Development of a novel assay for direct assessment of selective amylin receptor activation reveals novel differences in behaviour of selective and non-selective peptide agonists

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ABSTRACT

Dual amylin and calcitonin receptor agonists (DACRAs) show promise as efficacious therapeutics for treatment of metabolic disease, including obesity. However, differences in efficacy in vivo have been observed for individual DACRAs indicating that detailed understanding of the pharmacology of these agents across target receptors is required for rationale drug development. To date, such understanding has been hampered by lack of direct, subtype selective, functional assays for the amylin receptors (AMYRs). Here, we describe the generation of receptor-specific assays for recruitment of Venus tagged Gs protein through fusion of luciferase to either the human calcitonin receptor (CTR), human receptor activity-modifying protein (RAMP)-1, RAMP1 (AMY1R), human RAMP2 (AMY2R) or human RAMP3 (AMY3R). These assays revealed a complex pattern of receptor activation by calcitonin, amylin or DACRA peptides that was distinct at each receptor subtype. Of particular note, while both the CT-based DACRAs, sCT and AM1784, displayed relatively similar behaviours at CTR and AMY1R, they generated distinct responses at AMY2R and AMY3R. These data aid the rationalisation of in vivo differences in response to DACRA peptides in rodent models of obesity. Direct assessment of the pharmacology of novel DACRAs at AMYR subtypes is likely to be important for development of optimised therapeutics for treatment of metabolic diseases.

SIGNIFICANCE STATEMENT

Amylin receptors are important obesity targets. Here we describe a novel assay that allows selective functional assessment of individual amylin receptor subtypes that provides unique insight into the pharmacology of potential therapeutic ligands. Direct assessment of the pharmacology of novel agonists at AMYR subtypes is likely to be important for development of optimised therapeutics for treatment of metabolic diseases.
INTRODUCTION

Obesity is a major health burden with broad cardiovascular and metabolic co-morbidity, including hypertension, dyslipidaemia and type 2 diabetes (Nogueiras et al, 2023). Over the last 5 years, novel, efficacious, peptide therapeutics have been identified that are revolutionising the approach to treatment of overweight and obese individuals. These have centred around targeting of the glucagon-like peptide 1 receptor (GLP-1R), either alone (e.g. formulations of semaglutide) or via peptides activating the GLP-1R and related receptors, including the gastric inhibitory peptide receptor (e.g. tirzepatide) and/or glucagon receptor (Nogueiras et al, 2023). Despite these advances, there is a need for additional therapeutics, particularly those with an alternative mode of action that may be additive or synergistic with current drugs.

Selective targeting of amylin receptors (AMYRs), heterodimers of the calcitonin receptor (CTR) and one of 3 receptor activity-modifying proteins (RAMPs; (CTR+RAMP1=AMY1R; CTR+RAMP2=AMY2R; CTR+RAMP3=AMY3R) induces weight loss in both rodent models of obesity and humans, but the short acting AMYR selective peptides achieve only limited weight reduction in the clinic (Roth et al, 2006; Dehestani et al, 2021; Mathiesen et al, 2021). More recently, despite lack of weight loss with selective CTR agonists (Larsen et al, 2020), dual amylin and calcitonin receptor agonists (DACRAs) have emerged as novel, efficacious, potential therapeutics for treatment of obesity (Mathiesen et al, 2021; Sonne et al, 2021). In phase 2 clinical trials, the most advanced DACRA, cagrilintide, is highly efficacious as a monotherapy (Kruse et al, 2021; Lau et al, 2021) with additive benefit when co-administered with approved therapeutics such as semaglutide (Frias et al, 2023).

The first DACRA peptide identified was a teleost peptide, salmon CT (sCT), and DACRA peptides have been developed on either an amylin backbone (37 amino acids) or salmon CT backbone (32 amino acids). Recent structural data of peptides bound to Gs-coupled CTR and AMYRs revealed that sCT and amylin peptides induce distinct AMYR conformations (Cao et al, 2022). Moreover, there are potential distinctions in the behaviour of sCT-based DACRAs and amylin-based DACRAs in some preclinical models of obesity. While peptides based on both backbones have potent effects on acute inhibition of food intake in rodents and in weight reduction in rat models of obesity (Kruse et al, 2021; Larsen et al, 2022; Andressen et al, 2021; Mathiesen et al, 2021; Sonne et al, 2021), sCT has only a transient effect on weight loss in mouse diet induced obesity (DIO) models (Arrigoni et al, 2021). There are also potential differences in clinical efficacy of sCT-based DACRAs and amylin-based DACRAs (Mathiesen et al, 2021; Lau et al, 2021; NCT03907202; NCT00785408). Such data highlight the need to understand the relative activation of CTR and individual AMYRs by different DACRA peptides.

To date, in vitro pharmacological assessment of AMYRs has been indirect, primarily measuring Gs-dependent cAMP production as the canonical second messenger. In these systems, a background CTR only phenotype is present, and the readout in the presence of RAMP is a composite of CTR and AMYR activation (Hay et al, 2018). Thus, AMYR phenotype is inferred by comparison to CTR alone data. Moreover, the extent of phenotypic modulation by RAMP is also dependent on the cellular background and efficiency of transfection.
Given the high potential therapeutic importance of DACRAs as novel obesity medicines, there is a critical need for direct, and selective, assays of AMYR activation for the assessment of current and potential new peptide agonists.

Receptor-mediated activation of G proteins initiates GPCR signal transduction. As such, agonist-mediated recruitment of G proteins to activated GPCRs has been used as a direct, proximal, surrogate of receptor signalling, measured using resonance energy transfer between tagged receptor and G proteins (Olsen & English, 2023). We reasoned that a similar assay principle could be applied to measure selective G protein recruitment to individual CTR or AMYRs (by specifically labelling the RAMP subunit) to provide unique insight into the activity of peptide agonists across this receptor family (Fig. 1). In this study, we use these novel assays to measure -selective activation of CTR, AMY₁R, AMY₂R and AMY₃R in response to selective and non-selective peptide agonists of CT family receptors (Supp. Fig. 1).

**MATERIALS AND METHODS**

**Peptides**

Rat/mouse amylin (rAmy), salmon calcitonin (sCT), pramlintide, human α-calcitonin gene-related peptide (CGRP), human adrenomedullin (AM), human adrenomedullin 2 (AM2), semaglutide, cagrilintide (NNC0174-0833 or AM833), NNC0174-1213 (AM1213), and NNC0174-1784 (AM1784) were synthesized by Novo Nordisk (Copenhagen, Denmark). Human CT (hCT) was purchased from ChinaPeptides (Suzhou, China). All stock solutions of peptides were prepared in 0.05% acetic acid.

**Cell Culture**

COS-7 cells (American Type Culture Collection CRL-1651), an African green monkey (Cercopithecus aethiops), fibroblast-like, adherent, Simian Virus-40–transformed cell line, were used for experiments because of their lack of endogenous RAMP expression.

