

Themed Section: Secretin Family (Class B) G Protein-Coupled Receptors –  
from Molecular to Clinical Perspectives

## RESEARCH PAPER

# Pharmacological characterization of rat amylin receptors: implications for the identification of amylin receptor subtypes

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## BACKGROUND AND PURPOSE

Amylin (Amy) is an important glucoregulatory peptide and AMY receptors are clinical targets for diabetes and obesity. Human (h) AMY receptor subtypes are complexes of the calcitonin (CT) receptor with receptor activity-modifying proteins (RAMPs); their rodent counterparts have not been characterized. To allow identification of the most clinically relevant receptor subtype, the elucidation of rat (r) AMY receptor pharmacology is necessary.

## EXPERIMENTAL APPROACH

Receptors were transiently transfected into COS-7 cells and cAMP responses measured in response to different agonists, with or without antagonists. Competition binding experiments were performed to determine rAmy affinity.

## KEY RESULTS

rCT was the most potent agonist of rCT<sub>(a)</sub> receptors, whereas rAmy was most potent at rAMY<sub>1(a)</sub> and rAMY<sub>3(a)</sub> receptors. rAmy bound to these receptors with high affinity. Rat  $\alpha$ -calcitonin gene-related peptide (CGRP) was equipotent to rAmy at both AMY receptors. Rat adrenomedullin (AM) and rAM2/intermedin activated all three receptors but were most effective at rAMY<sub>3(a)</sub>. AC187, AC413 and sCT<sub>8-32</sub> were potent antagonists at all three receptors.  $\alpha$ CGRP<sub>8-37</sub> displayed selectivity for rAMY receptors over rCT<sub>(a)</sub> receptors. rAMY<sub>8-37</sub> was a weak antagonist but was more effective at rAMY<sub>1(a)</sub> than rAMY<sub>3(a)</sub>.

## CONCLUSIONS AND IMPLICATIONS

AMY receptors were generated by co-expression of rCT<sub>(a)</sub> with rRAMP1 or 3, forming rAMY<sub>1(a)</sub> and rAMY<sub>3(a)</sub> receptors, respectively. CGRP was more potent at rAMY than at hAMY receptors. No antagonist tested was able to differentiate the rAMY receptor subtypes. The data emphasize the need for and provide a useful resource for developing new CT or AMY receptor ligands as pharmacological tools or potential clinical candidates.

## LINKED ARTICLES

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## Abbreviations

AM, adrenomedullin; AM2, adrenomedullin 2/intermedin; Amy, amylin; AMY, amylin receptor; AP, area postrema; CT, calcitonin; CGRP, calcitonin gene-related peptide; CLR, calcitonin receptor-like receptor; IAPP, islet amyloid polypeptide; IAS, internal anal sphincter; RAMP, receptor activity-modifying protein; VD, vas deferens

## Introduction

The calcitonin (CT) peptide family is a group of therapeutically important peptides, which includes CT, amylin (Amy),  $\alpha$ - and  $\beta$ -calcitonin gene-related peptide (CGRP), adrenomedullin (AM) and AM2 (also known as intermedin). Despite sharing only limited amino acid sequence identity, key structural features are shared, including an N-terminal ring structure formed by a disulphide bond and an amidated C-terminus (Poyner *et al.*, 2002). Amy (also known as islet amyloid polypeptide; IAPP) is a 37-amino-acid peptide hormone, which plays an important role in regulating glucose homeostasis. Following nutrient influx, Amy is co-secreted with insulin from pancreatic islet beta cells (Ogawa *et al.*, 1990; Young, 2005a). This peptide may regulate blood glucose levels by inhibiting insulin secretion from the pancreas (Wang *et al.*, 1993; Young, 2005b). However, the primary sites of action for Amy appear to be brain nuclei that are devoid of the blood-brain barrier, such as the area postrema (AP) (Lutz, 2009; Potes and Lutz, 2010; Trevaskis *et al.*, 2010b). Here, Amy may act to regulate food intake by contributing to meal ending satiation (Lutz *et al.*, 2001; Roth *et al.*, 2012) and slow the rate by which nutrients are delivered into the circulation from the gastrointestinal tract (Clementi *et al.*, 1996). Amy may also act as an adiposity signal and a regulator of energy expenditure (Wielinga *et al.*, 2007; 2010; Zhang *et al.*, 2011).

In type I and the late stages of type II diabetes, pancreatic islet beta-cell mass is substantially reduced and there is correspondingly less insulin and Amy to be released into the circulation (Ogawa *et al.*, 1990; Makimattila *et al.*, 2000). Logically, dual treatment with both Amy and insulin offers promise for the treatment of diabetes. Therefore, Amy replacement therapy, in the form of a synthetic human Amy peptide analogue, Pramlintide ([Pro<sup>25,28,29</sup>]-human Amy), together with insulin is approved for assisting with the control of blood glucose in these patients (Ryan *et al.*, 2005). Further potential for Amy as a therapy is actively being explored, with recent data suggesting that combined Amy and leptin therapy may have profound effects on controlling body weight (Roth *et al.*, 2008; Ravussin *et al.*, 2009; Trevaskis *et al.*, 2010b).

There appears to be substantial complexity in the Amy receptor system. Amy binds with low affinity to the CT receptor, a G<sub>αs</sub>-coupled, family B GPCR. However, in the presence of receptor activity-modifying proteins (RAMPs), Amy affinity is substantially enhanced (Christopoulos *et al.*, 1999; Muff *et al.*, 1999; Leuthauser *et al.*, 2000; Kuwasako *et al.*, 2004; Hay *et al.*, 2005). Accordingly, co-expression of the CT receptor with the three RAMPs yields AMY<sub>1</sub>, AMY<sub>2</sub> and AMY<sub>3</sub> receptors, respectively (Poyner *et al.*, 2002). RAMPs can also interact with other receptors, such as the interaction of RAMP1 with the CT receptor-like receptor (CLR) to form the CGRP receptor. The Amy peptide and receptor (AMY) are distinguished by the use of lower- and uppercase letters, respectively. The human (h) CT receptor has two major splice variants; hCT<sub>(a)</sub> and hCT<sub>(b)</sub>, with the latter containing a 16-amino-acid insert in the first intracellular loop (Egerton *et al.*, 1995; Poyner *et al.*, 2002). Therefore, taking into account only the two major hCT receptor splice variants and their interactions with RAMPs, there are at least six potential

hAMY receptor subtypes (Tilakaratne *et al.*, 2000; Poyner *et al.*, 2002). The pharmacology and signalling of these receptors differ and are strongly influenced by cellular background; this effect is most evident for the hAMY<sub>2</sub> receptor (Tilakaratne *et al.*, 2000; Morfis *et al.*, 2008).

Despite the clear clinical relevance of Amy, it is not known which AMY receptor subtype mediates the therapeutically beneficial actions of Amy/pramlintide and whether there are different receptors at work in the CNS and peripheral tissues. For this reason, it is important to understand the pharmacology of Amy receptors and determine which subtype may be most usefully exploited in the clinic. In particular, non-injectable subtype-specific small-molecule agonists or positive allosteric modulators would be valuable additions to the pharmaceutical armoury used to treat diabetes.

Rodent models represent major systems for the physiological investigation of hormone action and pre-clinical validation of drugs. Substantial data for Amy were obtained in such systems, leading to the development of pramlintide. However, in accordance with the clinical situation, it is unknown which receptor Amy/pramlintide acts through in rodents. Receptor antagonists could be used in rodents to define the clinically relevant AMY receptor subtype(s) to enhance drug discovery efforts. Understanding rat (r) AMY receptors is particularly relevant as rat models display higher predictive value for therapies targeting AMY receptors on weight loss in humans than mouse models (Trevaskis *et al.*, 2010a). It has been assumed that rodent receptors for Amy are complexes of CT receptors and RAMPs, and rodent AMY receptors are named according to the human convention (Poyner *et al.*, 2002; Alexander *et al.*, 2011). However, this has never been experimentally tested and the only published pharmacological study of non-human CT receptor/RAMP combinations indicated that porcine (p) RAMPs did not enhance Amy affinity at pCT receptors (Kikumoto *et al.*, 2003). Therefore, it is vital to validate this nomenclature and determine the pharmacology of defined CT receptor/RAMP combinations in other species. Furthermore, there is reliance in the literature on the 'selective' Amy receptor antagonist, AC187, but there are little data at cloned receptors to support this. At hAMY receptors, the discrimination of this peptide between the AMY<sub>1(a)</sub> and CT<sub>(a)</sub> receptor was less than 10-fold. Therefore, the aim of this study was to characterize the pharmacology of rCT receptor/RAMP combinations. This will provide key information regarding the pharmacological discrimination of putative AMY receptor subtypes in rodents and a key resource for the further development of Amy agonists for the treatment of diabetes and obesity.

In rats, rCT<sub>(a)</sub> is equivalent to hCT<sub>(a)</sub> (Poyner *et al.*, 2002). We expressed rCT<sub>(a)</sub> with or without rRAMPs in COS-7 cells, which display a null phenotype for Amy and related receptor components (Hay *et al.*, 2006; Bailey and Hay, 2006), and screened a series of species-matched peptide agonists and antagonists. As CT<sub>(a)</sub>, RAMP1 and RAMP3 appear to be expressed in the rat AP, potentially a major site of Amy action, we have focussed on characterizing these combinations (Ueda *et al.*, 2001; Becskei *et al.*, 2004). This study has shown for the first time that the co-expression of rCT<sub>(a)</sub> with rRAMP1 or 3 produces functional AMY receptors in rats. Nevertheless, these receptors displayed distinct differences in

pharmacology compared with their human counterparts. This work has important implications for the discrimination of CT, AMY and CGRP receptors in rodents and highlights the need for selective ligands for these receptors.

## Methods

### DNA constructs

rCT<sub>(a)</sub> was kindly provided by Prof Patrick M Sexton (Monash Institute of Pharmaceutical Sciences, Australia). rRAMP3 and rCLR were previously described (Njuki *et al.*, 1993; Hay *et al.*, 2003). Mouse CLR was kindly provided by Prof Walter Born (University of Zurich, Switzerland). rRAMP1 was kindly provided by Dr Christopher Salvatore (Merck Research Laboratories, PA, USA). All constructs were untagged.

### Cloning of mouse RAMP3

Mouse RAMP3 (GenBank accession number AF146524) was cloned by reverse transcriptase-RT-PCR from total RNA (1 µg) extracted from mouse soleus muscle. RNA was heated at 65°C for 10 min and primed using random hexamers. Reverse transcription was performed using Expand™-RT (Roche, Auckland, New Zealand) for 10 min at 30°C, followed by 45 min at 42°C. Reaction conditions were as follows: Tris-HCl (50 mM); KCl (40 mM); MgCl<sub>2</sub> (5 mM); Tween-20 (0.5%); dithiothreitol (10 mM); RNase inhibitor (20 U); Expand-RT (50 U) (Boehringer Mannheim, Mannheim, Germany); dNTPs (1 mM); pH 8.3. Oligonucleotide primers for RAMP3 were 5-TAGGAT CCTGCATCTTAGTTGGCCATGA-3 (sense)/5-ATAGAATTCAT CCAGCAGATCCTCAAGC-3 (antisense). Underlined nucleotides, respectively, indicate *Bam*HI and *Eco*RI restriction sites introduced to facilitate cloning. PCR amplification was performed in an Eppendorf Mastercycler R gradient Thermal cycler for 40 cycles of 94°C (1 min), 55°C (2 min) and 72°C (3 min). Reaction conditions were: Tris/HCl (10 mM), KCl (50 mM), MgCl<sub>2</sub> (1.1 mM), oligonucleotides (0.5 µM), gelatin (0.01% w/v), dNTP's (200 µM), 2.5 U REDTaq™ DNA polymerase (Sigma, Saint Louis, MO), cDNA template (10 ng), at pH 8.3. PCR products were isolated and subcloned into pcDNA3.1 + (Invitrogen, Auckland, New Zealand) through the *Bam*HI and *Eco*RI restriction sites. Sequence analysis at the DNA sequencing facility, School of Biological Sciences, University of Auckland, confirmed the sequence.

