b]E_{max} values for IMDS were equivalent to those of hαCGRP assayed in parallel.

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Table 2. Comparison of IMDS and hαCGRP potency for stimulation of cAMP accumulation at human CT, AMY, CGRP and AM receptors. Values are mean ± standard error of the mean.

Receptor	Agonist	pEC_{50}	n
$CT_{(a)}$	IMDS	6.53 +/- 0.09	6
	hαCGRP	6.80 +/- 0.04	5
$AMY_{1(a)}$	IMDS	*8.07 +/- 0.19	6
	hαCGRP	8.70 +/- 0.17	10
$AMY_{2(a)}$	IMDS	6.25 +/- 0.26	6
	hαCGRP	7.24 +/- 0.19	5
$AMY_{3(a)}$	IMDS	†7.12 +/- 0.19	6
	hαCGRP	7.60 +/- 0.17	6
$CGRP_1$	IMDS	8.71 +/- 0.13	8
	hαCGRP	9.47 +/- 0.19	6
AM_1	IMDS	8.10 +/- 0.04	4
	hαCGRP	6.39 +/- 0.10	4
AM_2	IMDS	8.69 +/- 0.13	5
	hαCGRP	6.87 +/- 0.13	3

*$p<0.05$ vs $CT_{(a)}$, $AMY_{2(a)}$ and $AMY_{3(a)}$ receptors.

†$p<0.05$ vs $CT_{(a)}$, $AMY_{1(a)}$ and $AMY_{2(a)}$ receptors.

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Table 3. pK_B values for antagonists in antagonizing agonist-induced stimulation of cAMP accumulation at human CT and AMY receptor phenotypes. ND – not done, <5 – antagonist caused no significant shift of the agonist concentration effect curve at concentrations of 10^{-5}M.

Antagonist	Receptor	Agonist	pK_B (mean ± SEM)	n
sCT_{8-32}	$CT_{(a)}$	hCT	8.17 ± 0.17	7
	$CT_{(a)}$	rAMY	8.22 ± 0.26	7
	$AMY_{1(a)}$	hCT	7.95 ± 0.16	7
	$AMY_{1(a)}$	rAMY	7.78 ± 0.13	11
	$AMY_{1(a)}$	hαCGRP	7.80 ± 0.17	11
	$AMY_{1(a)}$	hβCGRP	7.68 ± 0.18	12
	$AMY_{1(a)}$	Tyr^0- hαCGRP	7.61 ± 0.17	4
	$AMY_{3(a)}$	hCT	7.87 ± 0.25	6
	$AMY_{3(a)}$	rAMY	7.92 ± 0.19	6
AC187	$CT_{(a)}$	hCT	7.15 ± 0.23	7
	$CT_{(a)}$	rAMY	6.89 ± 0.25	7
	$AMY_{1(a)}$	hCT	7.30 ± 0.11	7
	$AMY_{1(a)}$	rAMY	8.02 ± 0.18	7
	$AMY_{1(a)}$	hαCGRP	7.86 ± 0.20	11
	$AMY_{1(a)}$	hβCGRP	7.85 ± 0.26	4
	$AMY_{1(a)}$	Tyr^0- hαCGRP	7.73 ± 0.27	4
	$AMY_{3(a)}$	hCT	7.37 ± 0.33	6
	$AMY_{3(a)}$	rAMY	7.68 ± 0.22	5
AC413	$CT_{(a)}$	hCT	6.94 ± 0.13	7

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	$CT_{(a)}$	rAMY	7.48 ± 0.17	7
	$AMY_{1(a)}$	hCT	7.11 ± 0.27	5
	$AMY_{1(a)}$	rAMY	7.92 ± 0.23	4
	$AMY_{1(a)}$	hαCGRP	7.30 ± 0.24	10
	$AMY_{1(a)}$	hβCGRP	7.25 ± 0.21	2
	$AMY_{1(a)}$	Tyr^0- hαCGRP	7.44 ± 0.67	2
	$AMY_{3(a)}$	hCT	6.83 ± 0.27	8
	$AMY_{3(a)}$	rAMY	7.10 ± 0.14	8
hαCGRP$_{8-37}$	$CT_{(a)}$	hCT	< 5	5
	$CT_{(a)}$	rAMY	< 5	4
	$AMY_{1(a)}$	hCT	< 5	7
	$AMY_{1(a)}$	rAMY	6.62 ± 0.13	11
	$AMY_{1(a)}$	hαCGRP	6.79 ± 0.24	9
	$AMY_{1(a)}$	hβCGRP	6.78 ± 0.13	14
	$AMY_{1(a)}$	Tyr^0- hαCGRP	6.56 ±- 0.4	6
	$AMY_{3(a)}$	hCT	≤ 5	8
	$AMY_{3(a)}$	rAMY	6.17 ± 0.26	7
rAMY$_{8-37}$	$CT_{(a)}$	hCT	< 5	2
	$CT_{(a)}$	rAMY	< 5	2
	$AMY_{1(a)}$	hCT	< 5	4
	$AMY_{1(a)}$	rAMY	5.59 ± 0.24	3
	$AMY_{1(a)}$	hαCGRP	ND	
	$AMY_{1(a)}$	hβCGRP	ND	