Cell culture was performed in Class II biosafety cabinets under aseptic conditions. COS-7 cells were cultured in Dulbecco’s Modified Eagle's Medium (DMEM; Cat#11995, Invitrogen, Carlsbad, CA) supplemented with 5% v/v foetal bovine serum (Thermo Electron Corporation, Melbourne, VIC, Australia) at 37°C and 5% CO₂ in a humidified incubator. At the time of harvesting, cells were at approximately 80% confluency and washed with phosphate buffered saline (PBS) before dissociated from the culture surface using PBS supplemented with EDTA (0.5mM) and 0.025% trypsin (Invitrogen, Carlsbad, CA). When fully dissociated, cells were pelleted by centrifugation (350 x g, 3 min), then resuspended in culture media to be reseeded into a flask to maintain the cell line or to be transfected and plated for assay.

**Expression constructs**

The Gγ₂ construct in pcAGGS was kindly provided by Dr. Asuka Inoue (Tohoku University, Sendai, Japan). The constructs used to examine G protein activation (Gα₁/2/q/11-Rluc8, Gβ₃, and Gγ₉-GFP2, were each in pcDNAs5/FRT/TO; generously provided by Dr. Ryan Strachan (University of North Carolina, Chapel Hill) (Olsen et al., 2020). The Venus-tagged mini-Gs (NES-Venus-mini-Gs) in pVenus-C1 was provided by Dr. Nevin Lambert (Wan et al., 2018).
The cMyc-tagged hCTRaleu (hCTR) was a gift from Dr. Rasmus Just. c-myc-hCTRaleu-Rluc8 (hCTR-Rluc8) were generated by removal of the stop codon and subcloning it into a Rluc8 destination vector using Gateway technology (Invitrogen). Human RAMP1, RAMP2, RAMP3 and c-myc-tagged CLR were a gift from Dr. Steve Foord (McLatchie et al., 1998). RAMP1, RAMP2 and RAMP3 constructs with a N-terminal CD33 signal sequence and FLAG epitope tags (CD33-FLAG-RAMP1/2/3) were generated from constructs of human RAMP1, RAMP2 and RAMP3. From these CD33-FLAG-RAMP1/2/3 constructs, additional constructs with a C-terminal fusion of the NanoLuc® (Nluc) protein were generated. The myristoylated Gαs with a Venus fluorescent protein inserted after position 72 was provided by Dr. Nevin Lambert. The Gβ₁ was obtained from cdna.org. The vector pcDNA3.1 was used as a transfection control. Separate transfections on different cell passages constituted individual experiments.

cAMP accumulation
COS-7 cells were transiently transfected using polyethylenimine (PEI) Max (mol. wt. 40,000; Polysciences, Warrington, PA). Cells were transfected with a mixture of 32.5 ng per well of c-myc-CLR and 32.5 ng per well of either RAMP1, RAMP2 or RAMP3. For experiments using RAMP1/2/3-Nluc constructs, cells were transfected with a mixture of 10 ng per well of c-myc-CLR and 10 ng per well RAMP1/2/3 or RAMP1/2/3-Nluc DNA. DNA and PEI Max were each diluted in separate volumes of 150 mM NaCl per well, then combined in to yield a 1:6 DNA:PEI Max ratio before being briefly vortexed and incubated for at least 15 minutes at room temperature. The transfection mixture was added to the cells in suspension and was subsequently seeded into plates at 15,000 cells per well into 96-well clear plates (Corning) and incubated at 37°C in 5% CO₂.

After 48 hours of growth, the culture media was replaced with stimulation buffer (phenol red–free DMEM containing 25mM HEPES, 0.1% w/v ovalbumin and 0.5 mM 3-isobutyl-1-methylxanne, pH 7.4) and incubated for 30min at 37°C in 5% CO₂. Cells were then stimulated with the peptide. After 30 minutes, the reaction was terminated by aspiration of the stimulation buffer and addition of ice-cold ethanol. After evaporation of ethanol, the cells were lysed with 75 µL/well lysis buffer (5 mM HEPES, 0.1% w/v bovine serum albumin, 0.3% Tween 20, pH 7.4). The concentration of cAMP in the lysates was detected with the LANCE time-resolved Förster Resonance Energy Transfer kit (Perkin Elmer, Waltham, MA), as per the manufacturers' protocols and previous studies (Fletcher et al., 2021; Piper et al., 2022). The plates were read on an Envision multilabel plate reader (Perkin Elmer), and values were converted to an absolute concentration of cAMP using a cAMP standard curve detected in parallel.

TruPath G Protein Activation.
COS-7 cells were PEI-transfected with c-myc-CLR:pcDNA3/RAMP1/3:Gαₛ-Rluc8:Gβ₃:Gγ₉-GFP2 at a 1:1:1:1:1 ratio, giving 50 ng total DNA per well. Cells were plated at 15,000 cells per well into 96-well Greiner CELLSTAR white-walled plates (Sigma-Aldrich), and assays were performed 48 hours later. Growth medium was replaced with HBSS with 10 mM HEPES and 0.1% (w/v) ovalbumin and incubated at 37°C for 30 minutes. Prolume purple coelenterazine (Nanolight Technologies, Pinetop, AZ) was then added to the plate at a final concentration of 1.3 mM and incubated for a further 10 minutes at 37°C. BRET measurements were performed on a PHERASTAR plate reader (BMG Labtech) using 410/80 nm/515/30 nm filters,
with baseline measurements taken for 6 minutes before addition of vehicle or peptide and reading for a further 20 minutes. BRET signal was calculated as the ratio of the 515/30-nm emission over the 410/80-nm emission. This ratio was vehicle-corrected by subtracting the response of vehicle-treated wells from the same transfection ratio for the ligand-treated well and then baseline-corrected by subtracting to the mean BRET ratio of baseline values (prestimulation) for each well.

**Gs and mini-Gas BRET recruitment assay**

Following optimisation of DNA transfection ratios for peak signal to noise, G protein recruitment to individual AMYR subtypes was measured in COS-7 cells transfected with c-myc-hCTRaleu:RAMP1-/RAMP2-/RAMP3-Nluc:Gs-Venus:Gβ1:Gγ2 at a ratio of 2:0.1:1:1:1, giving 51ng total DNA per well. For comparisons with the CTR alone, COS-7 cells were similarly PEI transfected with c-myc-hCTRaleu-Rluc8:pcDNA3.1:Gs-Venus:Gβ1:Gγ2 at a ratio of 2:0.1:1:1:1. To study the recruitment of mini-Gαs, COS-7 cells were PEI transfected with c-myc-hCTRaleu:RAMP1-/RAMP2-/RAMP3-Nluc:NES-Venus-mGs (or hCTRaleu-Rluc8:pcDNA3.1: NES-Venus-mGs for CTR alone conditions) at a ratio of 2:0.1:1, giving 31ng total DNA per well. Cells were plated at 13,000 cells per well into 96-well Greiner CELLSTAR white-walled plates (Sigma-Aldrich), and assays were performed 48 hours later.

At the time of assay, the growth medium was replaced with HBSS, supplemented with 10mM HEPES and 0.1% (w/v) ovalbumin and incubated at room temperature for 30 min. Furimazine (NanoBRET™ Nano-Glo® substrate; Promega) or coelenterazine h (Nanolight Technologies, Pinetop, AZ) was then added to cells at a final concentration of 1:1000 or 5µM, respectively, and incubated for a further 10-15 min. Cell plates were transferred to a LUMIstar plate reader (BMG Labtech) at 30°C for BRET measurements. Luminescence measurements were performed using 475/30 nm and 535/30 nm filters with baseline measurements taken for approximately 3 min before addition of vehicle or peptide and reading resumed for a further 10 min. BRET signal was calculated as the ratio of the 535/30 nm emission over the 475/30 nm emission. This ratio was vehicle-corrected by subtracting the response of vehicle-treated wells from the ratio of ligand-treated wells, and then baseline-corrected by subtracting to the mean BRET ratio of baseline values (prestimulation) for each well.