### Cell culture and transfection

Culture of Cos 7 cells was performed as previously described (Bailey and Hay, 2006). Briefly, cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 8% heat inactivated fetal bovine serum and 5% v/v penicillin/streptomycin and kept in a 37°C humidified 95% air/5% CO<sub>2</sub> incubator. For cAMP assays, cells were plated into 96-well plates at a density of approximately 30 000 cells per well (or 10 000 for AlphaScreen), and for radioligand binding studies, cells were plated at a density of approximately 100 000 cells per well in 24-well plates. One day after plating, cells were transfected using polyethylenimine (PEI) as previously described (Bailey and Hay, 2006). Briefly, plasmid DNA (250 ng per well of a 96-well plate or 1 µg per well of a 24-well plate) at a 1:1 ratio of RAMP or vector to receptor DNA was

incubated with PEI, in 5% glucose for approximately 10 min before being added to complete growth medium and then to the wells. Cells were used for experimentation 36–48 h later.

### cAMP assay

Most cAMP assays were performed as described previously (Bailey and Hay, 2006). Briefly, transfected cells were serum-deprived in DMEM containing 1 mM IBMX and 0.1% BSA for 30 min. Agonists were then either added alone, or antagonists were added first followed immediately by agonists and incubated at 37°C for 15 min. cAMP was extracted with ice-cold ethanol and measured by competition between <sup>3</sup>H-cAMP and PKA as described previously (Bailey and Hay, 2006). For studies characterizing rAM2 potency at rodent AM<sub>2</sub> and CGRP receptors, cAMP was measured as per a modification to the standard AlphaScreen method (AlphaScreen cAMP assay kit; Perkin-Elmer Life and Analytical Sciences, Waltham, MA, USA) to permit use in adherent cells as described previously (Gingell *et al.*, 2010). The plates were then read using an Envision plate reader (Perkin-Elmer Life and Analytical Sciences).

### Radioligand binding

Cells were washed once with PBS (at room temperature) and then incubated for 2 h at room temperature in DMEM containing 0.1% BSA and ~100 pM [<sup>125</sup>I]-rAmy (Perkin-Elmer Life and Analytical Sciences) in the absence or presence of different concentrations of unlabelled rAmy or rαCGRP to a total volume of 500 µL. rAmy at a concentration of 1 µM was used to define non-specific binding. Following this incubation, the cells were washed once with ice-cold PBS before being lysed with 500 µL 0.2 M NaOH. The cell lysate was transferred to Eppendorf tubes and counted for 1 min per tube.

### Tissue collection

Two tissues that have been reported to express the CGRP<sub>2</sub> receptor phenotype were used (Hay *et al.*, 2008). Vas deferens (VD) was carefully dissected from four male Wistar rats, and internal anal sphincters (IAS) were carefully dissected from three male Wistar rats and immediately frozen at -80°C. All animal care and experimental procedures were conducted in accordance with the New Zealand animal welfare act (1999) and approved by the University of Auckland Animal Ethics Committee.

### RNA extraction

The VD and IAS tissues were individually ground under liquid nitrogen to a fine powder. Total RNA was extracted from the ground tissue using TRIZOL® Reagent (Invitrogen) according to the manufacturer's protocol, resuspended in 50 µL DEPC treated water and stored at -80°C. The concentration of total RNA was determined by a NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and RNA integrity was determined by gel electrophoresis.

### RT-PCR and primers

First strand cDNA was synthesized by using a SuperScript™ first-strand synthesis system (Invitrogen). Total RNA (5 µg) was incubated with or without SuperScript™ II reverse transcriptase in the presence of random hexamer primers. Primers

were developed for their specificity to rCLR, rCT receptors and rRAMP1. The sequences of the forward and reverse primers designed to target rCLR were CCAACGGATTACAT TGCATA and CAGTAAAGCAGCACAAATGG, rCT receptors were ATGAGGTTCTTCTCCTGAACAGG and CGGTCATAG CACTTGTACTGAGCA and to target rRAMP1 were GAGGA CATGGAGACCATAGG and CAGTCATGAGCAGTGTGACC, respectively. The product sizes were 397 base pairs for the rCLR primers, 267 base pairs for the rRAMP1 primers and 173 base pairs for rCT receptor. The annealing temperature used for all sets of primers was 57°C. PCR reactions were performed in a total volume of 20 µL per reaction, using *Pfu* polymerase (Promega, Madison, WI, USA) and containing 2 µL of the appropriate cDNA template. Reactions containing no template were also set up as controls. PCR cycling comprised a single step of 95°C for 2 min followed by 35 cycles of 95°C for 45 s, 56°C for 45 s, 72°C for 90 s and a single final extension step of 72°C for 7 min. PCR products (10 µL) were electrophoresed with a size marker for 60 min in a 2% (w/v) agarose gel containing SYBR Safe™ DNA gel stain (Invitrogen) and visualized on a Biorad™ imaging system under UV transillumination.

### Drugs chemicals and other materials

rAM, rCT,  $\alpha$ CGRP,  $\beta$ CGRP,  $\text{h}\alpha$ CGRP<sub>8-37</sub> and  $\text{r}\alpha$ CGRP<sub>8-37</sub> were purchased from American Peptide (Sunnyvale, CA, USA). rAM2 (47 amino acids), sCT (Cys(Et)<sup>2,7</sup>) $\text{h}\alpha$ CGRP (Cys(ACM)<sup>2,7</sup>) $\text{h}\alpha$ CGRP, sCT<sub>8-32</sub> and AC187 were purchased from Bachem (Bubendorf, Switzerland). rAmy and rAmy<sub>8-37</sub> were purchased from both American Peptide and Bachem. AC413 was kindly provided by Amylin Pharmaceuticals, Inc. (San Diego, CA) AC187 and AC413 are N-terminally acetylated and C-terminally amidated peptides; their sequences are shown in Figure 1. All peptides were dissolved in water to make 1 mM stock solutions and stored as aliquots in siliconized microcentrifuge tubes at -30°C. When making up these solutions, the peptide content was taken into account, but where no data sheet was supplied, content was assumed to be 80%. BSA, IBMX, PKA and activated charcoal were purchased from Sigma-Aldrich (St. Louis, MO, USA). DMEM

	8	32
sCT <sub>8-32</sub>	<u>VLGKLSQELHKLQTYPRNTGSGTP</u>	
AC187	<u>VLGKLSQELHKLQTYPRNTGSENTY</u>	
	8	32
sCT <sub>8-32</sub>	<u>VLGKLSQELHKLQTYPRNTGSGTP</u>	
AC413	<u>ATQRLANFLVRLQTYPRTNVGANTY</u>	
	8	37
rAmy <sub>8-37</sub>	<u>ATQRLANFLVRSSNNLGPVLPPTNVGSNTY</u>	
AC413	<u>ATQRLANFLVR---</u> <u>L-QTYPRTNVGANTY</u>	

### Figure 1

Amino acid sequences of rAmy<sub>8-37</sub>, sCT<sub>8-32</sub>, AC413 and AC187. Alignments were performed with ClustalW. Identical residues are underlined.

and TrypLE were purchased from Invitrogen, and forskolin was purchased from Tocris (Bristol, UK). All other reagents were of analytical grade.

### Data analysis

Data were analysed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). In each assay, cAMP data were first normalized to the maximal (100%) response obtained to 50 µM forskolin, and the minimum (no agonist/basal) that was present as a control on each plate or alternatively cAMP concentrations were determined from cAMP standard curves. For agonist responses, pEC<sub>50</sub> values were obtained by fitting a four-parameter logistic equation to the concentration-response curve data. To determine if the Hill slope was significantly different from one for agonist potency curves, *F*-tests were performed. For the majority of experiments, the Hill slope was not significantly different from unity. The agonist potency curves were therefore re-fitted with a Hill slope constrained to 1 and pEC<sub>50</sub>s obtained. For calculation of antagonist potency values (pA<sub>2</sub>), agonist concentration-response curves were fitted in the absence or presence of antagonist and analysed by Global Schild analysis as previously described (Hay *et al.*, 2005). To determine if Schild slopes were significantly different from unity within each experiment, *F*-tests were performed. In the majority of experiments, Schild slopes were not significantly different from one and were therefore constrained to 1; under these conditions the resulting estimate of pA<sub>2</sub> represents the pK<sub>B</sub>.

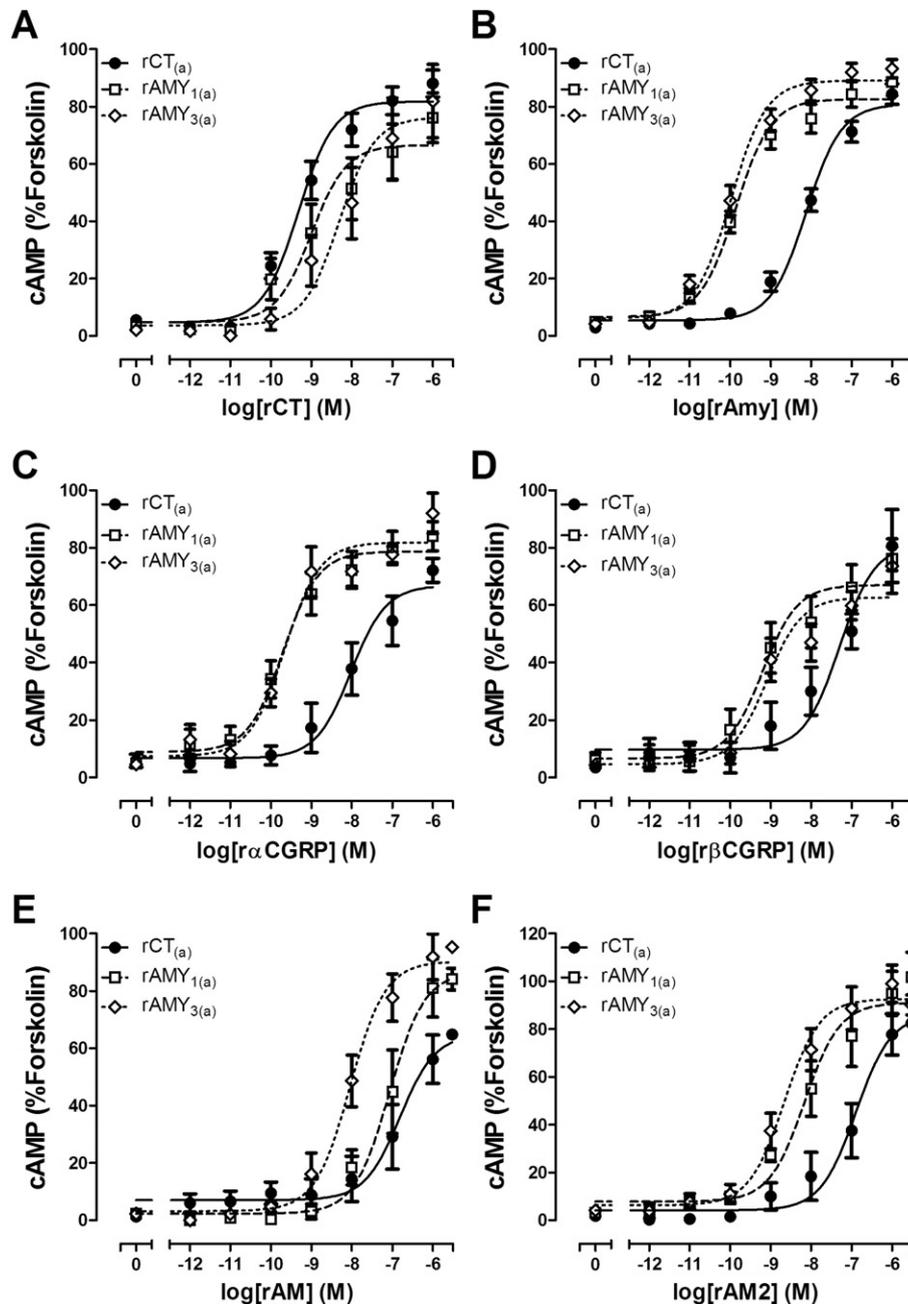
In radioligand binding experiments, the specific binding was first calculated for each receptor, and then data were normalized to this value before being fitted to a four-parameter logistic equation. As the Hill slopes were not significantly different from one by *F*-test, they were constrained to one for calculation of pIC<sub>50</sub> values.