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	$AMY_{1(a)}$	Tyr^0- hαCGRP	ND	
	$AMY_{3(a)}$	hCT	< 5	3
	$AMY_{3(a)}$	rAMY	< 5	4
hAM_{22-52}	$CT_{(a)}$	hCT	< 5	1
	$CT_{(a)}$	rAMY	< 5	1
	$AMY_{1(a)}$	hCT	< 5	3
	$AMY_{1(a)}$	rAMY	< 5	3
	$AMY_{1(a)}$	hαCGRP	< 5	1
	$AMY_{1(a)}$	hβCGRP	< 5	1
	$AMY_{1(a)}$	Tyr^0- hαCGRP	ND	
	$AMY_{3(a)}$	hCT	< 5	4
	$AMY_{3(a)}$	rAMY	< 5	4

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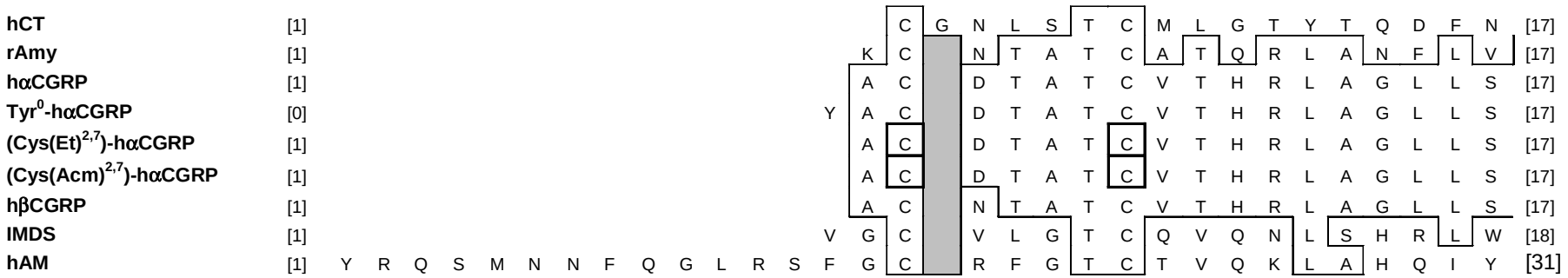
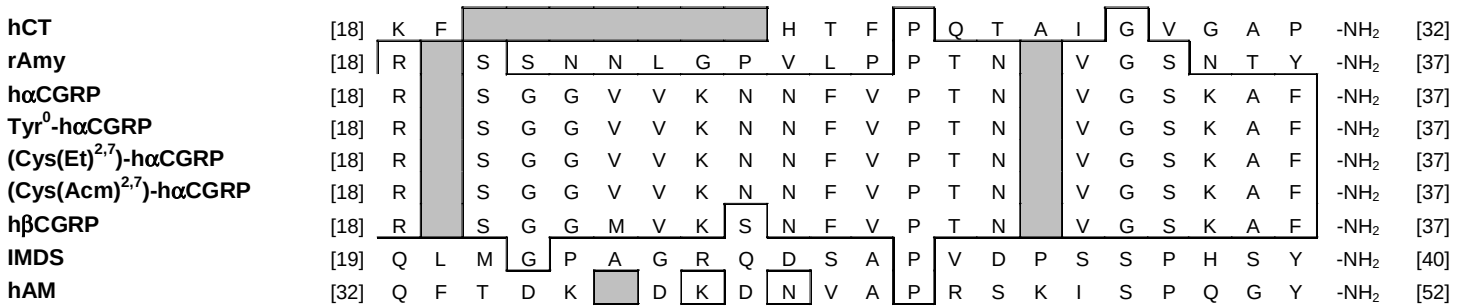
Table 4. Peptide affinity (pIC_{50} values) for inhibition of ^{125}I-rAMY binding to human AMY receptors. Values are mean ± standard error of the mean for 3 independent experiments each with 3 replicates.

	$AMY_{1(a)}$	**$AMY_{3(a)}$**
hCT	≤ 6	≤ 6
rAMY	8.76 ± 0.06	8.60 ± 0.09
hαCGRP	8.00 ± 0.08	6.97 ± 0.55
Tyr^0-hαCGRP	6.85 ± 1.05	6.73 ± 1.46
hβCGRP	8.80 ± 0.08	7.71 ± 0.07
$(Cys(Et)^{2,7})$hαCGRP	7.19 ± 0.06	6.96 ± 0.74
$(Cys(ACM)^{2,7})$hαCGRP	6.87 ± 1.08	6.45 ± 0.10
hAM	< 6	< 6
IMDS	6.93 ± 0.69	6.21 ± 0.26
sCT_{8-32}	8.52 ± 0.08	8.94 ± 0.04
AC187	8.62 ± 0.08	8.53 ± 0.05
AC413	8.59 ± 0.05	8.54 ± 0.06
$hαCGRP_{8-37}$	7.56 ± 0.16	7.51 ± 0.16
$rAMY_{8-37}$	≤ 6	6.67 ± 1.06
hAM_{22-52}	< 6	< 6