**Data Analysis**

All data were analysed using Prism 9 software (GraphPad Software Inc., San Diego, CA). Concentration-response signalling data were analysed using either a three-parameter logistic equation or a biphasic response equation, following an F test to determine the best fit. For the biphasic fits, the Hill slope for each phase was fixed to 1, -1 or -2, depending on the shape of the curve. From experimental data of kinetic measurements of BRET, following vehicle and baseline corrections, quantification of induced responses from individual peptide concentrations was determined by calculating the net area under curve (AUC).

For Gs and mini-Gs recruitment experiments, concentration-response data at the AMYRs were normalised to the maximal response of rAmy, or normalised to hCT for CTR, as determined from curve fits of plotted AUC data. Comparisons of pEC50 values determined from the separate Gs and mini-Gs recruitment experiments for each peptide were made by one-way ANOVA with Dunnett’s multiple comparisons post-test to either the reference
peptide (within receptor subtype comparisons) or CTR (for between receptor subtype comparisons). Emax values were analysed by “one sample t and Wilcoxon test” versus 100% (two-tailed). Statistical significance was accepted by rejection of the null hypothesis at $P < 0.05$.

To derive an integrated measure of the potency and Emax in the Gs-Venus recruitment assay, for peptides at individual receptors, the fractional difference in Emax from the reference peptide, where the reference Emax = 1, was subtracted from the potency ($pEC_{50}$) of the ligand. Within an individual receptor assay, these corrected values were also compared to the reference ligand.

**RESULTS**

To monitor selective receptor activation, either Rluc8 (CTR) or Nluc (AMYRs) was fused to the C-terminus of CTR (CTR-Rluc8) or the C-terminus of individual RAMPs (RAMP1-Nluc, RAMP2-Nluc, RAMP3-Nluc). Due to the short C-terminus of RAMPs, a 12 amino acid linker peptide was included between the far C-terminus of the RAMP and the luciferase, and the smaller, Nluc was used to minimise the potential impact of steric interference on function of the AMYR heterodimer.

The Rluc8 fusion to the CTR C-terminus does not alter CTR pharmacology, as previously validated (Fletcher et al, 2021). To confirm that the RAMP-Nluc constructs remained functional, the tagged RAMPs were co-transfected with the calcitonin receptor-like receptor (CLR) that requires RAMP for expression of functional CGRP (CLR:RAMP1) or adrenomedullin (CLR:RAMP2, AM1R; CLR:RAMP3, AM2R) receptors, and assayed for peptide-mediated cAMP production (Supp. Fig. 2). While the absolute potency of peptide responses was reduced, likely as a result of decreased expression of the tagged RAMP, the relative potency of CGRP and AM peptides was equivalent for each of the receptors, indicative of maintained function.

CTR and AMYRs couple predominantly to the stimulatory, Gs protein. Two different versions of the selective Gs BRET recruitment assays were established. The first utilised a Venus-tagged, full-length, Gαs protein that provides a measure of the relative ligand-induced G protein recruitment in a system where the full G protein activation cycle is intact, therefore allowing for G protein activation (which dissociates the G protein from the activated receptor) and turnover of the activated G protein (Fig. 1A). This is therefore a read out of net effect ligand-mediated recruitment vs dissociation. The second version utilised a Venus-tagged mini-Gαs protein that is guanine nucleotide insensitive and consequently is not released following binding to an activated receptor (Fig. 1B), leading to accumulation of BRET signal over time until all receptors in the system are occupied with Venus tagged G protein, or the labelled G protein is depleted. In both cases, the responses are proximal measures of receptor activation without downstream amplification that can occur for second messenger responses, including Gs-mediated cAMP production.

**Gs-Venus recruitment to CTR and AMYRs**
CTR-Rluc8 or CTR plus RAMP-Nluc constructs (AMYRs) were co-transfected with Gs-Venus into COS-7 cells that natively lack functional levels of either CTR or RAMP (Christopoulos et al., 1999), and the change in BRET ratio between donor and acceptor was monitored for 10 min following agonist peptide addition (Supp. Figs. 3-6). The following peptides were assessed, based on their reported pharmacological profiles in cAMP assays. Human CT (hCT), a selective CTR agonist; rat amylin (rAmy), pramlintide, and AM1213, AMYR selective agonists; and the DACRA peptides, sCT, and AM1784 (sCT-based). CGRP, reported as a high potency AMY1R agonist with selectivity over other CTR/AMYRs and AM, an endogenous agonist of AM receptors that has low potency for cAMP production at CTR/AMYRs were also included.

**CTR**

At the CTR, expressed in the absence of RAMPs, hCT and sCT had equivalent, high potency, and maximal response (Emax) for recruitment of Gs-Venus to CTR-Rluc8. AM1784 exhibited similar potency to the CT peptides, while pramlintide and rAmy had ~5-7-fold lower potency (Fig. 2A, 2B; Table 1). The Emax for these peptides was reduced relative to hCT but this was only significant for rAmy. The amylin analogue, AM1213, also had lower potency but with an Emax similar to the CT peptides. CGRP and AM were only weak agonists at this receptor (Fig. 2C; Table 1).

**AMY1R**

At the AMY1R (CTR-RAMP1-Nluc), the potency of sCT, AM1784 and the weak agonist, AM, were not significantly different when comparing Gs recruitment to CTR-Rluc8. In contrast, rAmy, pramlintide, AM1213 and CGRP were more potent (>10-fold), while hCT was less potent for Gs recruitment to CTR-RAMP1-Nluc, relative to CTR-Rluc8 (Fig. 2D-2F; Fig. 3A). Of note, sCT and AM1784 displayed lower potency than rAmy at the AMY1R, while, with the exception of pramlintide, the peptides had a lower Emax than rAmy; this was greatest for AM1784, and hCT, with <60% of the rAmy Emax (Table 1).

**AMY2R**

Due to the relatively weak induction of AMYR phenotype observed in cAMP assays, relative to CTR (Hay et al., 2018; Christopoulos et al., 1999; Tilakaratne et al., 2000), the pharmacology of the AMY2R is largely unexplored. In the Gs-Venus recruitment to CTR-RAMP2-Nluc assay, rAmy, pramlintide, AM1213 and CGRP had higher potency, relative to the CTR assay, hCT exhibited lower potency, while there was no significant difference in the potency of the other peptides (Fig. 2G-2I, Fig. 3B; Table 1). In contrast, there were stark differences in the maximal responses observed, where all CT-based peptides displayed higher Emax values than rAmy, which was greatest for sCT and AM1784. Pramlintide, CGRP and AM1213 had similar or slightly increased Emax, relative to rAmy (Table 1).

**AMY3R**

As with the other AMYRs, the profile of peptide responses for Gs recruitment to CTR-RAMP3-Nluc reflected higher potency of the selective amylin peptides, rAmy, pramlintide and AM1213, relative to the CTR selective agonist, hCT (Fig. 2J-2L; Fig. 3C; Table 1), typical of previously described phenotypic responses in cAMP assays (Fletcher et al, 2021). Despite relatively similar potency to rAmy for the other peptides, with the exception of AM, there were intriguing differences in the maximal responses. Pramlintide, AM1784 and hCT had
similar Emax values to rAmy, whereas AM1213 and CGRP had a lower Emax. In contrast, the maximal response to sCT was significantly higher than that of the other peptides (Fig. 2J-2L; Table 1).