For statistical analysis, pEC<sub>50</sub>, pIC<sub>50</sub> and pK<sub>B</sub> values from individual experiments were combined and compared using unpaired *t*-tests or one-way ANOVA followed by Tukey's *post hoc* test where appropriate. Unless stated otherwise, all potency and affinity values are expressed as logarithms, and all data are expressed as mean ± SEM. Significance was achieved at *P* < 0.05. *n* refers to the number of independent experiments (i.e. individual transient transfections and subsequent manipulations).

## Results

### Pharmacology of rat calcitonin receptors – cAMP assay

In order to determine the impact of RAMP co-transfection with CT receptors, it was important to characterize the pharmacology of the rat CT receptor in the absence of RAMPs. rCT potently stimulated cAMP production via rCT<sub>(a)</sub> and was significantly more potent than any other agonist tested (Figures 2 and 3A, Table 1). rAmy and  $\text{r}\alpha$ CGRP were approximately 20-fold less potent than rCT with  $\beta$ CGRP, rAM and rAM2 acting as weaker agonists. The pEC<sub>50</sub> values are displayed in Table 1, with corresponding curves in Figure 2 and summary graph with statistical analysis in Figure 3. (Cys(Et)<sup>2,7</sup>) $\text{h}\alpha$ CGRP and (Cys(ACM)<sup>2,7</sup>) $\text{h}\alpha$ CGRP failed to



**Figure 2**

Stimulation of cAMP production by (A) rat (r) CT, (B) rAmy, (C)  $\alpha$ CGRP, (D)  $\beta$ CGRP, (E) rAM and (F) rAM2 in COS-7 cells transfected to express either rCT<sub>(a)</sub> receptors, rAMY<sub>1(a)</sub> receptors or rAMY<sub>3(a)</sub> receptors as indicated. Data are expressed as a percentage of the cAMP response generated by 50  $\mu$ M forskolin and are mean  $\pm$  SEM of four to 23 combined experiments, performed in duplicate or triplicate.

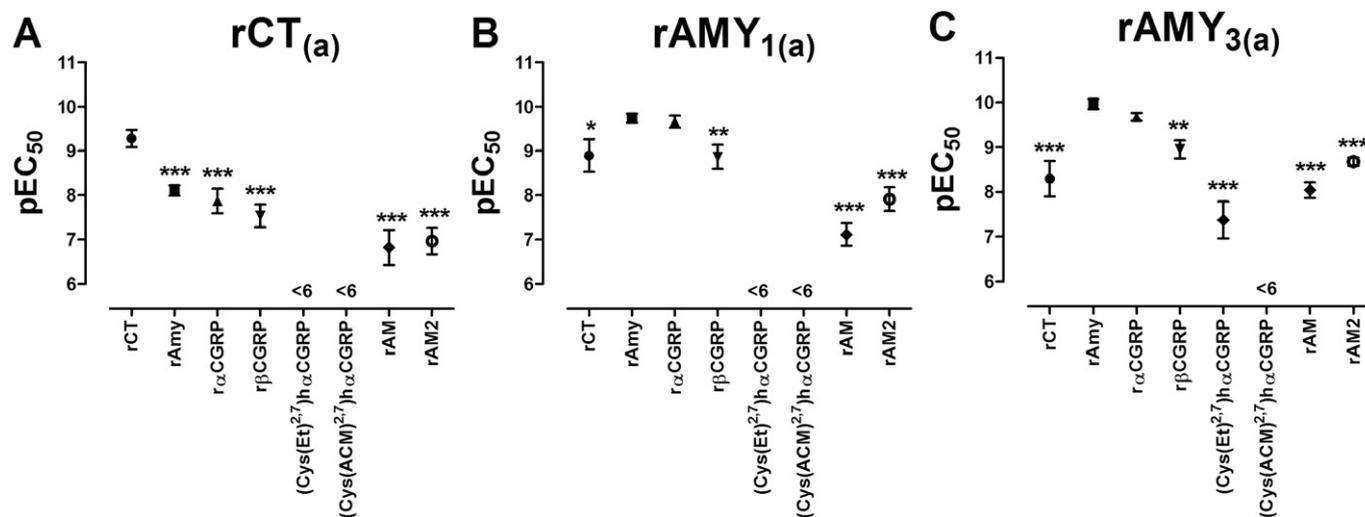
produce any elevation in cAMP over the baseline (data not shown). To provide further information from the agonist data, additional comparisons between agonists at this receptor are presented as supplementary data (Table S1). This revealed that rAmy was significantly more potent than rAM or rAM2. There were no other significant differences.

A series of peptide fragments, reported to be antagonists of CT, Amy and CGRP receptors were then profiled at rCT<sub>(a)</sub>. sCT<sub>8-32</sub>, AC187 and AC413 were similarly effective antagonists of rCT<sub>(a)</sub> receptors (Table 2, Figures 4A, 5A and 6A). In con-

trast, 10  $\mu$ M rAmy<sub>8-37</sub> and 10  $\mu$ M  $\alpha$ CGRP<sub>8-37</sub> were unable to produce a significant shift in the agonist curve at rCT<sub>(a)</sub> receptors (Table 2, Figures 7A and 8A). Statistical analysis is shown in Figure 9A.

### Pharmacology at rAMY<sub>1</sub> receptors – cAMP assay

rCT<sub>(a)</sub> and rRAMP1 were co-transfected to form putative rAMY<sub>1(a)</sub> receptors. rAmy and  $\alpha$ CGRP were effective agonists, which displayed almost identical potencies; there was no



**Figure 3**

Distribution of  $pEC_{50}$  values for the agonists rCT, rAmy,  $r\alpha$ CGRP,  $r\beta$ CGRP,  $(Cys(Et)^{2.7})h\alpha$ CGRP,  $(Cys(ACM)^{2.7})h\alpha$ CGRP, rAM and rAM2 in COS-7 cells transfected to express either (A)  $rCT_{(a)}$  receptors, (B)  $rAMY_{1(a)}$  receptors or (C)  $rAMY_{3(a)}$  receptors. Data are mean  $\pm$  SEM of four to 23 combined experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  by one-way ANOVA followed by Tukey's test. For simplification, only comparisons against the cognate agonist for each receptor are shown (rCT for  $CT_{(a)}$ , rAmy for  $rAMY_{1(a)}$  and  $rAMY_{3(a)}$ ); other comparisons are shown in supplementary Tables S1–3.  $(Cys(Et)^{2.7})h\alpha$ CGRP and  $(Cys(ACM)^{2.7})h\alpha$ CGRP were excluded from the analysis except in the  $rAMY_{3(a)}$  receptor, where  $(Cys(Et)^{2.7})h\alpha$ CGRP produced a sufficient response to be included.

**Table 1**

Agonist potencies ( $pEC_{50}$  values) for cAMP production at  $rCT_{(a)}$ ,  $rAMY_{1(a)}$  and  $rAMY_{3(a)}$  receptors

Agonist	$rCT_{(a)}$ receptor	$rAMY_{1(a)}$ receptor	$rAMY_{3(a)}$ receptor
rAmy	8.11 $\pm$ 0.12 (15)	9.74 $\pm$ 0.10 (23)***	9.97 $\pm$ 0.11 (18)***
rCT	9.28 $\pm$ 0.20 (11)	8.90 $\pm$ 0.37 (6)	8.30 $\pm$ 0.40 (4)*
$r\alpha$ CGRP	7.87 $\pm$ 0.28 (7)	9.66 $\pm$ 0.13 (13)***	9.68 $\pm$ 0.08 (6)***
$r\beta$ CGRP	7.54 $\pm$ 0.25 (7)	8.87 $\pm$ 0.27 (9)*	8.95 $\pm$ 0.20 (6)
$(Cys(ACM)^{2.7})h\alpha$ CGRP	<6 (4)	<6 (4)	<6 (4)
$(Cys(Et)^{2.7})h\alpha$ CGRP	<6 (4)	<6 (4)	7.70 $\pm$ 0.39 (4) <sup>a</sup>
rAM	6.82 $\pm$ 0.39 (6)	7.12 $\pm$ 0.26 (4)	8.04 $\pm$ 0.17 (5)* <sup>+</sup>
rAM2	6.97 $\pm$ 0.30 (6)	7.92 $\pm$ 0.27 (4)	8.68 $\pm$ 0.09 (5)***

Data are mean  $\pm$  SEM. Values in parentheses represent the number of individual experiments analysed. <sup>a</sup>Weak partial agonist. Comparisons between receptors were performed by one-way ANOVA followed by Tukey's test (vs.  $rCT_{(a)}$ , \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . vs.  $rAMY_{1(a)}$ , \* $P < 0.05$ ). Comparisons of agonists within each column can be found in Figure 3 and Tables S1–S3.

significant difference between them (Table 1, Figures 2B and C, 3B). All other agonists were significantly weaker compared with these two agonists; rCT and  $r\beta$ CGRP were approximately 10-fold weaker as agonists in cells transfected with these receptor components (Table 1, Figure 2A and D). rAM2 and rAM were approximately 100-fold and 1000-fold weaker than rAmy (Figures 2E and F, 3B) but were not significantly different from one-another (Table S2). Other comparisons are shown in Table S2.  $(Cys(Et)^{2.7})h\alpha$ CGRP and  $(Cys(ACM)^{2.7})h\alpha$ CGRP failed to produce any elevation in cAMP over the baseline.

AC413 was the most effective antagonist of  $rAMY_{1(a)}$  receptors, followed by AC187 and  $sCT_{8-32}$ . AC413 was significantly

more effective than  $sCT_{8-32}$  but not AC187 (Figures 4B, 5B, 6B and 9B, Table 2).  $rAmy_{8-37}$  was also able to antagonize this receptor but more weakly than the other antagonists (Table 2, Figures 7B and 9B).  $r\alpha$ CGRP<sub>8-37</sub> was as effective as  $sCT_{8-32}$  but weaker than AC187 and AC413 (Table 2, Figures 8B and 9B).  $h\alpha$ CGRP<sub>8-37</sub> was also an effective antagonist (Table 2). Figure 9B shows all statistical comparisons between these antagonists.

### Pharmacology at $rAMY_3$ receptors – cAMP assay

To examine the pharmacology of putative  $rAMY_{3(a)}$  receptor phenotype, cells were transfected with  $rCT_{(a)}$  and  $rRAMP3$ .

Table 2

Summary of  $pK_B$  values for antagonism of rAmy-induced cAMP production at  $rCT_{(a)}$ ,  $rAMY_{1(a)}$  and  $rAMY_{3(a)}$  receptors

Antagonist	$rCT_{(a)}$ receptor	$rAMY_{1(a)}$ receptor	$rAMY_{3(a)}$ receptor
sCT <sub>8-32</sub>	8.13 ± 0.23 (4)	7.42 ± 0.28 (4)	8.17 ± 0.16 (4)
AC187	7.78 ± 0.11 (4)	8.24 ± 0.36 (4)	8.11 ± 0.23 (3)
AC413	8.09 ± 0.15 (4)**	8.97 ± 0.14 (4)	8.60 ± 0.15 (4)
rAMY <sub>8-37</sub>	<5 (4) <sup>a</sup>	6.16 ± 0.14 (5)	5.53 ± 0.11 (4)*
rαCGRP <sub>8-37</sub>	<5 (3)	7.07 ± 0.15 (4)	7.00 ± 0.08 (3)
hαCGRP <sub>8-37</sub>	–	7.62 ± 0.14 (4)	–

Data are mean ± SEM. Values in parentheses represent the number of individual experiments analysed. <sup>a</sup>rCT was used as agonist. Comparisons between receptors were performed using unpaired *t*-tests or one-way ANOVA followed by Tukey's tests where appropriate (vs.  $rAMY_{1(a)}$ , \**P* < 0.05, \*\**P* < 0.01). Comparisons of antagonists within each column can be found in Figure 9.

