AM833 Is a Novel Agonist of Calcitonin Family G Protein–Coupled Receptors: Pharmacological Comparison with Six Selective and Nonselective Agonists[§]

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ABSTRACT

Obesity and associated comorbidities are a major health burden, and novel therapeutics to help treat obesity are urgently needed. There is increasing evidence that targeting the amylin receptors (AMRs), heterodimers of the calcitonin G protein–coupled receptor (CTR) and receptor activity-modifying proteins, improves weight control and has the potential to act additively with other treatments such as glucagon-like peptide-1 receptor agonists. Recent data indicate that AMR agonists, which can also independently activate the CTR, may have improved efficacy for treating obesity, even though selective activation of CTRs is not efficacious. AM833 (cagrilintide) is a novel lipidated amylin analog that is undergoing clinical trials as a nonselective AMR and CTR agonist. In the current study, we have investigated the pharmacology of AM833 across 25 endpoints and compared this peptide with AMR selective and nonselective lipidated analogs (AM1213 and AM1784), and the clinically used peptide

agonists pramlintide (AMR selective) and salmon CT (non-selective). We also profiled human CT and rat amylin as prototypical selective agonists of CTR and AMRs, respectively. Our results demonstrate that AM833 has a unique pharmacological profile across diverse measures of receptor binding, activation, and regulation.

SIGNIFICANCE STATEMENT

AM833 is a novel nonselective agonist of calcitonin family receptors that has demonstrated efficacy for the treatment of obesity in phase 2 clinical trials. This study demonstrates that AM833 has a unique pharmacological profile across diverse measures of receptor binding, activation, and regulation when compared with other selective and nonselective calcitonin receptor and amylin receptor agonists. The present data provide mechanistic insight into the actions of AM833.

Introduction

Obesity is a major international health burden that has increased in prevalence nearly 3-fold from 1975 to 2016 to over 650 million obese adults (<http://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight>). Overweight and obesity

are major risk factors for other diseases, including heart disease and stroke, osteoarthritis, and some cancers, and are among the largest contributors to morbidity and mortality (Blüher, 2019). As such, there is an urgent need for drugs as an additional treatment option alongside lifestyle intervention and surgery. However, few drugs have been approved for the treatment of obesity, and most have limited efficacy, promoting only around 3%–7% weight loss, although higher efficacy is reported for high-dose formulation of the glucagon-like peptide-1 (GLP-1) receptor (GLP-1R) agonist semaglutide (Srivastava and Apovian, 2018; Williams et al., 2020; Wilding et al., 2021). Therefore, there is a need for the development of more efficacious antiobesity drugs and, consequently, further investigation into novel mechanisms of treatment.

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ABBREVIATIONS: AMY, amylinrGFP Green Fluorescent Protein, recombinant CAAX plasma membrane targeting motif FYVE FYVE domain of human Early Endosome Antigen 1 Rab Ras-related protein; AMYR, amylin receptor; AUC Area under the curve BRET, bioluminescence resonance energy transfer; CT, calcitonin; CTR, calcitonin receptor; cMyc cMyc epitope tag CTR_{aleu} "a" isoform and "leu" polymorphism of the CTR; DMEM, Dulbecco's modified Eagle's medium; E_{max} Maximum effect GLP-1, glucagon-like peptide-1; GLP-1R, GLP-1 receptor; GPCR, G protein–coupled receptor; h, human; HBBS, Hanks' balanced salt solution; HEK, human embryonic kidney; PEI, polyethylenimine; r, rat; RAMP, receptor activity-modifying protein; Rluc8, *Renilla* luciferase version 8; s, salmon; t_{1/2}, half-life.