**Mini-Gs-Venus recruitment to CTR and AMYRs**

Overall, the mini-Gs-Venus recruitment assay, as expected, generated substantially larger BRET signals and parallel enhancements in the magnitude of ligand-induced change in BRET, as well as the signal to noise window (Supp. Figs. 7-10). However, while distinct receptor phenotypes were clearly discernible, the differences in relative responses of peptides were generally smaller, both at individual receptors and between receptors, compared with profiles in the full-length Gs-Venus assay. As such, the mini-Gs assay may be better suited for identification of weak agonists but may have limitations in discriminating between different ligand pharmacology. Nonetheless, the improved signal to noise in the mini-Gs assay was associated with lower inter-assay variance and consequently the ability to resolve smaller differences in peptide responses.

**CTR**

For mGs recruitment to CTR-Rluc8, there was <3-fold difference in potency between hCT and rAmy, with sCT and AM1784 having equivalent potency to hCT, while pramlintide had a slightly lower potency, but was also not significantly different (Fig. 4A, 4B; Table 2). AM1213, and CGRP had similar potency to rAmy, with AM a relatively less potent agonist (Fig. 4C). There were only small differences in peptide Emax, with lower responses, relative to hCT, for rAmy, CGRP and AM, and no difference for sCT, AM1784 or AM1213 (Fig. 4A-4C; Table 2).

**AMY\(_1\)R**

At the AMY\(_1\)R, rAmy, pramlintide, CGRP and sCT exhibited similar potency and Emax (Fig. 4D-4F) for mGs recruitment to RAMP-1-Nluc, with rAmy and CGRP also having higher potency relative to mGs recruitment to the CTR (Fig. 3A; Table 2). AM1213, and AM had lower potency than rAmy, with similar Emax values that were >90% of the rAmy Emax. AM1784 and hCT had lower potencies for mGs recruitment to the AMY\(_1\)R, relative to CTR, with hCT also displayed lower potency relative to rAmy at the AMY\(_1\)R, however, both peptides exhibited a lower Emax relative to rAmy at this receptor (Fig. 3A, 4D-4F; Table 2).

**AMY\(_2\)R**

The profile of peptide response at the AMY\(_2\)R was generally similar to that observed at the AMY\(_1\)R, with selective lower Emax of hCT and AM1784, relative to rAmy and other peptides, and a similar lower relative potency of hCT, AM1784, and AM, compared to rAmy (Fig. 4G-4I; Table 2). This was, in part, due to a lower relative potency at AMY\(_2\)R for hCT and AM1784, but higher potency for AM, compared to assessment of mGs recruitment at the CTR. Of note, the sCT potency at the AMY\(_2\)R was also lower relative to measurements at the CTR but was not significantly different from rAmy at AMY\(_2\)R (Fig. 3B; Fig. 4I; Table 2).

**AMY\(_3\)R**

With the exception of AM, peptide potencies were similar at the AMY\(_3\)R, albeit that AM1213 had a small but significantly lower potency than rAmy at this receptor (Fig. 4J-4L; Table 2). Emax values were also similar, with the exception of CGRP and AM that had a lower Emax
than other peptides, and sCT that had a significantly higher Emax than rAmy (Fig. 4J-4L; Table 2). Relative to responses at CTR, the potencies of hCT, sCT and AM1784 were lower, whereas potencies for the other agonists were similar across the two receptor assays (Fig. 3C; Table 2).

**DACRA peptides are selective for CTR-based heteromeric receptors**

In addition to establishing a direct assay of AMYR function, the G protein recruitment data illustrate the complexity of peptide responses at CTR-based heteromeric receptors and the potential for cross-specificity of peptide interaction between related peptides and receptors within the broader calcitonin subfamily. Moreover, previous work has indicated the potential for alternate profiles of peptide selectivity at CGRP and AM receptors, if measured across multiple pathways (Clark et al, 2021; Weston et al, 2016).

Unlike AMYRs, where the observed agonist signalling phenotype is a composite of background CTR and the AMYR-specific response, CGRPR (CLR+RAMP1), AM1R (CLR+RAMP2) and AM2R (CLR+RAMP3) can be interrogated without the complication of background phenotype as CLR alone is poorly expressed at the cell surface and does not respond to peptide agonists (McLatchie et al, 1998).

To determine whether any of the selective or DACRA peptides could also activate the related CGRP and AM receptors, we assessed both canonical cAMP production and proximal measures of G protein activation for representative members of Gs, Gi/o, and Gq/11 and protein subfamilies (Gs, Gi2, Gq and G11) using the TruPath assay (Olsen et al, 2020).

**cAMP accumulation assay**

The canonical agonists for the CLR-based receptors exhibited the expected rank order of potency for cAMP production. CGRP was 23-fold and 126-fold more potent than AM peptides, AM2 and AM, respectively, at the CGRPR, the AM peptides were ~100-fold, or greater, more potent than CGRP at the AM2R, and AM had the highest potency, followed by AM2 at the AM1R, where CGRP had low potency (Fig. 5A, 5D, 5G; Supp. Table 1). At the CGRPR, the CTR/AMYR peptide agonists had only weak potency (pramlintide, AM1213, sCT) or no response (AM1784), and only pramlintide was a full agonist (Fig. 5B, 5C; Supp. Table 1). Neither of the CT-based peptides (sCT, AM1784) produced any substantive response at the AM1R or AM2R (Fig. 5F, 5I), whereas pramlintide and AM1213 were low potency weak partial agonists at the AM2R (Fig 5H), and did not elicit responses at the AM1R (Fig. 5E; Supp. Table 1).

**TruPath G protein dissociation assay**

The TruPath assay detects the BRET signal between a donor (Rluc8) fused to the Gα subunit and the acceptor (GFP2) fused to the Gγ of the obligate Gβγ dimer, providing a real-time proximal measurement considered to represent net G protein dissociation (decreased BRET) as a surrogate for G protein activation (Olsen et al, 2020). Nonetheless, conformational changes in the G protein that alter the position of the donor and acceptor can also alter the measured BRET signal leading to increased or decreased BRET (Fletcher et al, 2021), potentially contributing to complexity in agonist response (see Supp. Discussion). Agonist-induced BRET changes were assessed over 20 min for each G protein (Supp. Fig. 11-14).
Gs protein assay
All 3 receptors displayed unexpected profiles, exhibiting agonist-induced changes in BRET for the Gs protein that were biphasic, with increases in BRET that were either sustained (AM1R) or subsequently decreased at higher ligand concentrations (CGRPR, AM2R), however, in all cases the net change in BRET did not decrease below the baseline for any of the peptides (Fig. 6A-6I). Nonetheless, the overall pattern of peptide response across the receptors was similar to observed in the cAMP assay (Fig. 5C, 5F; Fig. 6). Initial increases in BRET at low agonist concentrations have previously been observed with the Gs sensor, including in assays of CTR and AMYRs (Fletcher et al, 2021), with the relative prevalence of the two phases in those assays being receptor expression dependent.