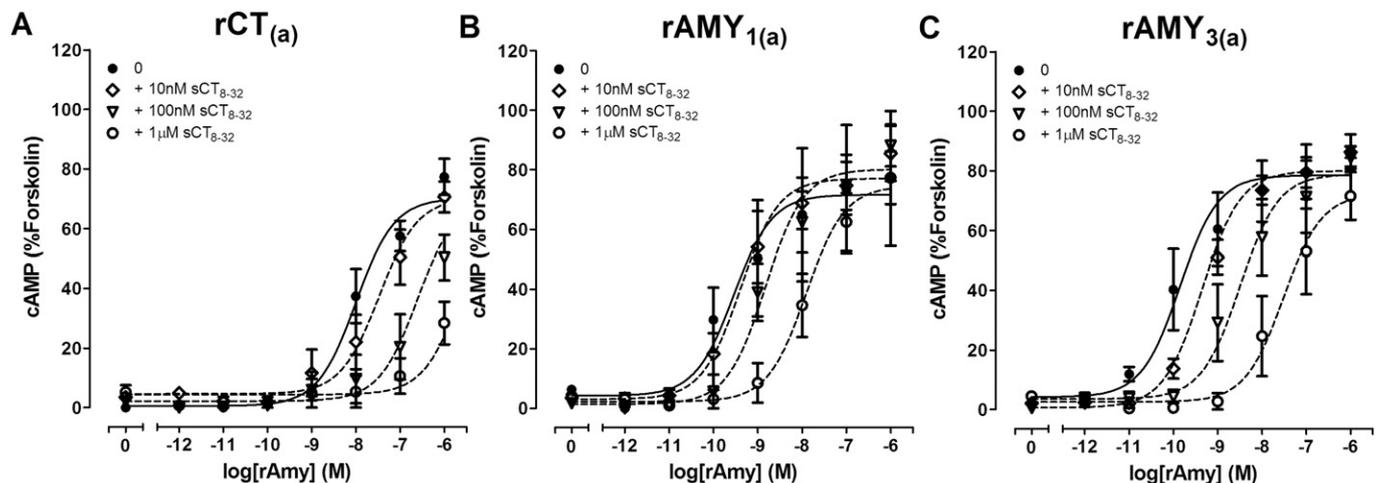


Figure 4

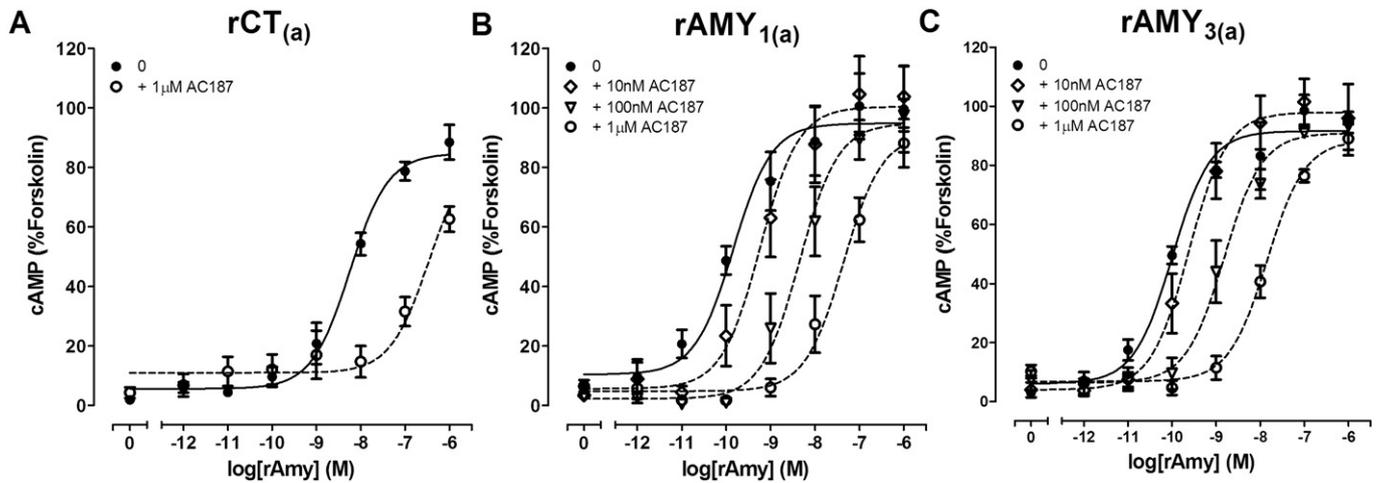
Antagonism of rAmy-induced cAMP production by the indicated concentrations of sCT<sub>8-32</sub> in COS-7 cells transfected to express either (A)  $rCT_{(a)}$  receptors, (B)  $rAMY_{1(a)}$  receptors or (C)  $rAMY_{3(a)}$  receptors. Data are expressed as a percentage of the cAMP response generated by 50 µM forskolin. Data are mean ± SEM of four combined experiments, performed in triplicate or quadruplicate.

rAmy potently stimulated cAMP production in these cells (Table 1, Figures 2B and 3C); rαCGRP was similarly potent (Table 1, Figure 2C). All other agonists were significantly weaker at this receptor (Figure 3C). Whilst (Cys(ACM)<sup>2,7</sup>) hαCGRP was without measurable effect (Cys(Et)<sup>2,7</sup>) hαCGRP behaved as a weak partial agonist compared with rAmy and the other agonists tested ( $E_{max}$ , 18.8% ± 2.6 forskolin, *n* = 4). The statistical comparison between agonists is shown in Table S3.

AC187, AC413 and sCT<sub>8-32</sub> were equally effective antagonists of  $rAMY_{3(a)}$  receptors, followed by rαCGRP<sub>8-37</sub> (Table 2, Figures 4C, 5C, 6C and 8C). rAMY<sub>8-37</sub> acted as a weak antagonist of this receptor and was significantly less effective than the other antagonists tested (Table 2, Figure 7C). Figure 9C shows all statistical comparisons between these antagonists.

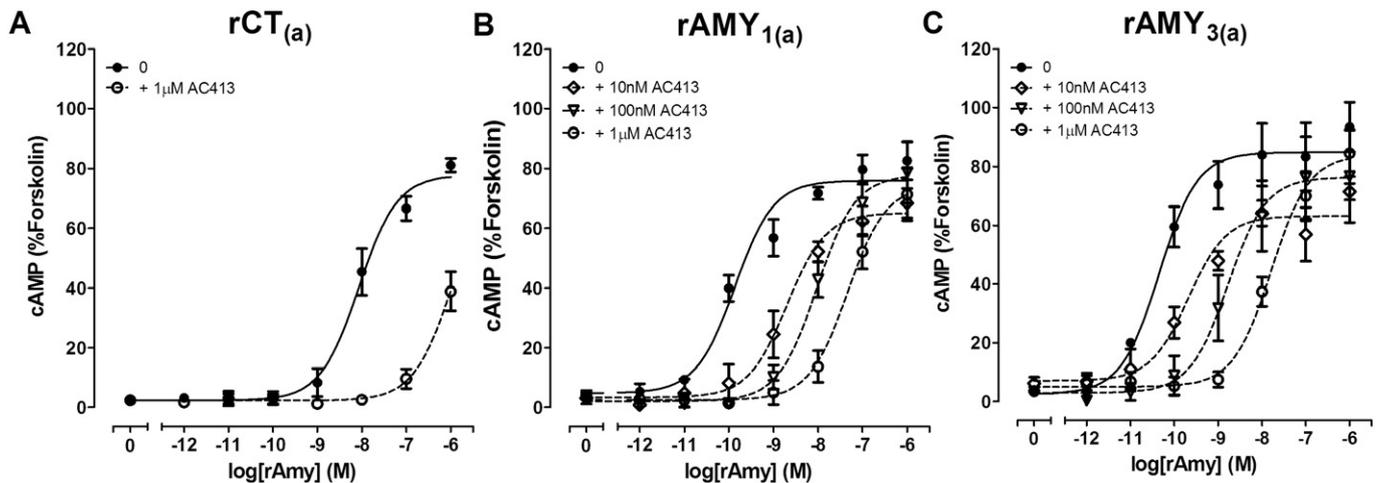
### Comparison of pharmacology across rat $CT_{(a)}$ , $AMY_{1(a)}$ and $AMY_{3(a)}$ receptor subtypes – cAMP assay

Comparison of agonist potencies between receptors by one-way ANOVA followed by Tukey's tests (Table 1) revealed that rCT potency was significantly reduced at  $rAMY_{3(a)}$  compared with  $rCT_{(a)}$ . It was also reduced at  $rAMY_{1(a)}$ , but this did not reach statistical significance. rAmy potency was significantly increased at both  $rAMY_{1(a)}$  and  $rAMY_{3(a)}$  compared with  $rCT_{(a)}$ , although there was no difference between  $rAMY_{1(a)}$  and  $rAMY_{3(a)}$ . rαCGRP and rβCGRP behaved similarly to rAmy, although significance was not reached for  $rAMY_{3(a)}$  with rβCGRP. rAM potency was not significantly enhanced at the  $rAMY_{1(a)}$  receptor compared with the  $rCT_{(a)}$  receptor but was enhanced at the  $rAMY_{3(a)}$  receptor. This was reflected by sig-



**Figure 5**

Antagonism of rAmy-induced cAMP production by the indicated concentrations of AC187 in COS-7 cells transfected to express either (A) rCT<sub>(a)</sub> receptors, (B) rAMY<sub>1(a)</sub> receptors or (C) rAMY<sub>3(a)</sub> receptors. Data are expressed as a percentage of the cAMP response generated by 50 μM forskolin. Data are mean ± SEM of three to four combined experiments, performed in triplicate or quadruplicate.



**Figure 6**

Antagonism of rAmy-induced cAMP production by the indicated concentrations of AC413 in COS-7 cells transfected to express either (A) rCT<sub>(a)</sub> receptors, (B) rAMY<sub>1(a)</sub> receptors or (C) rAMY<sub>3(a)</sub> receptors. Data are expressed as a percentage of the cAMP response generated by 50 μM forskolin. Data are mean ± SEM of four combined experiments, performed in triplicate or quadruplicate.

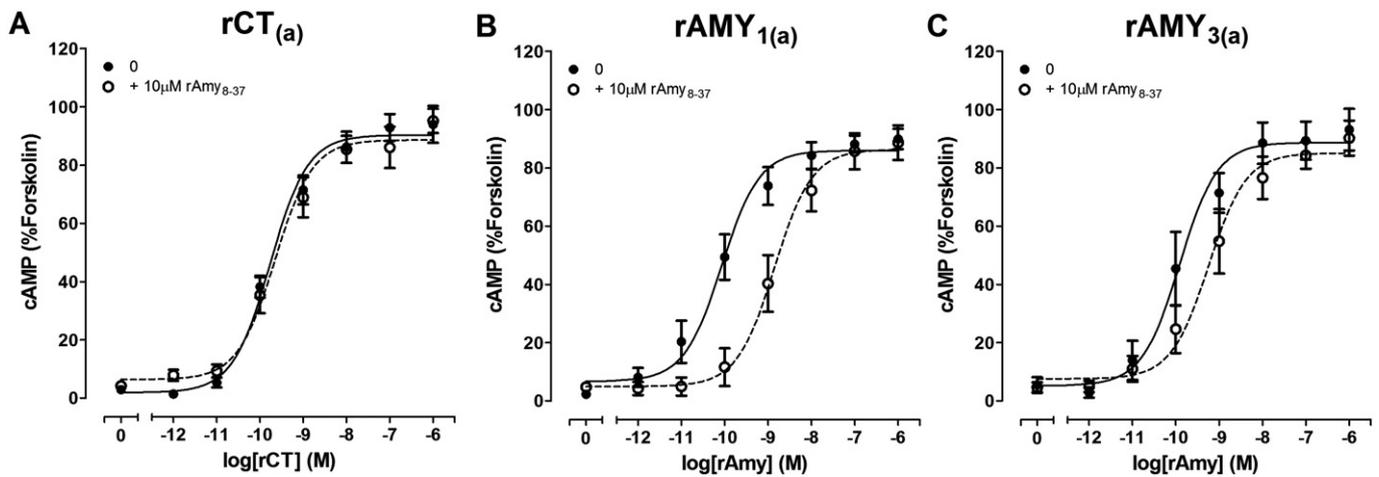
nificant differences between rAMY<sub>1(a)</sub> and rAMY<sub>3(a)</sub> receptors, with rAM exhibiting higher potency at rAMY<sub>3(a)</sub> than rAMY<sub>1(a)</sub>. For rAM2, the potency of this peptide was also enhanced at rAMY<sub>3(a)</sub> relative to rCT<sub>(a)</sub>, but in this case, the difference in potency between rAMY<sub>1(a)</sub> and rAMY<sub>3(a)</sub> did not reach statistical significance.