Among the targets currently being investigated are the amylin receptors (AMyRs). The roles of AMyRs in glucose regulation have been well established, with a modified version of the endogenous (amylin) hormone, pramlintide, approved as a treatment of type 1 diabetes (Young et al., 1996; Gingell et al., 2014). However, amylin also regulates energy homeostasis, acting as a satiation signal, reducing food intake, and increasing energy expenditure while decreasing adiposity (Lutz, 2010; Zakariassen et al., 2020a). Although pramlintide was not optimized for weight reduction, patients treated with this drug exhibited small but significant weight loss, providing proof of principle that AMyRs could be targeted in humans (Aronne et al., 2007, 2010). This has led to an increased interest in the development of AMyR agonists for the treatment of obesity, as they could provide a novel mechanism of action relative to other proposed anorectic agents, including those acting at the GLP-1 receptor (Mathiesen et al., 2021).

AMyRs are heterodimers of the class B calcitonin (CT) G protein-coupled receptor (CTR) and receptor activity-modifying proteins (RAMPs). All three RAMPs can interact with the CTR, and this gives rise to three discrete AMyR receptor phenotypes, AMyR₁, AMyR₂, and AMyR₃, with RAMP1, RAMP2, and RAMP3, respectively (Hay et al., 2015). The CTR is expressed at the cell surface independently of RAMPs and has a distinct phenotype, exhibiting potent responses to human, and other species, of CT peptides but weak responses to amylin, whereas when CTRs interact with a RAMP, they form AMyRs that have high affinity and potency in response to amylin peptides but weak response to human CT (hCT). However, salmon CT (sCT) is a nonselective agonist and has high affinity and potency across both CTR and AMyRs (Hay et al., 2005; Udawela et al., 2006a,b; Morfis et al., 2008; Qi et al., 2013; Gingell et al., 2014).

Both selective AMyR agonists and nonselective agonists, such as sCT, have demonstrated efficacy in controlling appetite and body weight; however, this is not seen with the selective CTR agonist hCT (Lutz et al., 2000; Chelikani et al., 2007; Feigh et al., 2011). These observations have led to the hypothesis that the anorectic actions of these peptides are mediated by one or more of the AMyR subtypes.

As pramlintide only promoted limited weight loss in clinical trials, the focus has turned to optimizing amylin peptides for weight loss to enhance their *in vivo* efficacy. One approach is modification of peptides to extend their plasma half-lives, as pramlintide and nonamyloidogenic species of amylin (such as rat) have only a relatively short half-life of ~13 minutes in rodents and <50 minutes in healthy humans (Kolterman et al., 1996; Young et al., 1996). Enhanced plasma half-lives have been successfully achieved for GLP-1R agonists through modifications that increase plasma albumin binding or decrease metabolism and clearance (Iepsen et al., 2015; Lau et al., 2015). Modifications of amylin and calcitonin peptides have also been explored, including PEGylation, incorporation of albumin binding moieties, or glycosylation (Guerreiro et al., 2013; Tomabechi et al., 2013; Kowalczyk et al., 2014).

Another approach to extending the *in vivo* activity of peptides that has been investigated by multiple groups is the generation of chimeras of amylin and sCT. In contrast to amylin peptides, sCT has a prolonged duration of action that is related to its very slow off-rates from CT family receptors (Lutz et al., 2000). Both selective and nonselective AMyR

chimeric peptide agonists have been synthesized that may have improved efficacy in reducing body weight (Hilton et al., 2000; Mack et al., 2010; Andreassen et al., 2014a; Furness et al., 2016; Gydesen et al., 2016, 2017a; Larsen et al., 2019, 2020b). For example, davalintide, a chimera of sCT and rat amylin, provided long-term weight loss in rodent models and prolonged *in vivo* action over rat amylin, despite having a similar plasma half-life to amylin (Mack et al., 2010). This was attributed to the slow dissociation of davalintide from AMyRs that had been engineered into the peptide (Mack et al., 2011).