AGONISTS





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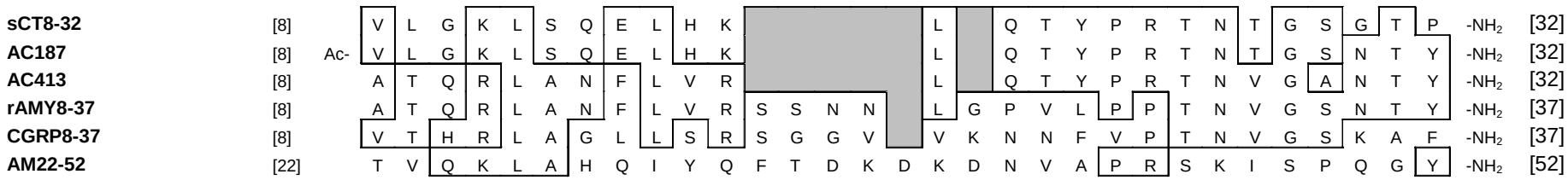

Figure 1

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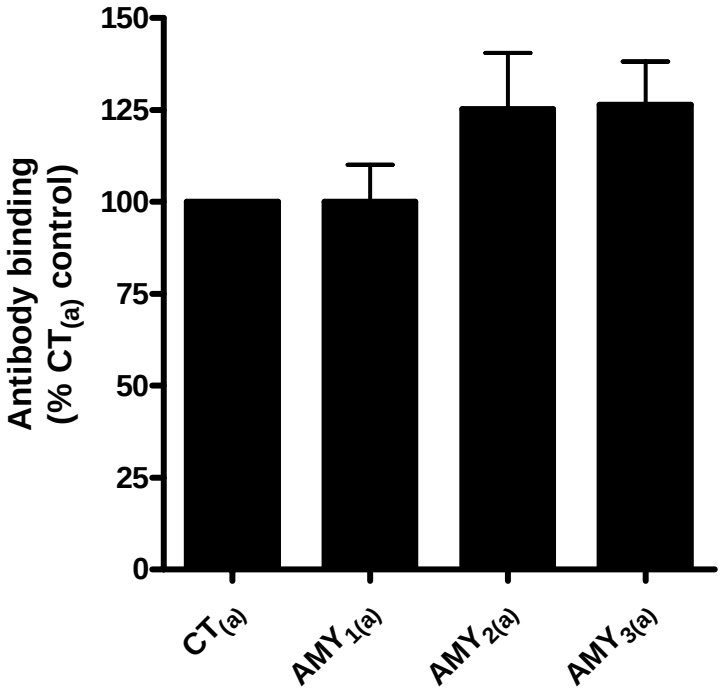

Figure 2

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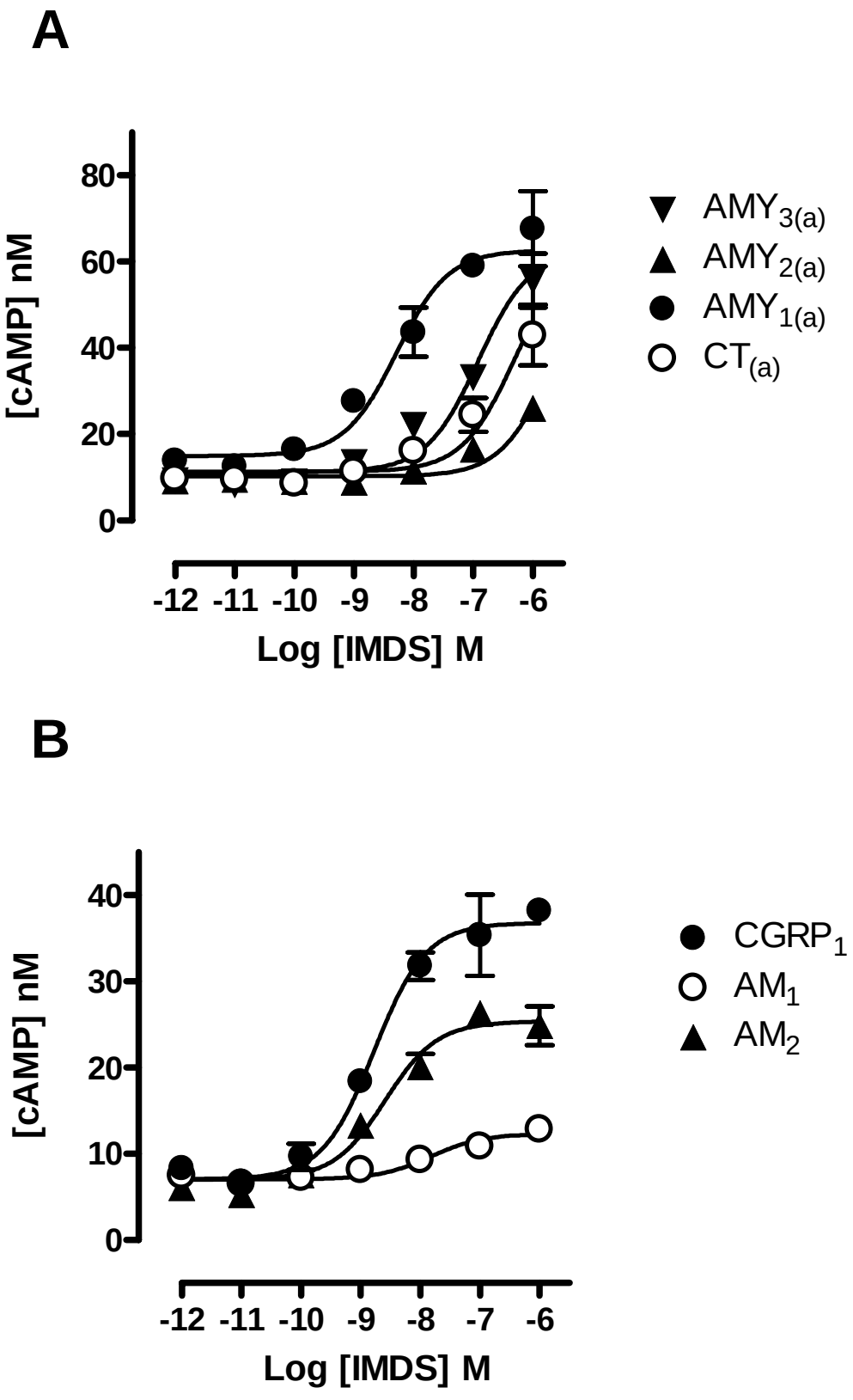