Comparisons of the antagonist potency data across the three receptors showed that rAmy<sub>8-37</sub> was a selective antagonist of the rAMY<sub>1(a)</sub> receptor, compared with rAMY<sub>3(a)</sub> and rCT<sub>(a)</sub> where no measurable shift in the concentration–response curve to rCT was detected (Table 2). rαCGRP<sub>8-37</sub> was without effect at rCT<sub>(a)</sub> but was able to antagonize rAmy responses at rAMY<sub>1(a)</sub> and rAMY<sub>3(a)</sub>, although there was no significant difference between these receptors. There were no

significant differences in sCT<sub>8-32</sub> or AC187 potency among the three receptors, although there was a trend towards a decrease in sCT<sub>8-32</sub> potency at rAMY<sub>1(a)</sub> compared with the other two receptors and a trend towards increased AC187 potency at rAMY<sub>1(a)</sub> and rAMY<sub>3(a)</sub> versus rCT<sub>(a)</sub>. AC413 had higher potency at the rAMY<sub>1(a)</sub> and rAMY<sub>3(a)</sub> receptors compared with rCT<sub>(a)</sub>, but this only reached statistical significance for rAMY<sub>1(a)</sub>.

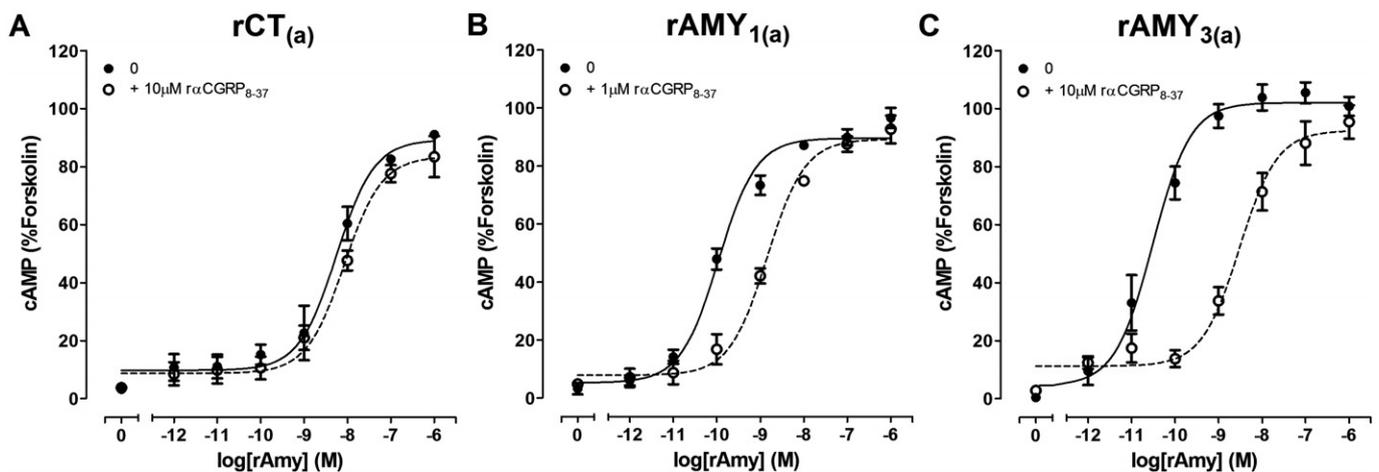
### Pharmacology of rat CT<sub>(a)</sub>, AMY<sub>1(a)</sub> and AMY<sub>3(a)</sub> receptor subtypes – radioligand binding

The co-transfection of rCT<sub>(a)</sub> with either rRAMP1 and rRAMP3 resulted in significant enhancement in rAmy



**Figure 7**

Antagonism of rCT or rAmy-induced cAMP production by 10 μM rAmy<sub>8-37</sub> in COS-7 cells transfected to express either (A) rCT<sub>(a)</sub> receptors, (B) rAMY<sub>1(a)</sub> receptors or (C) rAMY<sub>3(a)</sub> receptors. Data are expressed as a percentage of the cAMP response generated by 50 μM forskolin. Data are mean ± SEM of four to five combined experiments, performed in triplicate or quadruplicate.



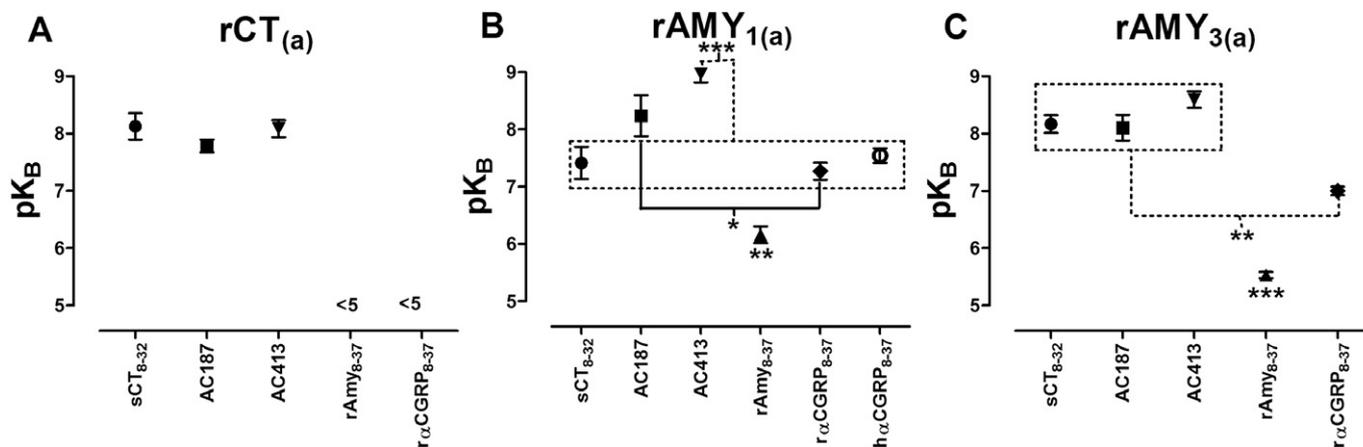
**Figure 8**

Antagonism of rAmy-induced cAMP production by the indicated concentrations of rαCGRP<sub>8-37</sub> in COS-7 cells transfected to express either (A) rCT<sub>(a)</sub> receptors, (B) rAMY<sub>1(a)</sub> receptors or (C) rAMY<sub>3(a)</sub> receptors. Data are expressed as a percentage of the cAMP response generated by 50 μM forskolin. Data are mean ± SEM of three to four combined experiments, performed in triplicate or quadruplicate.

potency. To confirm the induction of Amy receptor phenotype, radioligand binding studies were conducted. There was negligible specific binding of [<sup>125</sup>I]-rAmy to cells transfected with rCT<sub>(a)</sub> alone under these experimental conditions (Figure 10A). However, the specific binding of [<sup>125</sup>I]-rAmy was increased when rCT<sub>(a)</sub> was co-transfected with rRAMP1 ( $P < 0.05$ ) or rRAMP3 ( $P < 0.01$ ) (Figure 10A). In competition binding assays, rAmy displaced [<sup>125</sup>I]-rAmy from rAMY<sub>1(a)</sub> and rAMY<sub>3(a)</sub> receptors with pIC<sub>50</sub>s of  $8.62 \pm 0.01$  and  $8.64 \pm 0.02$  ( $n = 3$ ), respectively (Figure 10B). Similarly, rαCGRP displaced [<sup>125</sup>I]-rAmy from rAMY<sub>1(a)</sub> receptors with a pIC<sub>50</sub> of  $8.43 \pm 0.09$  ( $n = 3$ ) (Figure 10C). At rAMY<sub>3(a)</sub> receptors, rαCGRP displaced [<sup>125</sup>I]-rAmy with a pIC<sub>50</sub> of  $7.92 \pm 0.03$  ( $n = 2$ ).

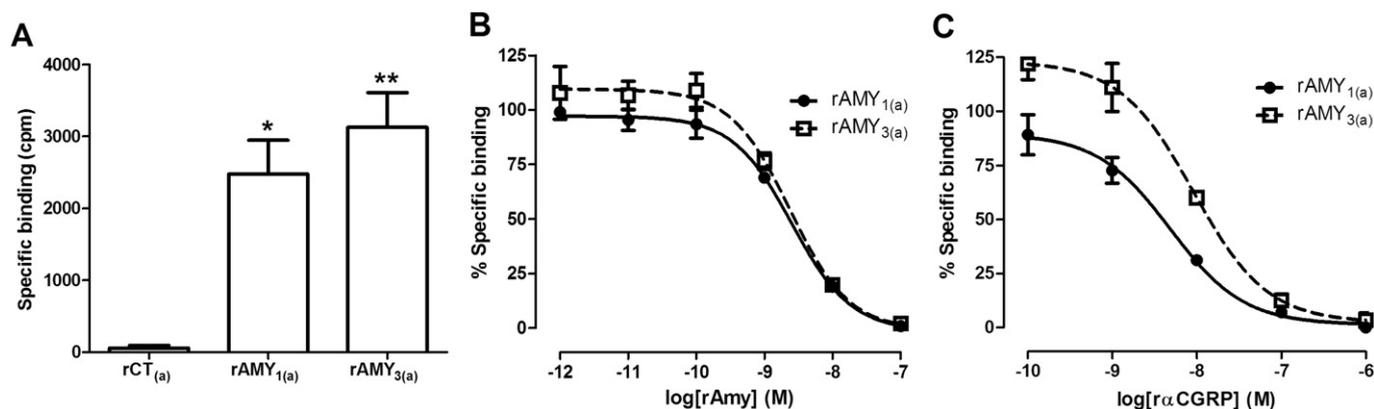
### Pharmacology of CGRP-responsive receptors – cAMP assay

The AMY<sub>1</sub> receptor has been suggested to represent a major molecular correlate responsible for observations of relatively CGRP<sub>8-37</sub>-insensitive CGRP-responsive receptors in the literature, known formerly as 'CGRP<sub>2</sub> receptors' (Hay *et al.*, 2008). This explanation is based on studies with human receptors whilst the reported heterogeneity is from rodents or other species. Therefore, we compared the antagonist potency of αCGRP<sub>8-37</sub> at rAMY<sub>1(a)</sub> and rCGRP receptors stimulated by rαCGRP. rαCGRP potently stimulated cAMP production in cells transfected with rCGRP receptors (pEC<sub>50</sub>;  $10.50 \pm 0.16$ ,  $n = 7$ ) and rAMY<sub>1(a)</sub> receptors (pEC<sub>50</sub>;  $9.66 \pm 0.13$ ,  $n = 13$ ).



**Figure 9**

Distribution of  $pK_B$  values for the antagonists sCT<sub>8-32</sub>, AC187, rAmy<sub>8-37</sub>, rαCGRP<sub>8-37</sub> and hαCGRP<sub>8-37</sub> in COS-7 cells transfected to express either (A) rCT<sub>(a)</sub> receptors, (B) rAMY<sub>1(a)</sub> receptors or (C) rAMY<sub>3(a)</sub> receptors. Data are mean  $\pm$  SEM of three to five combined experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  by one-way ANOVA followed by Tukey's tests. Data points in boxes are significantly different, as indicated, from the comparator.