Moreover, nonselective pharmacology has become a desired feature of peptide drug discovery programs that target CT family receptors. Despite the assumption that the primary targets mediating the weight loss are AMyRs, nonselective agonists are reported to have higher efficacy than amylin mimetics (Andreassen et al., 2014a; Gydesen et al., 2016, 2017a; Larsen et al., 2019, 2020b). Emerging data suggest that coactivation of both CTRs and AMyRs may confer greater efficacy in metabolic control than selective activation of AMyRs, despite observations that selective activation of CTRs does not robustly affect weight loss in rodents (Larsen et al., 2020a). Importantly, this synergistic benefit did not necessarily require the extended duration of action associated with sCT-like peptides (Larsen et al., 2020a).

Although controversial, meta-analysis of clinical data on the use of sCT for bone disease has suggested a weak association with cancer risk. This, together with lack of sCT effect on bone fracture in patients with osteoporosis, led to an unfavorable risk-benefit profile and, consequently, regulatory restrictions on the duration and nature of sCT treatment (<http://www.fda.gov/drugs/postmarket-drug-safety-information-patients-and-providers/questions-and-answers-changes-indicated-population-miacalcin-calcitonin-salmon>; Wells et al., 2016). Although it is unclear what the underlying mechanism may be for the potential cancer risk, ligands that mimic the pharmacological behavior of sCT, including the prolonged binding and duration of action, have not been proven to be superior to amylin analogs with respect to body weight in clinical trials (Ravussin et al., 2009; Mack et al., 2011). Therefore, an amylin analog that does not exhibit prolonged binding may be a better candidate for treatment of obesity.

In the current study, we have investigated the *in vitro* pharmacology of the lipidated amylin analog AM833 (cagrilintide) (Supplemental Fig. 1), which has recently completed a phase 2 clinical trial for obesity and a phase 1 combination trial with the GLP-1 receptor agonist semaglutide (<http://www.globenewswire.com/news-release/2020/06/18/2050266/0/en/Novo-Nordisk-successfully-completes-AM833-phase-2-trial-and-phase-1-combination-trial-with-AM833-and-semaglutide-in-obesity.html>). AM833 was developed by Novo Nordisk as a long-acting (once-weekly administration) and nonselective AMyR and CTR receptor agonist. We also compared AM833 with other AMyR selective and nonselective lipidated analogs—AM1213 and AM1784, respectively—as well as with the clinically used peptide agonists pramlintide (AMyR selective) and sCT (nonselective) and with native hCT and rat amylin as prototypical selective agonists of CTR and AMyR, respectively (Supplemental Fig. 1). Our results demonstrate that AM833 has a unique pharmacological profile compared with the other peptides across diverse measures of receptor binding, activation, and regulation.

Materials and Methods

Cell Lines and Culture. COS-1 cells (American Type Culture Collection CRL-1650), an African green monkey (*Cercopithecus aethiops*), fibroblast-like, adherent, Simian Virus-40–transformed cell line, were used for most experiments because of their lack of endogenous RAMP expression. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) with 5% heat-inactivated FBS (Thermo Electron Corporation, Melbourne, VIC, Australia). COS-7 cells (American Type Culture Collection CRL-1651) stably transfected with pEF-IRESpuro6 expression vector containing cMyc-tagged hCTR_{aleu} (Furness et al., 2016) were used for the cAMP duration-of-action assays. COS-7 cMyc-hCTR_{aleu} cells were maintained in DMEM (Invitrogen) with 2 µg/ml puromycin (Invivogen, San Diego, CA) and 5% FBS and cultured at 37°C and 95% O₂/5% CO₂ in a humidified incubator. Upon reaching ~80% confluency, cells were washed with PBS, harvested from tissue culture flasks using versene (PBS with EDTA at 0.196 g/l), pelleted by centrifugation at 350g for 3 minutes, and then resuspended in media to be reseeded into a flask to maintain the cell line or transfected and plated for an assay. HEK FreeStyle 293-F (ThermoFisher Scientific, Waltham, MA) cells were used for the membrane-based kinetic competition binding assays and were maintained in suspension in Freestyle Expression Medium.