Figure 3.

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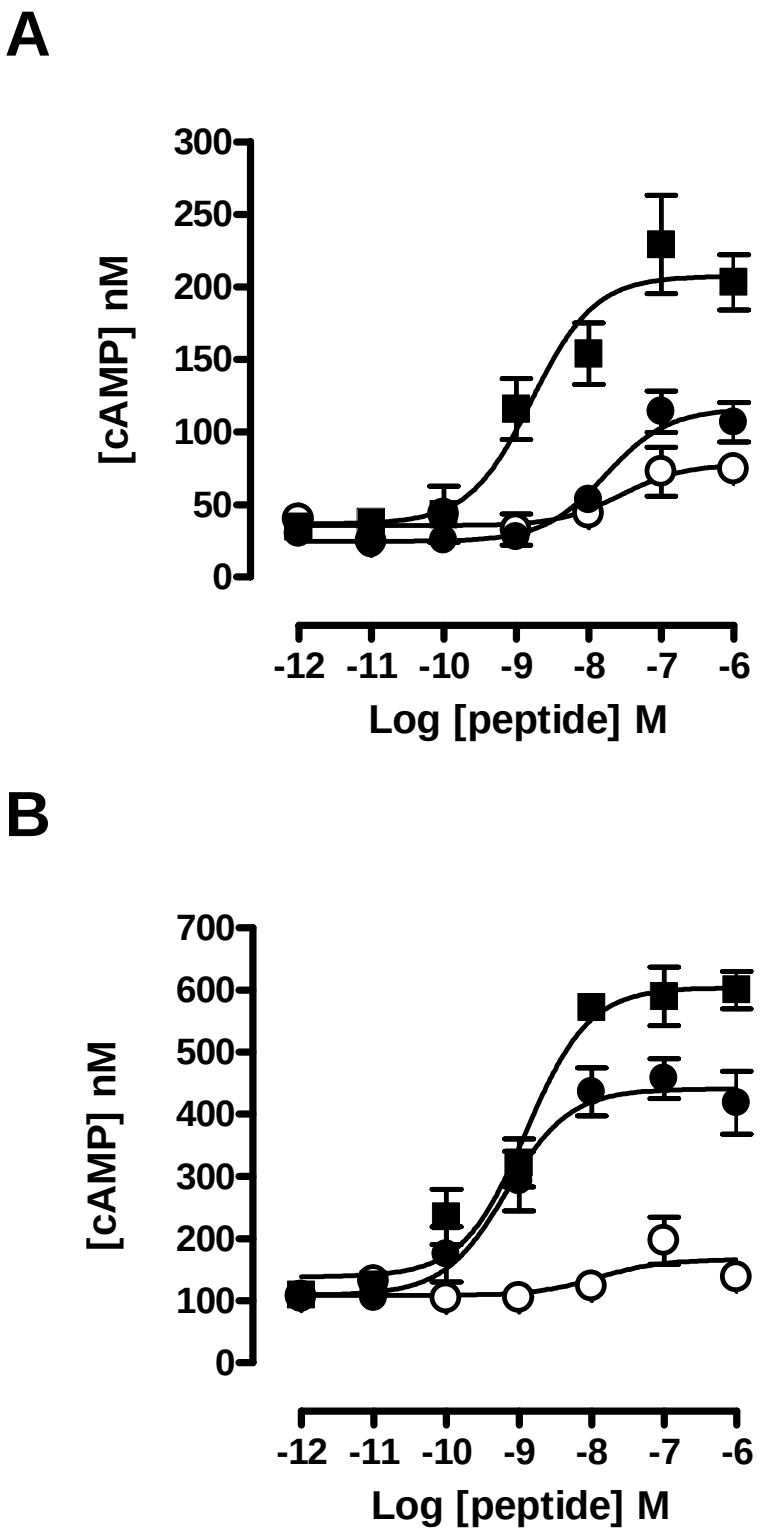