**Figure 10**

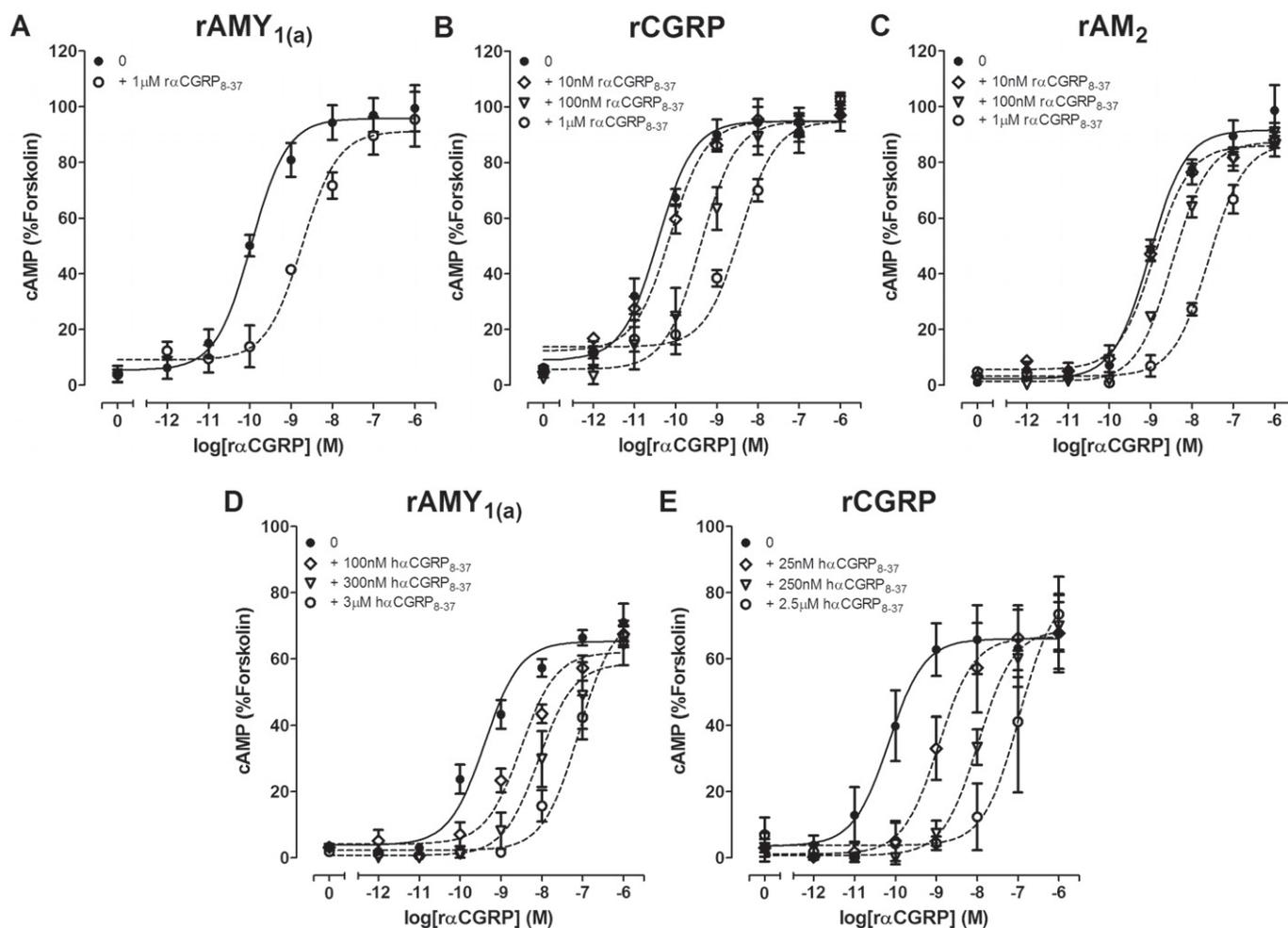
Specific binding of 100 pM [<sup>125</sup>I]-rAmy in (A) Cos-7 cells transfected to express either rCT<sub>(a)</sub> receptors, rAMY<sub>1(a)</sub> receptors or rAMY<sub>3(a)</sub> receptors and competitive displacement of 100 pM [<sup>125</sup>I]-rAmy by (B) rAmy or (C) rαCGRP in COS-7 cells transfected to express either rat AMY<sub>1(a)</sub> receptors or rat AMY<sub>3(a)</sub> receptors expressed as a percentage maximum of 100 pM [<sup>125</sup>I]-rAmy specific binding. Data are mean  $\pm$  SEM of three to five (A) or two to three (B and C) combined experiments, performed in duplicate or triplicate.

rαCGRP displayed higher potency at rCGRP receptors, compared with the rAMY<sub>1(a)</sub> receptor ( $P < 0.01$  by one-way ANOVA followed by Tukey's test). Antagonism of rαCGRP-mediated cAMP stimulation by rαCGRP<sub>8-37</sub> was significantly greater ( $P < 0.01$  by one-way ANOVA followed by a Tukey's test) at the rCGRP receptor ( $pK_B$ ;  $8.12 \pm 0.27$ ,  $n = 3$ ) than at the rAMY<sub>1(a)</sub> receptor ( $pK_B$ ;  $7.20 \pm 0.16$ ,  $n = 3$ ) (Figures 11A and B, 12A). The AM<sub>2</sub> receptor has also been suggested as a molecular correlate for the 'CGRP<sub>2</sub> receptor' and so rαCGRP<sub>8-37</sub> was also compared at this receptor. rAM<sub>2</sub> receptors were effectively activated by rαCGRP with a  $pEC_{50}$  of  $8.96 \pm 0.13$ ,  $n = 3$  ( $P < 0.001$  vs. rCGRP and rAMY<sub>1(a)</sub> receptors by one-way ANOVA followed by a Tukey's test). This receptor was also antagonized by rαCGRP<sub>8-37</sub> ( $pK_B$ ;  $7.32 \pm 0.19$ ,  $n = 3$ ) but less potently than the rCGRP receptor, although this difference did not reach statistical significance (Figures 11C and 12A).

As many researchers use hαCGRP<sub>8-37</sub>, we also compared antagonism by this peptide at rCGRP and rAMY<sub>1(a)</sub> receptors; a similar pattern to rαCGRP<sub>8-37</sub> was observed (Figure 11D and E, with hαCGRP<sub>8-37</sub> being significantly less effective ( $P < 0.01$  by Student's *t*-test) at the rAMY<sub>1(a)</sub> ( $pK_B$ ;  $7.47 \pm 0.23$ ,  $n = 4$ ) than CGRP receptor ( $pK_B$ ;  $8.76 \pm 0.18$ ,  $n = 4$ ) (Figure 12B). Both antagonists behaved in an equivalent manner using rAmy or rαCGRP as agonists (Table 2, Figure 12).

#### Expression of rCTR, rCLR and rRAMP1

Observations of CGRP<sub>8-37</sub>-insensitive receptors have been made in tissues such as the internal anal sphincter (IAS) and vas deferens (VD). In order to assess the potential physiological contribution of rAMY<sub>1(a)</sub> receptors to this pharmacology, the expression of rAMY<sub>1(a)</sub> receptor components was examined in these two tissues. We found that IAS and VD from



**Figure 11**

Antagonism of  $r\alpha$ CGRP-induced cAMP production by the indicated concentrations of  $r\alpha$ CGRP<sub>8-37</sub> or  $h\alpha$ CGRP<sub>8-37</sub> in COS-7 cells transfected to express either (A) and (D)  $rAMY_{1(a)}$  receptors or (B) and (E)  $rCGRP$  receptors or (C)  $rAM_2$  receptors. Data are expressed as a percentage of the cAMP response generated by 50  $\mu$ M forskolin. Data are mean  $\pm$  SEM of three to four combined experiments, performed in triplicate or quadruplicate.

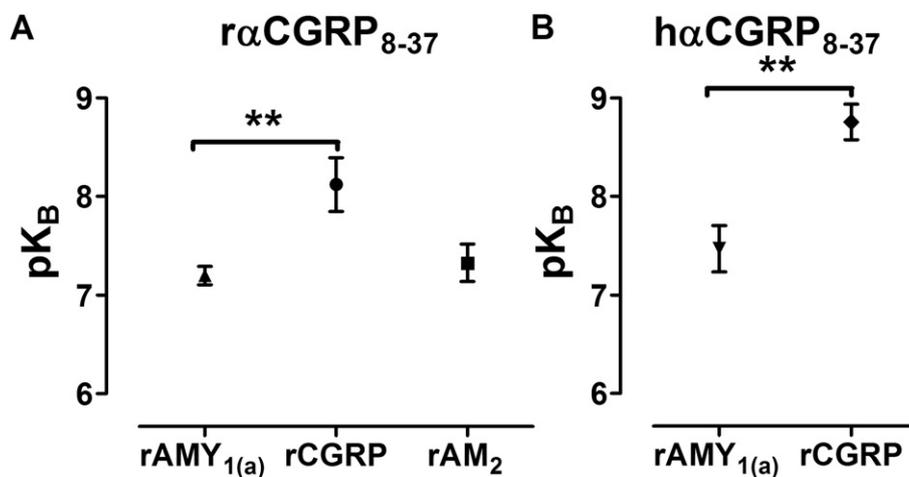
Wistar rats both expressed mRNA for  $rCLR$ ,  $rCT$  receptors and  $rRAMP1$ , although the band intensity of  $rCT$  receptors in IAS in our example was weaker than the others (Figure 13). However, as this is not a quantitative technique and the band intensities of each product varied between each individual preparation (data not shown), we cannot estimate relative abundance. A further study would be required to do this.

### *The pharmacology of $AM_2$ at CGRP and $AM_2$ receptors, compared with $AMY$ and $CT$ receptors*

The pharmacology of  $AM_2$  is ill-defined and has not been reported at rodent CT family receptors. Therefore, it was important to compare our observations of  $rAM_2$  potency at  $rAMY$  and  $rCT_{(a)}$  receptors with  $rCGRP$  and  $rAM_2$  receptors under similar conditions. To examine the pharmacology of the  $rAM_2$  peptide at  $rCGRP$  and  $rAM_2$  receptors, the ability of cells transfected with  $rCLR$  and  $rRAMP1$  or  $rRAMP3$  to stimu-

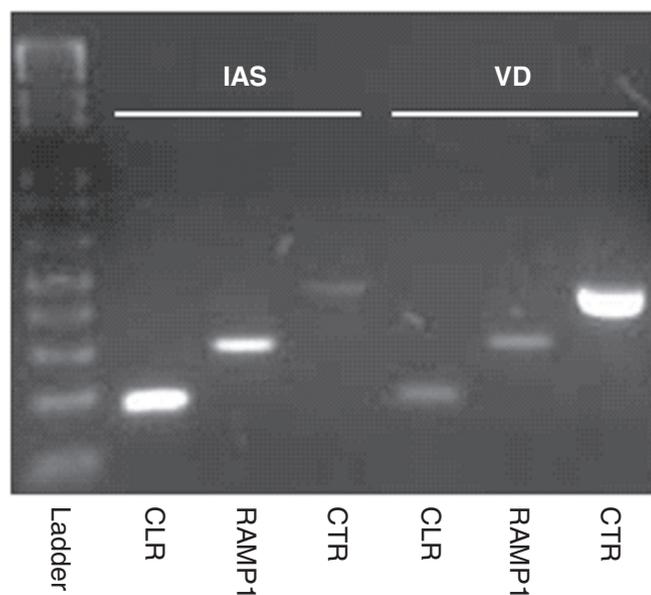
late cAMP production with  $rAM$ ,  $r\alpha$ CGRP or  $rAM_2$  was determined. All potently stimulated cAMP production at  $rCGRP$  and  $rAM_2$  receptors (Table 3, Figure 14).  $r\alpha$ CGRP was more potent at  $rCGRP$  compared to  $rAM_2$  receptors and  $rAM$  was more potent at  $rAM_2$  compared with  $rCGRP$  receptors. Interestingly,  $rAM_2$  displayed highest potency at  $rAM_2$  compared with  $rCGRP$  receptors. At  $rCGRP$  receptors  $r\alpha$ CGRP displayed higher potency than  $rAM$  or  $rAM_2$ ;  $rAM$  and  $rAM_2$  were equipotent (Figure 14A). The converse was observed at  $rAM_2$  receptors with  $r\alpha$ CGRP displaying lower potency than  $rAM$  or  $rAM_2$ ;  $rAM$  and  $rAM_2$  were equipotent (Figure 14B). Similar pharmacology to  $rAM_2$  receptors was observed for mouse  $AM_2$  receptors (Figure 14C).