Gi2 protein assay
For the control agonists, the relative potencies of CGRP, AM and AM2 were consistent with Gs and cAMP assays, with CGRP>AM>AM2 at the CGRPR, AM>AM2>CGRP at the AM1R and AM=AM2>CGRP at the AM2R (Fig. 7). While there was a potential biphasic signal for CGRP at the AM1R and AM2R, with a low fraction, high potency response (Fig. 7D, 7G) a single site model was a statistically better fit for the data (F-test). None of the CT-based peptides elicited a significant change in the BRET signal at CGRPR, AM1R or AM2R, while AM1213 was a low potency partial agonist, selectively for the CGRPR, with no activity at AM1R and AM2R (Fig. 7B). Interestingly, pramlintide exhibited a robust response at all 3 CLR-based receptors, with a similar potency to the weaker control agonist peptides (AM at CGRPR, CGRP at AM1R and AM2R), and with a similar Emax to control peptides at CGRPR and AM1R, but partial agonism at AM2R (Fig. 7B, 7E, 7H).

Gq and G11 protein assays
Peptide responses were qualitatively similar in the Gq and G11 TruPath assays, and generally similar to the observations for the Gi2 assay (Fig. 8A-8I; Fig. 9A-9I), with control peptides having the expected rank order of potency and only pramlintide of the CT-based and Amy-based peptides able to induce a robust signal. Interestingly, while pramlintide was a partial agonist in the AM1R Gq assay (Fig. 8E), it was a full agonist, relative to the canonical agonists in the CGRPR and AM2R assays (Fig. 8B, 8H) and a full agonist at all 3 receptors in the G11 assay (Fig. 9B, 9E, 9H). Similar to the Gi2 assay, there was an indication of a biphasic response for CGRP at AM1R and AM2R for both pathways (Fig. 8D, 8G; Fig 9D, 9G), albeit that the high potency fraction was small, and a biphasic fit was only statistically better for the AM2R Gq data; here the high potency response was similar to that observed for CGRP activation of the CGRPR. Also of note, AM and AM2 demonstrated similar potencies for activation of the Gq/11 proteins at the AM1R (Fig. 8D; Fig. 9D), in contrast to the greater potency of AM observed in each of the other assays.
DISCUSSION

In this study, we generated novel AMYR subtype selective BRET assays using Venus fused to either mini-Gs or full-length Gs protein that provide measures of Gs recruitment without G protein dissociation, or integrated net recruitment when the G protein is able to dissociate/turnover, respectively. Collectively, these assays provide the first direct measurement of functional activity at AMYRs, including investigation of the phenotype of AMY$_2$R that has not been robustly studied previously due to limited induction of phenotype relative to CTR alone in classical measures of signalling, such as second messenger assays (Hay et al, 2018; Christopoulos et al, 1999; Tilakaratne et al, 2000).

Assessment of the relative potencies of peptides at the CTR, across different assays linked to Gs-mediated activation or signaling (cAMP accumulation (Fletcher et al, 2021), TruePath Gs activation (Fletcher et al, 2021)) versus full-Gs recruitment, mini-Gs recruitment) provides validation of the current assay systems. While differences in the absolute change in potency may be expected to occur due to assay amplification or receptor reserve, relative potencies of peptides across the different assay formats should be similar. This was indeed the case (Supp. Fig. 15A, 15B), with the magnitude of differences reflective of the absolute potencies of peptides in the individual assay formats. (Supp. Fig. 15A, 15B). Nonetheless, across the 2 BRET G protein recruitment assays, unique patterns of responses were observed for each peptide that could be sub-grouped for different sets of peptides.

Within individual receptor subtypes, potencies were similar for full-length Gs and mini-Gs assays, with a number of exceptions (Supp. Fig. 16A-16D); these differences were amplified when potencies in the full-length Gs assay were normalised for differences in relative Emax of responses (Supp. Fig. 16E-16H). In general, peptides exhibiting high potency at the receptor subtypes exhibited either similar potencies across the Gs and mini-Gs assays or higher potency in the Gs assay. In contrast, peptides with relatively low potency generally displayed higher potencies in the mini-Gs assay, including CGRP and the AMYR-selective peptides at the CTR (Supp. Fig. 16A), and hCT at the AMYRs (Supp. Fig. 16B-16D). This disparity likely reflects a dissociation of the ability of individual peptides to promote recruitment of Gs protein versus G protein dissociation/turnover.

There were marked differences in Emax, in a peptide- and receptor- specific manner, particularly evident in the full-length Gs assay; this may reflect a higher rate of Gs protein dissociation/turnover for peptides with a lower Emax, and consequently, lower turnover with a higher Emax, albeit the efficiency of recruitment will also influence this Emax. In contrast, in the mini-Gs assay, a lower Emax could reflect a decreased efficiency of Gs protein recruitment (and vice versa).

In the full length Gs assay, divergent patterns of response were observed. Pramlintide had a similar Emax, relative to control peptides (hCT for CTR and rAmy for AMYRs) for all receptor subtypes (Fig. 10A-10D). In contrast, sCT was the only peptide with a higher Emax at the AMY$_3$R (Fig. 10D), which was also a unique feature in the AMY$_3$R mini-Gs assay, albeit less pronounced (Fig. 10H). With the exception of pramlintide, all peptides had a lower Emax than rAmy at the AMY$_3$R in the full length Gs assay, with hCT and AM1784 lower than the other peptides (Fig. 10B), and this latter trend was also apparent in the mini-Gs assay, albeit
again to a lesser degree (Fig. 10F). At the AMY₂R, a higher Emax relative to rAmy was observed for all CT-based peptides in the full-length G protein assay, and was greatest for sCT (Fig. 10C). Given the similar Emax values for these peptides and rAmy in the mini-Gs assay, it is possible that these peptides are less efficient at inducing G protein dissociation/turnover relative to rAmy, thus contributing to the higher Emax in the full length Gs assay.

Intriguingly, while the potency of AM1784 was similar to sCT at all receptor subtypes, the Emax tracked with that of hCT in both assays, and these were the only peptides with a lower Emax at the AMY₂R for the mini-Gs assay (Fig. 10A-10H). While speculative, this may relate to the stability of the N-terminal activation domain (residues 1-7) of these peptides. At the CTR, MD simulations of hCT and sCT bound to active-state (Gs coupled) receptor predicted greater stability of the sCT activation domain within the receptor core, compared to hCT (Dal Maso et al, 2018). This is consistent with the requirement for presence of the disulfide bond (Cys1-Cys7) in hCT for maintenance of potency in cAMP accumulation assays in receptor overexpression systems where potency is composite of affinity and efficacy due to the presence of receptor reserve, whereas this disulfide was not required for sCT in these systems (Sexton et al, 1999). AM1784, which retains high affinity and cAMP potency for CTR when co-expressed with and without RAMPs (giving a mixed phenotype of CTR and AMYRs) (Fletcher et al, 2021), lacks a N-terminal disulfide (Ala1, Ala7 substitution for the native Cys1, Cys7)), and this might be expected to increase N-terminal dynamics, and thus result in differences in maximal response observed in the BRET assays.

As a potential measure of relative signaling efficiency, we corrected the potencies using the difference in Emax values observed for each receptor, for the full-length Gs assay (Fig. 3D; Supp. Fig. 17). In this analysis, among the DACRAs, the CT-template peptides displayed apparent decreased signaling efficiency for AMY₂R and AMY₃R, relative to CTR, while having no change (sCT) or increased efficiency (AM1784) at AMY₁R (Fig. 3D).