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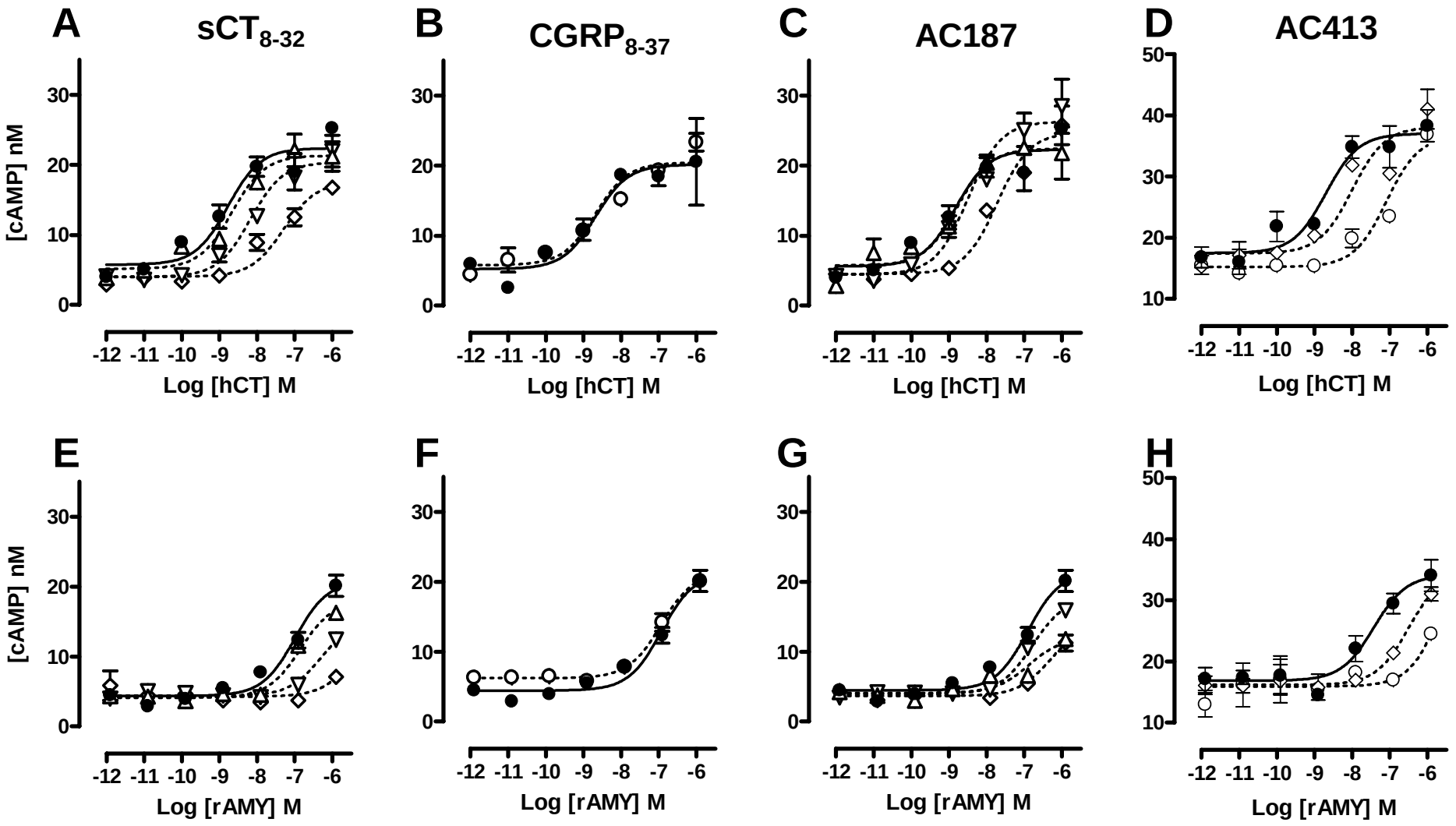