Expression Constructs and Peptides. All constructs were in pcDNA3.1. cMyc-tagged hCTR_{aleu} was a gift from Dr. Rasmus Just. c-myc-hCTR_{aleu}-Rluc8 was generated by removal of the stop codon and subcloning it into a Rluc8 destination vector using Gateway technology (Invitrogen). Human RAMP1 and RAMP3 were a gift from Dr. Steve Foord (McLatchie et al., 1998). The vector pcDNA3.1 was used as a transfection control for RAMPs. The BRET sensors rGFP-CAAX, rGFP-FYVE, tandem rGFP-Rab4, and tandem rGFP-Rab11 were generously provided by Dr. Michel Bouvier (University of Montreal) and have been previously described (Namkung et al., 2016). The Gα_{i1/11/12}-Rluc8, Gβ₃, and Gγ₉-GFP2 constructs were generously provided by Dr. Ryan Strachan (University of North Carolina, Chapel Hill) (Olsen et al., 2020). hCT, rAmy, NNC0174-0833 (AM833, or cagrilintide, its proposed International Nonproprietary Name for pharmaceutical substances) (https://www.who.int/medicines/publications/druginformation/issues/INN_List-123.pdf?ua=1), NNC0174-1213 (AM1213), and NNC0174-1784 (AM1784) were synthesized by Novo Nordisk (Copenhagen). sCT was purchased from Sigma-Aldrich, St. Louis, MO, and pramlintide was purchased from ChemPep, Inc. (Wellington, FL). All peptides were dissolved in 0.05% acetic acid. sCT(R11, R18, K14)8-32:AF568 was generated as previously described (Furness et al., 2016).

Membrane-Based Kinetic Competition Binding. Fifty-milliliter cultures of HEK293F cells were transiently transfected with 25 µg cMyc-hCTR_{aleu} and 25 µg hRAMP1, hRAMP3, or pcDNA using LipofectAMINE 2000 (Thermo Fisher). DNA and LipofectAMINE were diluted in 2.5 ml of Opti-MEM and combined in a 1:2 ratio and incubated for 20 minutes before adding to the cells. Cells were harvested after 24 hours, and crude plasma membranes were prepared by sucrose density centrifugation as described in Furness et al. (2016). Membrane kinetic competition fluorescent ligand binding assays were performed in HBSS + 10 mM HEPES + 0.1% ovalbumin (pH 7.4) in black polypropylene round-bottom 96-well plates (Corning, NY). Assays were read in a kinetic competition mode at 30°C on a PHERAstar plate reader (BMG Labtech, Offenbourg, Germany), with each well containing buffer and 10 nM sCT8-32:AF568, with or without the indicated concentration of competing unlabeled control peptide. Control wells containing 1 µM of unlabeled sCT and 10 nM sCT8-32:AF568 were used to define unspecific binding. After baseline acquisition, membrane was added, and data were collected for 60 minutes. Graphs of the kinetic response were generated by normalizing the means from the replicates of individual experiments to 100% using the last time point from the vehicle well.

cAMP Accumulation Assays. COS-1 cells were transiently transfected using 1 mg/ml polyethylenimine (PEI) Max (mol. wt. 40,000; Polysciences, Warrington, PA). Cells were transfected with

32.5 ng per well of C-myc-CTR and 32.5 ng per well of either pcDNA, RAMP1, or RAMP3. DNA and PEI, each diluted in 10 µl of 150 mM NaCl per well, were combined in a 1:6 ratio before being briefly vortexed and incubated for 15 minutes at room temperature. The transfection mixture was added dropwise to the cells and was subsequently seeded into plates at 35,000 cells per well into 96-well clear plates (Corning) coated with poly(D-lysine) (Sigma-Aldrich) and incubated at 37°C in 5% CO₂.