Our study provides the first direct assessment of functional response at the AMY₂R, the least well characterised AMYR subtype. In the Gs assay, with the exception of hCT (and AM), all peptides displayed similar potency to rAmy, but very distinct Emax values. Relative to Gs recruitment to the CTR, the CTR selective peptide, hCT, had lower potency, while the potency of AMYR selective peptides was higher (Fig. 3B). There was little difference in the potency of any of the DACRA peptides at the AMY₂R, relative to CTR. Use of the Emax corrected Gs potencies as a guide to potential signaling efficiency, further differentiated the peptides where only pramlintide had increased potency relative to CTR, with similar large decreases in corrected potency for each of the CT-based peptides (Fig. 3D). Similarly, the corrected Gs potency values were also indicative of differences in signaling efficiency between the 32 amino acid CT-based peptides and the longer, 37 amino acid peptides, including CGRP (Fig. 3D; Supp. Fig. 17C).

Structural studies of CTR and AMYRs, have revealed differences in the conformation of AMYRs when bound to rAmy relative to CT peptides (Cao et al, 2022), with the shorter CT peptides constraining the position of the receptor extracellular domain that binds the peptide C-terminus, to a similar orientation to that of CTR in the absence of RAMPs. Interestingly, despite having low potency for Gs recruitment, a complex between hCT and
AMY$_2$R could be purified for structure determination, enabled by the relatively weak transmembrane domain interface between RAMP2 and CTR. This could explain the relatively high affinity of hCT in competition for $^{125}$I-rAmy in cells expressing CTR and RAMP2 that would contain both AMY$_2$R and CTR, but where the probe selectively labels AMY$_2$Rs at the concentrations used, suggesting high affinity of hCT for AMY$_2$R (Tilakaratne et al, 2000). However, our current data reveal that this is not sufficient to drive higher potency in surrogate functional assays. Given the prior relative paucity of pharmacological characterisation of the AMY$_2$R, the relevance of this receptor to amylin physiology has been largely ignored. Intriguingly, sCT produced a more sustained reduction of food intake and body weight in transgenic mice lacking RAMP1 and RAMP3 than in WT animals, suggesting that the AMY$_2$R may have underappreciated effects on metabolism (Arrigoni et al, 2021).

Of note, while the overall potencies of peptides were generally lower at AMY$_3$R than at the other receptor subtypes in the Gs protein recruitment assays, sCT had differentiated behaviour relative to other peptides. In this context, it is interesting to note that the biochemical yields for generation of an active complex were lower than seen with the other AMYRs (Cao et al, 2022), and the distinct pharmacology of sCT could potentially contribute to that observation. However, further research is required to test this hypothesis.

Our study also revealed that the CT-based or Amy-based DACRA peptides had weak or no activity at CGRP or AM receptors, either in assays of second messenger cAMP production, or in TruPath G protein “activation” assays for Gs, Gi and Gq/11 proteins. This indicates that it is unlikely that DACRA peptides will modulate these receptors when administered therapeutically.

**Conclusion**

Direct measurement of G protein recruitment at CTR and AMYRs, either cumulatively or at steady state, revealed novel pharmacological differences in responsiveness of subtype selective and non-selective peptides. With increasing evidence supporting potential clinical benefit of targeting CTR and AMYRs, direct assessment of the pharmacology of novel DACRAs at AMYR subtypes is likely to be important for development of optimised therapeutics for treatment of metabolic diseases.
ACKNOWLEDGEMENTS
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AUTHORSHIP CONTRIBUTIONS
Participated in research design: Wootten D, Sexton P and Glendorf T
Conducted experiments: Keov P and Hick C
Contributed new reagents or analytical tools: Christopoulos G
Wrote or contributed to writing of the manuscript: Keov P, Sexton P, Wootten D, Ballarín-González B, Glendorf T
Review and editing of the manuscript: All authors
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https://classic.clinicaltrials.gov/ct2/show/NCT00785408


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FOOTNOTES

Conflict of interest statement
PMS is a co-founder and P.M.S. and D.W. are shareholders of Septerna Inc. P.M.S. and D.W. are co-founders and shareholders of DACRA Tx. T.G. and B.B.-G. are employees and shareholders of Novo Nordisk. The work was funded, in part, by Novo Nordisk.

Data availability statement
All data are available upon reasonable request.
LEGENDS FOR FIGURES

Figure 1. Schematic of CTR and AMYR subtype selective G protein recruitment assays. A. Full-length Gαs-Venus. Agonist binding to luciferase tagged CTR (left panel) initiates the recruitment of Venus-Gs heterotrimer generating a BRET signal (green). Following recruitment, the G protein is activated and released from the receptor with the assay signal at net equilibrium of the recruitment and activation cycles. In the right panel, the luciferase is fused to the RAMP. While Venus-Gs is recruited agonist bound to either CTR, or AMYRs (CTR+RAMP), only Gs protein recruited to the AMYR generates a BRET response. B. mini-Gαs-Venus assay. The mini-Gs lacks guanine nucleotide binding and is not released from agonist-bound receptors following recruitment, leading to a cumulative increase in BRET signal to either CTR-Rluc8 (left panel) or AMYR (CTR+RAMP-Nluc).

Figure 2. Peptide-mediated recruitment of Gαs-Venus to CTR-Rluc8 (A-C), AMY1R (RAMP1-Nluc; D-F), AMY2R (RAMP2-Nluc; G-I), AMY3R (RAMP3-Nluc; J-L). Data represent the area-under-curve (AUC) of induced BRET (corrected for baseline and vehicle responses) measured over 11 min of receptor stimulation. Data were normalised to the maximal response of rAmy (or hCT for CTR-Rluc8). Data were fit with a three-parameter sigmoidal curve. Values are mean ± SEM from 5-10 independent experiments performed in duplicate. The reference peptides, rAmy and hCT, are displayed as dashed lines in each graph.

Figure 3. Peptide potency change from CTR for each of AMY1R (A), AMY2R (B) and AMY3R (C). Gs-Venus recruitment assay, changes in potency (corrected for Emax differences as described in Methods) from CTR. Data for potency changes in cAMP accumulation response or Gs TruPath assay for CTR, AMY1R and AMY3R were from Fletcher et al, 2021.

Figure 4. Peptide-mediated recruitment of mini-Gαs-Venus to CTR-Rluc8 (A-C), AMY1R (RAMP1-Nluc; D-F), AMY2R (RAMP2-Nluc; G-I), AMY3R (RAMP3-Nluc; J-L). Data represent the area-under-curve (AUC) of induced BRET (corrected for baseline and vehicle responses) measured over 11 min of receptor stimulation. Data were normalised to the maximal response of rAmy (or hCT for CTR-Rluc8). Data were fit with a three-parameter sigmoidal curve. Values are mean ± SEM from 5-10 independent experiments performed in duplicate. The reference peptides, rAmy and hCT, are displayed as dashed lines in each graph.

Figure 5. Peptide-mediated cAMP accumulation in COS-7 cell transiently expressing CGRPR (A-C), AM1R (D-F) or AM2R (G-I). Data shown are mean ± SEM from 3 independent experiments performed in duplicate. The reference peptides (CGRP, CGRPR; AM and AM2 at the AM1R and AM2R) are shown as dashed lines.