Comparison of  $rAM_2$  potencies by one-way ANOVA followed by Tukey's test revealed that  $rAM_2$  was more potent at  $rAM_2$  receptors compared with  $rCGRP$  ( $P < 0.01$ ),  $rCT_{(a)}$   $rAMY_{1(a)}$  ( $P < 0.001$ ) and  $rAMY_{3(a)}$  ( $P < 0.05$ ) receptors. The rank order of potency for  $rAM_2$  across these receptors was  $rAM_2 > rCGRP = rAMY_{3(a)} > rAMY_{1(a)} > rCT_{(a)}$  (Table 1, Table 3).



**Figure 12**

Distribution of  $pK_B$  values for (A)  $r\alpha\text{CGRP}_{8-37}$  or (B)  $h\alpha\text{CGRP}_{8-37}$  in COS-7 cells transfected to express either  $r\text{AMY}_{1(a)}$  receptors,  $r\text{CGRP}$  receptors or  $r\text{AM}_2$  receptors using  $r\alpha\text{CGRP}$  as the agonist. Data are mean  $\pm$  SEM of three to five combined experiments. Comparisons were performed by (A) one-way ANOVA followed by Tukey's tests or (B) unpaired  $t$ -test (\*\* $P < 0.01$ ).



**Figure 13**

Representative gel electrophoresis image showing the expression of mRNA encoding  $r\text{CLR}$ ,  $r\text{RAMP1}$  and  $r\text{CT}$  receptor in rat IAS and VD. Similar results were obtained from tissue derived from three to four adult male Wistar rats.

## Discussion and conclusions

Additional Amy receptor modulators could be valuable medicines. However, their development depends on determination of the Amy receptor subtypes involved in the specific biological processes of this peptide. This is not known in humans or rodent models of disease. To complement molecular methods of identifying AMY receptor subtypes, we herein report the pharmacological characterization of  $r\text{AMY}$  recep-

tors. We provide a valuable resource; allowing the complex overlapping pharmacology previously observed in many rodent tissues to be better understood (Sexton *et al.*, 1994; Riediger *et al.*, 1999).

As observed for recombinant human receptors (Christopoulos *et al.*, 1999; Muff *et al.*, 1999; Hay *et al.*, 2005),  $r\text{CT}_{(a)}$  alone produced a  $r\text{CT}$  receptor, whereas the co-expression of  $r\text{CT}_{(a)}$  with either  $r\text{RAMP1}$  or  $r\text{RAMP3}$  produced AMY receptors. This is defined by significant enhancement in  $r\text{Amy}$  potency and induction of high affinity  $r\text{Amy}$  binding (Poyner *et al.*, 2002). This contrasts with  $p\text{CT}_{(a)}$  also transfected into COS-7 cells, where  $h\text{Amy}$  potency in cAMP assays was not increased by the presence of any  $p\text{RAMP}$  (Kikumoto *et al.*, 2003). Enhancement in  $p\text{CGRP}$  potency in the presence of  $p\text{RAMP1}$  was observed, consistent with our data and earlier studies (Christopoulos *et al.*, 1999; Kuwasako *et al.*, 2004; Hay *et al.*, 2005). The apparent disparity in pharmacology between human, rat and  $p\text{CT}_{(a)}/\text{RAMP}$  complexes are not obviously explained by methodological differences. A contributing factor could be the species of Amy used. Alternatively, Amy potency might have been enhanced if an alternative signalling molecule to cAMP was measured (Morris *et al.*, 2008).

$r\alpha\text{CGRP}$  potency at  $r\text{CT}_{(a)}$ ,  $r\text{AMY}_{1(a)}$  and  $r\text{AMY}_{3(a)}$  receptors mirrored that of  $r\text{Amy}$ , displaying equivalent potency to  $r\text{Amy}$  at all receptors. This differs from  $h\text{AMY}$  receptors, where there was greater discrimination between  $h\alpha\text{CGRP}$  and  $r\text{Amy}$  at  $h\text{AMY}_{3(a)}$  receptors (Hay *et al.*, 2005).  $r\beta\text{CGRP}$  tended to be less potent than  $r\alpha\text{CGRP}$  at  $r\text{CT}_{(a)}$ ,  $r\text{AMY}_{1(a)}$  and  $r\text{AMY}_{3(a)}$  receptors. This also contrasts with human receptors, which displayed a reversal of this trend at  $h\text{CT}_{(a)}$  and  $h\text{AMY}_{1(a)}$  and similar potency for  $h\alpha\text{CGRP}$  and  $h\beta\text{CGRP}$  at  $h\text{AMY}_{3(a)}$  (Hay *et al.*, 2005). Some of these differences may be explained by sequence differences between peptides and/or receptor subunits. However, no clear pattern is apparent, and further work is needed to understand these differences.

The linear CGRP analogues (Cys(Et)<sup>2,7</sup>) $h\alpha\text{CGRP}$  and (Cys(ACM)<sup>2,7</sup>) $h\alpha\text{CGRP}$ , both failed to significantly enhance

Table 3

Agonist potencies (pEC<sub>50</sub> values) for cAMP production at rCGRP, rAM<sub>2</sub> and mouse (m) AM<sub>2</sub> receptors

Agonist	rCGRP receptor	rAM <sub>2</sub> receptor	mAM <sub>2</sub> receptor
rαCGRP	10.53 ± 0.04 (5)	8.59 ± 0.13 (8) <sup>++</sup>	8.65 ± 0.10 (4)
rAM	8.50 ± 0.43 (5) <sup>**</sup>	10.15 ± 0.26 (7) <sup>***,+++</sup>	10.24 ± 0.08 (3) <sup>***</sup>
rAM2	8.79 ± 0.02 (3) <sup>**</sup>	9.93 ± 0.26 (3) <sup>***,+</sup>	10.05 ± 0.21 (3) <sup>***</sup>

Data are mean ± SEM. Values in parentheses represent the number of individual experiments analysed. In these experiments, cAMP concentrations were determined by AlphaScreen. Comparisons between rCGRP and rAM<sub>2</sub> receptors were performed by Student's *t*-test (<sup>+</sup>*P* < 0.05; <sup>++</sup>*P* < 0.01; <sup>+++</sup>*P* < 0.001). Comparisons between peptides within each receptor were performed by one-way ANOVA followed by Tukey's tests. Comparisons shown are against rαCGRP (<sup>\*\*</sup>*P* < 0.01; <sup>\*\*\*</sup>*P* < 0.001). No significant differences between rAM and rAM<sub>2</sub> were observed.

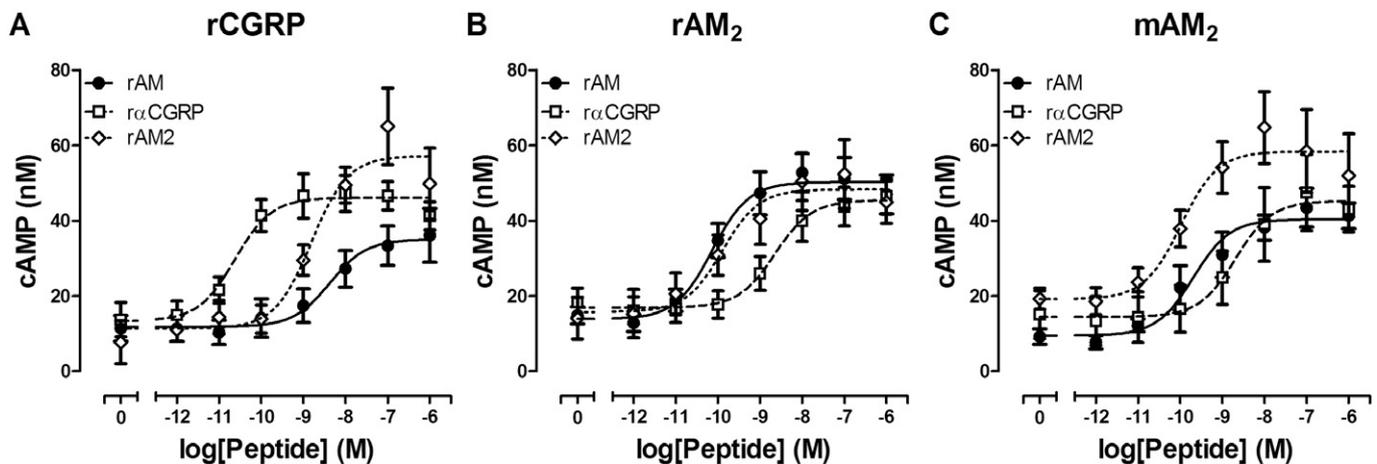


Figure 14

Stimulation of cAMP production by rAM, rAM<sub>2</sub> or rαCGRP in COS-7 cells transfected to express either (A) rCGRP receptors, (B) rAM<sub>2</sub> receptors or (C) mouse (m) AM<sub>2</sub> receptors. Data are expressed as the concentration of cAMP generated and are mean ± SEM of three to eight combined experiments, performed in triplicate.

cAMP production at either rCT<sub>(a)</sub> or rAMY<sub>1(a)</sub> receptors. (Cys(ACM)<sup>2-7</sup>)hαCGRP was also without effect at rAMY<sub>3(a)</sub>, although (Cys(Et)<sup>2-7</sup>)hαCGRP behaved as a weak partial agonist. This may reflect their nature as partial agonists. However, it is again interesting that there was a reversal in trend at the rat versus human receptors. Both linear analogues were capable of activating hAMY<sub>1(a)</sub> but not hAMY<sub>3(a)</sub> receptors under similar experimental conditions. However, the use of transient transfections and slightly different transfection ratios of RAMP to CT<sub>(a)</sub> may have resulted in differences in expression and therefore receptor number between the studies.

rCT potency was reduced with rRAMP co-expression, consistent with earlier observations at human receptors and was most noticeable with AMY<sub>3(a)</sub>. This is consistent with the suggestion that less 'free' CT<sub>(a)</sub> is available to be expressed in the presence of RAMP3 and indicates that RAMP3 may interact more strongly with rCT<sub>(a)</sub> than RAMP1 (Hay *et al.*, 2005). 'Free' CT<sub>(a)</sub> may occur as it can be expressed independent of RAMP at the cell surface, but it is difficult to quantify the potential different populations of each receptor. It is interesting that we still observed this with equal transfection of

RAMP and CT<sub>(a)</sub>, whereas previously RAMP was in slight excess in the transfection protocol (Hay *et al.*, 2005). The potencies of most other peptides were enhanced with RAMP expression, suggesting that RAMP is either directly involved in their binding or generates favourable conformations of CT<sub>(a)</sub> for binding. In contrast, the presence of RAMP does not influence CT interactions in the same way. hCT has relatively low affinity and weakly competes with [<sup>125</sup>I]-rAmy binding in cells transfected with CT<sub>(a)</sub> and RAMP1 or RAMP3 (Tilakaratne *et al.*, 2000).

rAM potency was substantially enhanced at rAMY<sub>3(a)</sub> receptors over rCT<sub>(a)</sub>. This has previously been observed for human receptors in HEK293 cells but not in COS-7 cells (Kuwasako *et al.*, 2003; Hay *et al.*, 2005). It is unclear how important the AMY<sub>3(a)</sub> receptor might be to AM physiology or pathophysiology, but our observation, together with the earlier work, does emphasize the need to use multiple probes wherever possible to assist with receptor identification.

We also observed enhanced rAM<sub>2</sub> potency at rAMY<sub>3(a)</sub>, compared with rCT<sub>(a)</sub>. AM<sub>2</sub> was most potent at rAMY<sub>3(a)</sub> versus rAMY<sub>1(a)</sub> and rCT<sub>(a)</sub>. This contrasts with earlier work for human receptors where either hAM<sub>2</sub> (47 amino acids as used

here) or the shorter form (40 amino acids) were both more potent at hAMY<sub>1(a)</sub> (Takei *et al.*, 2004; Hay *et al.*, 2005). To investigate this further, we compared rAM2 potency at rCGRP and rAM<sub>2</sub> receptors. These data support a RAMP3 bias, with rAM2 showing highest potency at the rAM<sub>2</sub> versus the rCGRP receptor. Several studies in the literature with AM2 support this (Chang *et al.*, 2004; Takei *et al.*, 2004; Qi *et al.*, 2008; Hong *et al.*, 2012; Kuwasako *et al.*, 2011). Unfortunately, there is little quantification of potency in many studies, and so it is difficult to draw firm conclusions. Nevertheless, accumulating data seem to suggest that AM2 is a potent agonist of the AM<sub>2</sub> receptor. This is also supported by our observations at the mouse AM<sub>2</sub> receptor.