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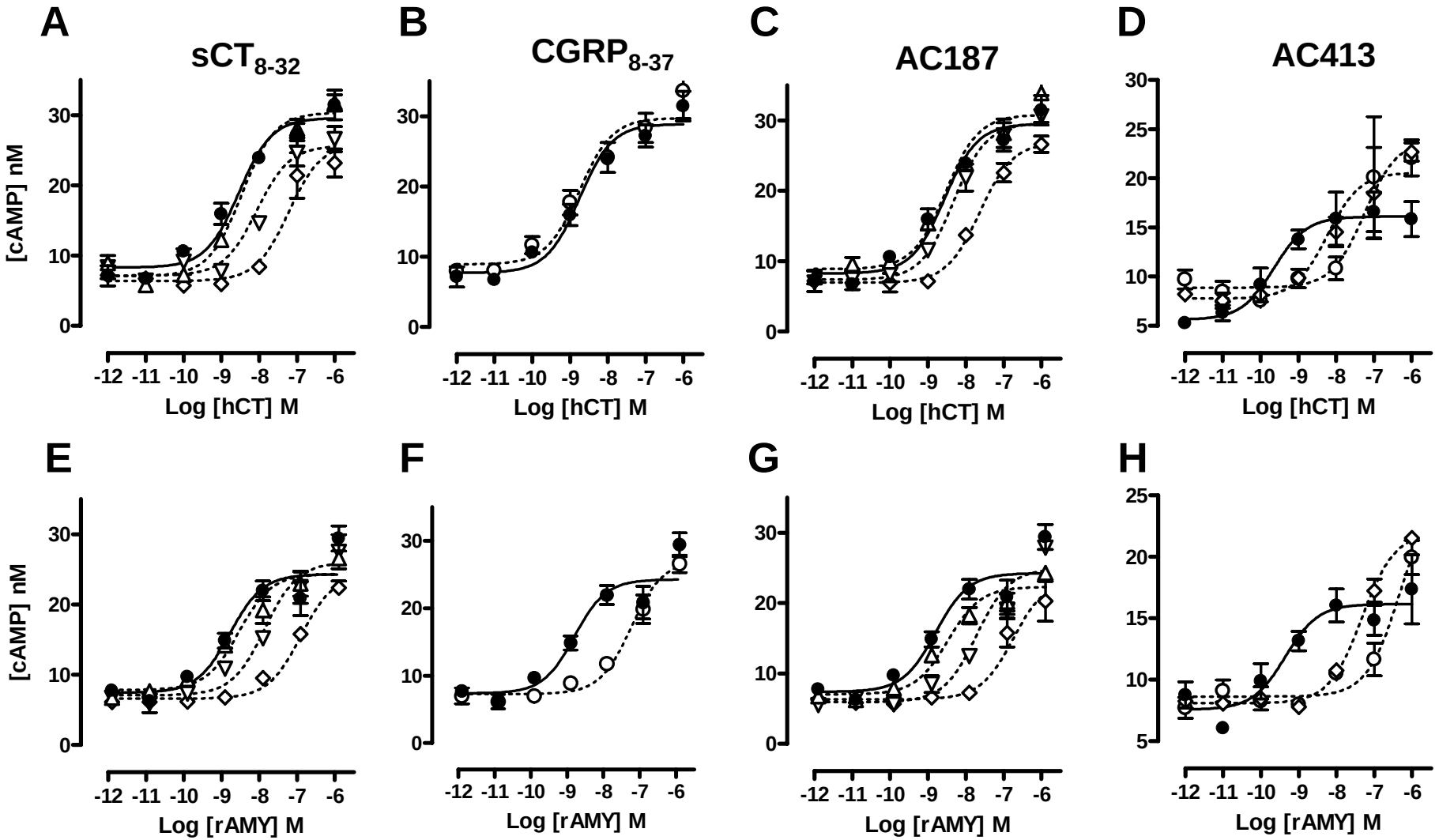