Figure 6. Peptide-mediated TruPath Gs protein activation in COS-7 cells transiently expressing CGRPR (A-C), AM1R (D-F) or AM2R (G-I). Data represent the area under the curve (AUC) of ligand-induced BRET over 20 min (corrected for baseline and vehicle) and were fit to either a three-parameter logistic equation or a biphasic equation, with Hill slopes of 1, -1 or -2, as appropriate. Values are mean ± SEM from 3 independent experiments. The reference peptides (CGRP, CGRPR; AM and AM2 at the AM1R and AM2R) are shown as dashed lines.
Figure 7. Peptide-mediated TruPath Gi2 protein activation in COS-7 cell transiently expressing CGRPR (A-C), AM1R (D-F) or AM2R (G-I). Data represent the area under the curve (AUC) of ligand-induced BRET over 20 min (corrected for baseline and vehicle) and were fit to a three-parameter logistic equation. Values are mean ± SEM from 3 independent experiments. The reference peptides (CGRP, CGRPR; AM and AM2 at the AM1R and AM2R) are shown as dashed lines.

Figure 8. Peptide-mediated TruPath Gq protein activation in COS-7 cell transiently expressing CGRPR (A-C), AM1R (D-F) or AM2R (G-I). Data represent the area under the curve (AUC) of ligand-induced BRET over 20 min (corrected for baseline and vehicle) and were fit to either a three-parameter logistic equation or a biphasic equation, with a Hill slopes of -1. Values are mean ± SEM from 3 independent experiments. The reference peptides (CGRP, CGRPR; AM and AM2 at the AM1R and AM2R) are shown as dashed lines.

Figure 9. Peptide-mediated TruPath G11 protein activation in COS-7 cell transiently expressing CGRPR (A-C), AM1R (D-F) or AM2R (G-I). Data represent the area under the curve (AUC) of ligand-induced BRET over 20 min (corrected for baseline and vehicle) and were fit to a three-parameter logistic equation. Values are mean ± SEM from 3 independent experiments. The reference peptides (CGRP, CGRPR; AM and AM2 at the AM1R and AM2R) are shown as dashed lines.

Figure 10. Peptide response Emax differences in the Gs-Venus (A-D) or mini-Gs-Venus (E-H) recruitment assays. The Emax responses were normalised to maximum response of the reference peptides, hCT (CTR) or rAmy (AMYRs).
Reference peptide data are underlined. ^One-way ANOVA with Dunnett’s multiple comparison post-test to either hCT (CTR) or rAmy (AMYRs) for $pEC_{50}$ differences within individual receptors. * and bold type: One-way ANOVA with Dunnett’s multiple comparison post-test to CTR for $pEC_{50}$ differences between receptor subtypes. **Emax values were analysed by “one sample t and Wilcoxon test” versus 100% (two-tailed). Significance was accepted at P<0.05.

**Tables**

**Table 1.** Quantitative pharmacological parameters (from Fig. 2) for peptide-mediated Gs-Venus recruitment to CTR and AMYR subtypes transiently transfected into COS-7 cells.

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**Table 2.** Quantitative pharmacological parameters (from Fig. 4) for peptide-mediated mini-Gs-Venus recruitment to CTR and AMYR subtypes transiently transfected into COS-7 cells.

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<td>AM</td>
<td>6.34 ± 0.05</td>
<td>86 ± 3**</td>
<td>5</td>
<td>6.59 ± 0.05</td>
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Reference peptide data are underlined. ^One-way ANOVA with Dunnett’s multiple comparison post-test to either hCT (CTR) or rAmy (AMYRs) for $pEC_{50}$ differences within individual receptors. * and bold type: One-way ANOVA with Dunnett’s multiple comparison post-test to CTR for $pEC_{50}$ differences between receptor subtypes. **Emax values were analysed by “one sample t and Wilcoxon test” versus 100% (two-tailed). Significance was accepted at P<0.05.
FIGURES
Figure 2
Figure 3

A. Change potency CTR-AMY₁R

B. Change potency CTR-AMY₂R

C. Change potency CTR-AMY₃R

D. Change potency from CTR [corrected pEC₅₀]
Figure 5
Figure 6
Figure 7
Figure 9
Figure 10
Development of a novel assay for direct assessment of selective amylin receptor activation reveals novel differences in behaviour of selective and non-selective peptide agonists

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Supplementary discussion .......... page 2
Supplementary table 1 ...... page 3
Supplementary figures 1 – 17 ...... pages 4 - 20
SUPPLEMENTARY DISCUSSION

Complexity of TruPath pharmacological responses.
Our data does support complexity in the response to AM, AM2 and CGRP peptides in a pathway and receptor specific manner, consistent with previous reports (Clark et al, 2021; Weston et al, 2016). The TruPath assay measures distance between a BRET donor on the Ga and acceptor on the Gγ subunit of the obligate Gβγ dimer, and is generally interpreted as activation when the signal is decreased, indicative of agonist-receptor mediated subunit dissociation. While signals for Gi2, Gq and G11 followed the expected pattern of net G protein dissociation for G protein activation, the Gs sensor displayed a unique response profile with either an increase in BRET signal followed by a decline (CGRPR, AM2R), or a rise in signal without decline (AM1R), however, in all cases the signal did not fall below the starting baseline. While this might be an artefact of the G protein tagging and/or the expression levels of the G protein relative to the receptor in the cells, activation by other classes of Gs coupled receptors leads to the expected reduction in BRET signal (Olsen et al, 2020; Cary et al, 2023), although an initial rise in signal has been observed with some GPCRs, particularly within the class B1 GPCR subfamily, including the CTR and AMYRs (Fletcher et al, 2021). While most data support subunit dissociation as an element of cellular signaling, dissociation is not absolutely required for downstream signaling (e.g. Bondar and Lazar, 2014). Efficient Gs-mediated signaling from CGRP and AMRs is reported to require presence of the intracellular accessory protein, receptor component protein (RCP) (Dickerson, 2013), and it is possible that this contributes to the apparent tethering of the Gs subunits.

Supplementary discussion specific references


SUPPLEMENTARY TABLES

Supplementary Table 1. Quantitative pharmacological parameters for peptide-mediated cAMP accumulation in COS-7 cells transiently transfected with CGRPR, AM1R or AM2R. Data are mean ± sem from n=3 independent experiments. Emax values are cAMP levels expressed as nM concentration.