N-terminally truncated CT-family peptides act as antagonists (Alexander *et al.*, 2011). Therefore, we compared the antagonist potencies of sCT<sub>8-32</sub>, rAmy<sub>8-37</sub> and rαCGRP<sub>8-37</sub> at rCT<sub>(a)</sub>, rAMY<sub>1(a)</sub> and rAMY<sub>3(a)</sub>. In addition, we used AC187 and AC413, which are modified forms of sCT<sub>8-32</sub> and rAmy<sub>8-37</sub>. AC187 is predominantly sCT<sub>8-32</sub> but has two substitutions with the equivalent rAmy residues at positions 30 and 32 (Figure 1). On the other hand, AC413 is mostly rAmy<sub>8-37</sub> but has the central portion replaced with a sequence of sCT residues. AC413 also has an additional serine to alanine substitution towards the peptide C-terminus.

sCT<sub>8-32</sub> was an effective but non-selective antagonist across these three receptors, though it trended towards being weaker at rAMY<sub>1(a)</sub> receptors. This pattern is consistent with studies in human receptors. sCT<sub>8-32</sub> is therefore unlikely to be a useful tool for discriminating between rat CT and AMY receptor subtypes.

AC187 is commonly used as an antagonist of Amy and has been reported to be a selective AMY receptor antagonist (Young *et al.*, 1994). For example, AC187 administration has been shown to reduce glucagon secretion (Gedulin *et al.*, 2006) and increase food intake (Rushing *et al.*, 2001). However, where tested at cloned receptors its ability to distinguish between hAMY<sub>1(a)</sub> and hAMY<sub>3(a)</sub> over hCT<sub>(a)</sub> receptors was limited (~10-fold) (Hay *et al.*, 2005). We now report that AC187 was an effective antagonist across all three rat receptors examined, but it did not display significantly selective antagonism between AMY and CT receptors. A trend towards greater effectiveness at rAMY<sub>1(a)</sub> was observed, but the effect was very small. This may relate to the two amino acids of rAmy that have been incorporated (Figure 1). Therefore, AC187 displays significant antagonism at rCT<sub>(a)</sub>, rAMY<sub>1(a)</sub> and rAMY<sub>3(a)</sub> but is unable to discriminate between them. Thus, AC187 displays similar limitations to sCT<sub>8-32</sub> and should also be used with caution. Its usefulness is likely to depend upon the concentration of agonist present: antagonism of a low Amy concentration would suggest AMY receptor involvement but would not distinguish between AMY<sub>1(a)</sub> and AMY<sub>3(a)</sub> receptors. Conversely, this antagonist may be a useful tool for discriminating between CT<sub>(a)</sub>-based and CLR-based receptors, particularly with respect to agonism by CGRP, which can effectively activate both CGRP and AMY receptors (Muff *et al.*, 1999; Leuthauser *et al.*, 2000; Kuwasako *et al.*, 2004; Bailey and Hay, 2006). AC187 is a weak antagonist of CGRP receptors (Bailey and Hay, 2006).

Amy<sub>8-37</sub> is generally considered a weak antagonist of rAmy and rαCGRP responses (Poyner *et al.*, 1998; Hay *et al.*, 2005;

Bailey and Hay, 2006), with some notable exceptions (Cornish *et al.*, 1999; Danaher *et al.*, 2008). Here, rAmy<sub>8-37</sub> was also a weak antagonist, with no measurable affinity at rCT<sub>(a)</sub>, but measurable antagonism at rAMY<sub>1(a)</sub> and rAMY<sub>3(a)</sub> receptors. The pK<sub>B</sub> we observed at rAMY<sub>1(a)</sub> is higher than previous observations at hAMY<sub>1(a)</sub>; 6.16 versus 5.59, respectively (both using rAMY<sub>8-37</sub>) (Hay *et al.*, 2005). Similarly, rAmy<sub>8-37</sub> was more effective at rAMY<sub>3(a)</sub> than hAMY<sub>3(a)</sub>; pK<sub>B</sub> 5.53 versus <5, respectively. This difference between human and rat receptor systems could explain why in some rodent studies effective antagonism by Amy<sub>8-37</sub> is observed (Wang *et al.*, 1993; Ye *et al.*, 2001), despite reports of negligible potencies or affinities (Aiyar *et al.*, 1995; Hay *et al.*, 2005). However, we acknowledge that the antagonist is still very weak, even at the rat receptors. Importantly, rAmy<sub>8-37</sub> exhibited selective antagonism for rAMY<sub>1(a)</sub> over rAMY<sub>3(a)</sub> receptors. Thus, rAmy<sub>8-37</sub> displays some characteristics necessary to effectively discriminate rAMY receptor subtypes. Therefore, whilst rAmy<sub>8-37</sub> is unlikely to be useful as a practical tool for distinguishing AMY receptor subtypes in its own right, it could serve as a basis for developing other peptides.

AC413 has also been reported as an amylin receptor antagonist (Young, 2005c). This peptide was the most effective antagonist of rAMY<sub>1(a)</sub> receptors, but the discrimination against rCT<sub>(a)</sub> receptors was less than 10-fold. This peptide was also slightly more effective at inhibiting rAmy responses at hAMY<sub>1(a)</sub> receptors than hCT<sub>(a)</sub> or hAMY<sub>3(a)</sub>. This suggests that AC413 does have an AMY<sub>1(a)</sub> receptor bias, but it does not have sufficient selectivity to be a useful tool. AC413 shares most sequence identity with rAmy<sub>8-37</sub> and also shows a preference for rat and hAMY<sub>1(a)</sub> receptors over AMY<sub>3(a)</sub> and CT<sub>(a)</sub> receptors. Therefore, the incorporation of the sCT sequence appears to confer increased affinity with the limited degree of AMY<sub>1(a)</sub> receptor selectivity being retained in the remaining Amy sequence. There may be scope for further modifications to the AC413 sequence that improve the separation between receptors; such tools are urgently needed.

Of the antagonists tested, rαCGRP<sub>8-37</sub> displayed the greatest selectivity for rAMY receptors over rCT<sub>(a)</sub> (>100-fold) but was unable to distinguish between AMY receptor subtypes. However, αCGRP<sub>8-37</sub> antagonizes rαCGRP activity at other CGRP-responsive receptors; it displays significantly higher potency at CGRP receptors and similar antagonism at AM<sub>2</sub> receptors to AMY receptors. This indicates that rαCGRP<sub>8-37</sub> would need to be used in concert with other antagonists to effectively distinguish CGRP-responsive receptor subtypes. These studies were expanded to include hαCGRP<sub>8-37</sub> for compatibility with our earlier work and because it is used interchangeably with rαCGRP<sub>8-37</sub> in rodent studies. hαCGRP<sub>8-37</sub> antagonized rAmy and rαCGRP-mediated rAMY<sub>1(a)</sub> receptor activation with similar potency to rαCGRP<sub>8-37</sub>.

Historically, two distinct subtypes of the CGRP receptor were described; CGRP<sub>1</sub> and CGRP<sub>2</sub>. These receptor subtypes were defined based on relative sensitivity to CGRP<sub>8-37</sub>. This classification has been rendered obsolete by the molecular and pharmacological characterization of the CGRP and related receptors (Hay *et al.*, 2008). The CGRP receptor (formerly CGRP<sub>1</sub>) is a complex of CLR and RAMP1, whereas the molecular composition of the CGRP<sub>2</sub> receptor phenotype most likely comprises other CGRP-responsive receptors such as AMY<sub>1</sub> and AM<sub>2</sub> receptors (Hay *et al.*, 2008). Nevertheless,

this explanation has been based on pharmacological characterization of human receptors. Thus, it was important to confirm this in rodents, which is principally where the heterogeneity is reported. Both  $h\alpha$ CGRP<sub>8-37</sub> and  $r\alpha$ CGRP<sub>8-37</sub> displayed weaker antagonism of  $r\alpha$ CGRP-stimulated cAMP accumulation at rAMY<sub>1(a)</sub> compared with rCGRP receptors. For completeness, the rAM<sub>2</sub> receptor was also included and behaved in a similar manner to the rAMY<sub>1(a)</sub> receptor. The potential physiological contribution of rAMY<sub>1(a)</sub> receptors to CGRP receptor heterogeneity was also examined by determining the expression of rAMY<sub>1(a)</sub> receptor subunits in two rat tissues (VD and IAS), which have been historically reported to contain 'CGRP<sub>2</sub>' receptors (Hay, 2007). Rat vas deferens RAMP3 expression has been reported to be extremely low, suggesting that AM<sub>2</sub> receptors are unlikely to contribute to the 'CGRP<sub>2</sub>' receptor phenotype in this tissue (Hay and Smith, 2001). From our observation of PCR products relating to the different subunits, we conclude that it is plausible that these tissues have the capacity to express CGRP and AMY<sub>1</sub> receptors, although further quantitative and co-localization studies are needed to confirm this. Furthermore, rat VD has been reported to express an Amy-sensitive receptor that is antagonized by CGRP<sub>8-37</sub> (Wisskirchen *et al.*, 1998; Poyner *et al.*, 1999). The data confirm that the 'CGRP<sub>2</sub>' receptor phenotype is most likely to be explained by CGRP-responsive receptors such as AMY<sub>1</sub> or AM<sub>2</sub> receptors.

In conclusion, this study has shown for the first time that the co-expression of rCT<sub>(a)</sub> with rRAMP1 or 3 produces functional AMY receptor subtypes in rats, which display equivalent potency for rAmy and  $r\alpha$ CGRP. Some differences in pharmacology were noted between the rodent receptors and previous work with human receptors, and so it is important not to assume that there will always be a direct translation between species. The work has reinforced the urgent need for new tools with which to distinguish CT from AMY receptors and AMY receptor subtypes from one-another. The most commonly used tools at present are not adequate and caution should be applied when interpreting data that use them. Nevertheless, with careful use of combinations of existing agonists and antagonists, it should be possible to distinguish receptors, but this may not always be practical. The data presented provide a useful resource that could be employed in developing new CT or AMY receptor ligands. These would be valuable pharmacological tools or leads for developing new treatments for diabetes, obesity and bone disorders.

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## Conflicts of interest

The authors have no conflicts of interest to declare.

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## Supporting information

Additional Supporting Information may be found in the online version of this article:

**Table S1** Statistical comparison of agonist potencies ( $pEC_{50}$  values) for cAMP production at  $rCT_{(a)}$  receptors. Comparisons between agonists were performed using one-way ANOVA followed by Tukey's tests (N.S.  $P > 0.05$ ; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ).  $pEC_{50}$  values could not be determined for (Cys(ACM)<sup>2,7</sup>)h $\alpha$ CGRP or (Cys(Et)<sup>2,7</sup>)h $\alpha$ CGRP

**Table S2** Statistical comparison of agonist potencies ( $pEC_{50}$  values) for cAMP production at  $rAMY_{1(a)}$  receptors. Comparisons between agonists were performed using one-way ANOVA followed by Tukey's tests (N.S.  $P > 0.05$ ; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ).  $pEC_{50}$  values could not be determined for (Cys(ACM)<sup>2,7</sup>)h $\alpha$ CGRP or (Cys(Et)<sup>2,7</sup>)h $\alpha$ CGRP

**Table S3** Statistical comparison of agonist potencies ( $pEC_{50}$  values) for cAMP production at  $rAMY_{3(a)}$  receptors. Comparisons between agonists were performed using one-way ANOVA followed by Tukey's tests (N.S.  $P > 0.05$ ; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ).  $pEC_{50}$  values could not be determined for (Cys(ACM)<sup>2,7</sup>)h $\alpha$ CGRP

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