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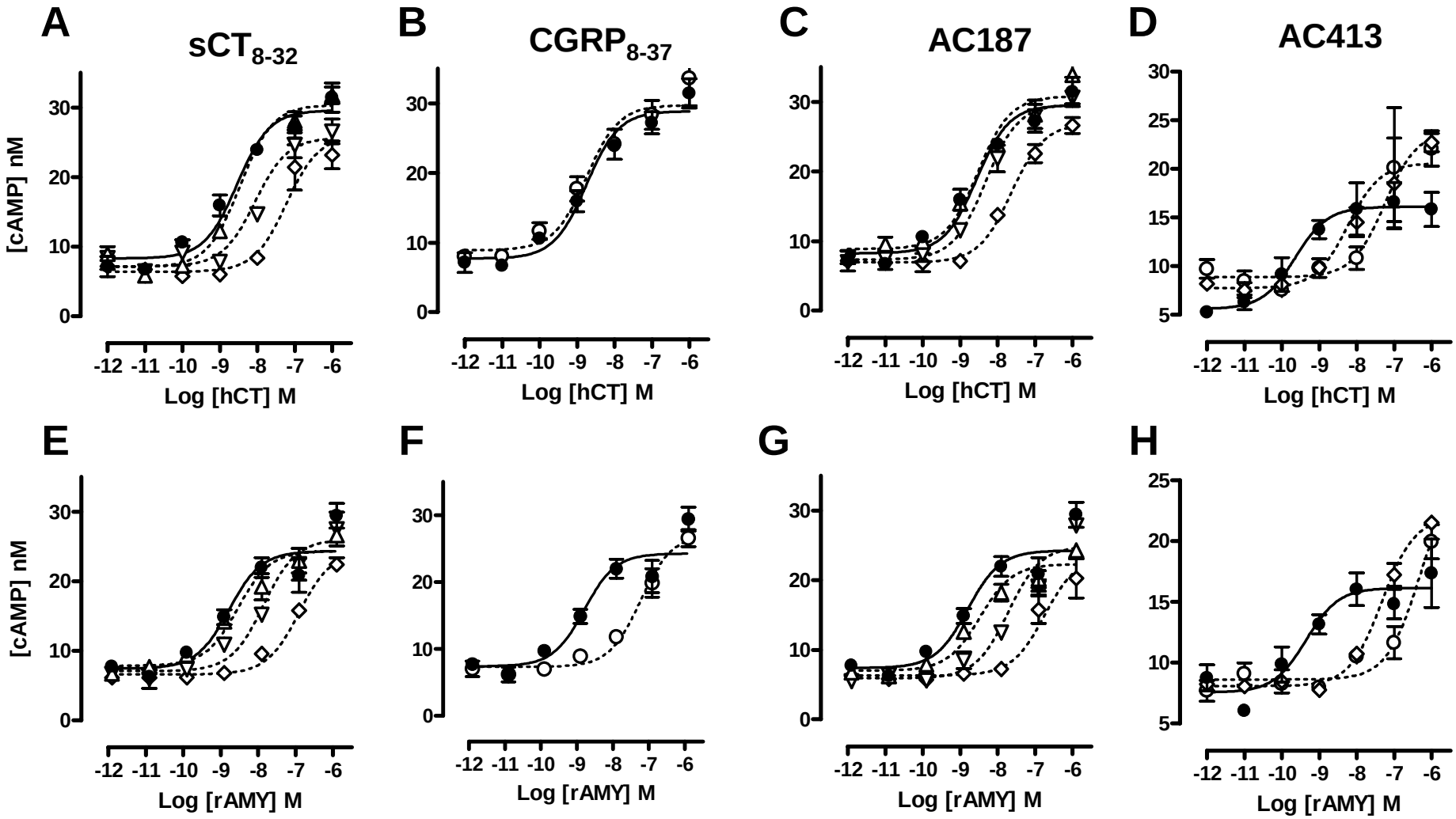

Figure 7.

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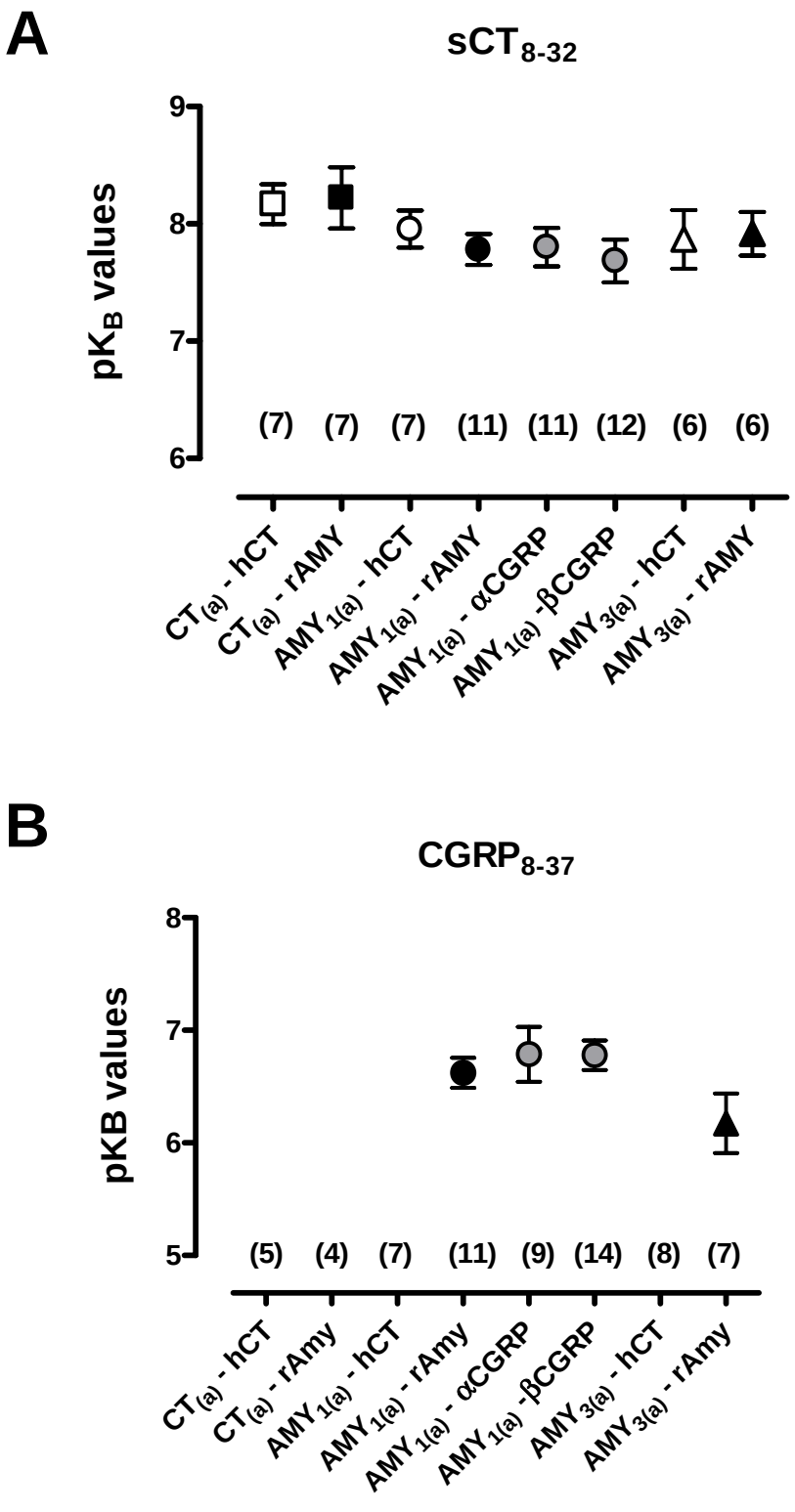