<table>
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<td>AM1784</td>
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### Supplementary Figure 1

Schematic of peptide sequences, illustrating homology between amylin-based peptides (A), calcitonin-based peptides (B) or CGRP and adrenomedullin peptides (C). Amino acids are shown in single letter code. All peptides contain a C-terminal amide. AM1213 and AM1784 have lipid modifications at the N-terminus. Disulphide bonded cysteines are present in the N-terminus of all peptides except AM1784. Shaded amino acids indicate conservation relative to rAmy. Amino acids in red indicate conservation relative to sCT.
Supplementary Figure 2. Construct validation at CGRPR, AM\textsubscript{1}R and AM\textsubscript{2}R in transiently transfected COS-7 cells assayed for cAMP accumulation. Values are mean ± SEM response, normalised to the maximum response of CGRP (left panel) or AM (middle and right panels) at the control receptor (CLR + untagged RAMP), determined from 4 individual experiments performed in duplicate.
Supplementary Figure 3. Kinetic traces of peptide-mediated recruitment of Gs-Venus to CTR. Kinetics of peptide concentration-dependent recruitment of Gs-Venus to CTR-Rluc8. The BRET ratio was measured for three baseline readings before the cells were stimulated with peptide and then read for a further 11 min. The data were corrected by subtraction of the vehicle data, followed by subtraction of the mean average of respective pre-stimulation baseline measurements (ΔΔ BRET). Values are mean ± SEM from 5-10 independent experiments.
Supplementary Figure 4. Kinetic traces of peptide-mediated recruitment of Gs-Venus to AMY1R. Kinetics of peptide concentration-dependent recruitment of Gs-Venus to AMY1R-Nluc (CTR+RAMP1-Nluc). The BRET ratio was measured for three baseline readings before the cells were stimulated with peptide and then read for a further 11 min. The data were corrected by subtraction of the vehicle data, followed by subtraction of the mean average of respective pre-stimulation baseline measurements (ΔΔ BRET). Values are mean ± SEM from 5-10 independent experiments.
Supplementary Figure 5. Kinetic traces of peptide-mediated recruitment of Gs-Venus to AMY2R. Kinetics of peptide concentration-dependent recruitment of Gs-Venus to AMY2R-Nluc (CTR+RAMP2-Nluc). The BRET ratio was measured for three baseline readings before the cells were stimulated with peptide and then read for a further 11 min. The data were corrected by subtraction of the vehicle data, followed by subtraction of the mean average of respective pre-stimulation baseline measurements (ΔΔ BRET). Values are mean ± SEM from 5-10 independent experiments.
Supplementary Figure 6. Kinetic traces of peptide-mediated recruitment of Gs-Venus to AMY$_3$R. Kinetics of peptide concentration-dependent recruitment of Gs-Venus to AMY$_3$R-Nluc (CTR+RAMP3-Nluc). The BRET ratio was measured for three baseline readings before the cells were stimulated with peptide and then read for a further 11 min. The data were corrected by subtraction of the vehicle data, followed by subtraction of the mean average of respective pre-stimulation baseline measurements (ΔΔ BRET). Values are mean ± SEM from 5-10 independent experiments.
Supplementary Figure 7. Kinetic traces of peptide-mediated recruitment of mini-Gs-Venus to CTR. Kinetics of peptide concentration-dependent recruitment of mini-Gs-Venus to CTR-Rluc8. The BRET ratio was measured for three baseline readings before the cells were stimulated with peptide and then read for a further 11 min. The data were corrected by subtraction of the vehicle data, followed by subtraction of the mean average of respective pre-stimulation baseline measurements ($\Delta\Delta$ BRET). Values are mean ± SEM from 5-10 independent experiments.
Supplementary Figure 8. Kinetic traces of peptide-mediated recruitment of mini-Gs-Venus to AMY₁R. Kinetics of peptide concentration-dependent recruitment of mini-Gs-Venus to AMY₁R-Nluc (CTR+RAMP1-Nluc). The BRET ratio was measured for three baseline readings before the cells were stimulated with peptide and then read for a further 11 min. The data were corrected by subtraction of the vehicle data, followed by subtraction of the mean average of respective pre-stimulation baseline measurements (ΔΔ BRET). Values are mean ± SEM from 5-10 independent experiments.
Supplementary Figure 9. Kinetic traces of peptide-mediated recruitment of mini-Gs-Venus to AMY2R. Kinetics of peptide concentration-dependent recruitment of mini-Gs-Venus to AMY2R-Nluc (CTR+RAMP2-Nluc). The BRET ratio was measured for three baseline readings before the cells were stimulated with peptide and then read for a further 11 min. The data were corrected by subtraction of the vehicle data, followed by subtraction of the mean average of respective pre-stimulation baseline measurements (ΔΔ BRET). Values are mean ± SEM from 5-10 independent experiments.
**Supplementary Figure 10. Kinetic traces of peptide-mediated recruitment of mini-Gs-Venus to AMY3R.** Kinetics of peptide concentration-dependent recruitment of mini-Gs-Venus to AMY3R-Nluc (CTR+RAMP3-Nluc). The BRET ratio was measured for three baseline readings before the cells were stimulated with peptide and then read for a further 11 min. The data were corrected by subtraction of the vehicle data, followed by subtraction of the mean average of respective pre-stimulation baseline measurements (ΔΔ BRET). Values are mean ± SEM from 5-10 independent experiments.
Supplementary Figure 11. Kinetics of peptide-mediated change in TruPath Gs BRET signal at CGPR, AM1R and AM2R. The BRET ratio was measured for three baseline readings before the cells were stimulated with peptide and then read for a further 20 min. The data were corrected by subtraction of the vehicle data, followed by subtraction of the mean average of respective pre-stimulation baseline measurements (ΔΔ BRET). Data are mean ± SEM from 3 independent experiments.
Supplementary Figure 12. Kinetics of peptide-mediated change in TruPath Gi2 BRET signal at CGRPR, AM\textsubscript{1}R and AM\textsubscript{2}R. The BRET ratio was measured for three baseline readings before the cells were stimulated with peptide and then read for a further 20 min. The data were corrected by subtraction of the vehicle data, followed by subtraction of the mean average of respective pre-stimulation baseline measurements (\(\Delta \Delta \text{ BRET}\)). Data are mean ± SEM from 3 independent experiments.
Supplementary Figure 13. Kinetics of peptide-mediated change in TruPath Gq BRET signal at CGPRR, AM\textsubscript{1}R and AM\textsubscript{2}R. The BRET ratio was measured for three baseline readings before the cells were stimulated with peptide and then read for a further 20 min. The data were corrected by subtraction of the vehicle data, followed by subtraction of the mean average of respective pre-stimulation baseline measurements (ΔΔ BRET). Data are mean ± SEM from 3 independent experiments.
Supplementary Figure 14. Kinetics of peptide-mediated change in TruPath G11 BRET signal at CGRPR, AM₁R and AM₂R. The BRET ratio was measured for three baseline readings before the cells were stimulated with peptide and then read for a further 20 min. The data were corrected by subtraction of the vehicle data, followed by subtraction of the mean average of respective pre-stimulation baseline measurements (ΔΔ BRET). Data are mean ± SEM from 3 independent experiments.
Supplementary Figure 15. Peptide potency differences relative to reference peptides. hCT (A, B) or sCT (B-D) at CTR (A, B), AMY1R (C), or AMY3R (D), for Gs recruitment, mini-Gs recruitment, TruPath Gs activation or cAMP accumulation assays (Fletcher et al, 2021).
Supplementary Figure 16. Correlation between peptide potency in the Gs-Venus and mini-Gs-Venus assays. A-D. Uncorrected peptide potencies. E-F. Using Gs-Venus potencies adjusted for the difference in response Emax. A, E. CTR; B, F. AMY1R; C, G. AMY2R; D, H. AMY3R.
Supplementary Figure 17. Potency differences of peptides in the Gs-Venus recruitment assay, relative to sCT, at CTR (A), AMY1R (B), AMY2R (C) and AMY3R (D). Potency differences were calculated using both uncorrected potencies (navy blue bars) or potencies that were adjusted for the difference in response Emax (light blue bars). Data are differences in the mean potency values.