Figure 8.

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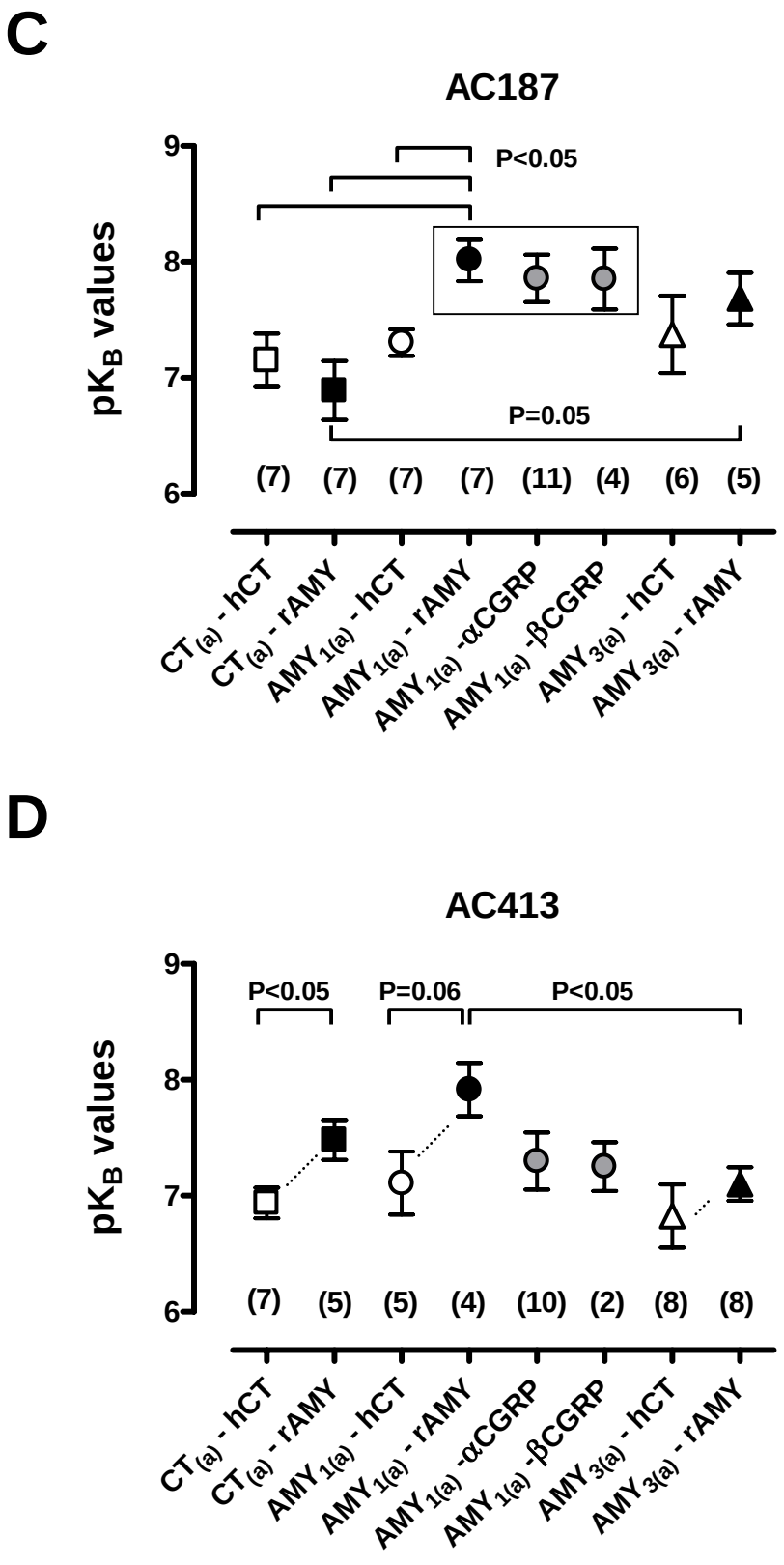

Figure 8.