After 48 hours, cells were washed and changed into stimulation buffer (phenol red-free DMEM containing 0.1% w/v ovalbumin and 0.5 mM 3-isobutyl-1-methylxanthine, pH 7.4) and incubated for 30 minutes in 37°C, 5% CO₂, before cells were stimulated with the peptide. After 30 minutes, the reaction was terminated by the aspiration of the buffer and addition of 50 µl of ice-cold absolute ethanol. Upon the evaporation of ethanol, the cells were lysed with 75 µl of 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 Forster Resonance Energy Transfer kit (Perkin Elmer, Waltham, MA). 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.

cAMP Duration of Action. COS-7 cells stably expressing CTR_{aleu} were seeded at 15,000 cells per well in 5% FBS DMEM onto poly(D-lysine)-coated 96-well plates and incubated overnight at 37°C, 5% CO₂. The addition of reagents for the assay was performed in laminar flow hoods under sterile conditions. Cells were washed and changed into stimulation buffer (phenol red-free DMEM containing 1% FBS, 0.1% w/v ovalbumin, pH 7.4) and incubated for 30 minutes in 37°C, 5% CO₂, before cells were stimulated with peptide. For the buffer wash plates, the cells were washed twice with stimulation buffer before replacement with drug-free buffer 1 hour after the addition of drug. After the indicated time, the reaction was terminated by the aspiration of the buffer and addition of 50 µl of ice-cold absolute ethanol. Upon the evaporation of ethanol, the cells were lysed with 75 µl of 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 Forster Resonance Energy Transfer kit. 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 performed in parallel. Data were subsequently normalized to the response of 100 µM forskolin at 10 minutes.

Calcium Mobilization. COS-1 cells were transiently transfected with CTR and pcDNA/RAMPs, as for the cAMP accumulation assay, except cells were plated into clear-bottomed, black-walled ViewPlates (Perkin Elmer). Cells were washed and changed into stimulation buffer [150 mM NaCl, 2.6 mM KCl, 1.18 mM MgCl₂·6H₂O, 2.2 mM CaCl₂·2H₂O, 10 mM D-glucose, 10 mM HEPES, 0.5% (w/v) ovalbumin, 4 mM probenecid] with 10 µM Fluo8 (Abcam, Cambridge, UK) and incubated at 37°C for 1 hour. The plates were read on the Functional Drug Screening System (FDSS)/µcell (Hamamatsu, Japan) with an excitation wavelength at 480 nm and an emission wavelength at 542 nm, with reads every 1 second for 2.5 minutes, including a 30-second baseline. All raw data were baseline- and vehicle-subtracted and then normalized to 100% of the 10 µM ATP response and 0% vehicle response, and the concentration-response data of the peptides were analyzed for AUC of 40 seconds poststimulation and time to peak.

G Protein Activation. COS-1 cells were PEI-transfected with hCTR_{aleu}:pcDNA3/RAMP1/3:Gα_x-Rluc8:Gβ₃:Gγ₉-GFP2 at a 1:1:1:1:1 ratio, giving 50 ng total DNA per well. Cells were plated at 30,000 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 µM and incubated for a further 10 minutes at 37°C. BRET measurements were performed on

a PHERAstar or LUMIstar 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

Fig. 1. Second messenger signaling of COS-1 cells transiently transfected with CTR or AMYRs. (A–C) Concentration-response curves of 30-minute cAMP accumulation at CTR (A), AMY₁R (B), and AMY₃R (C). Data were normalized to the maximal peptide response and fit with a biphasic equation in which the top was shared and Hill slopes for the two phases were set to 1. Values are means ± S.E.M. of three to four experiments performed in triplicate. (D–F) Concentration-response curves for the iCa²⁺ mobilization peak response CTR (D), AMY₁R (E), and AMY₃R (F). Data were corrected for baseline and vehicle responses and normalized to the response of 10 μ M ATP. Data points are means ± S.D. of three to four experiments performed in duplicate. (G and H) pEC₅₀ values of iCa²⁺ mobilization data measured at peak (G) or as AUC of the 40 seconds poststimulation (H). Values are means ± S.D., calculated from three to four individual experiments. Differences were assessed by two-way ANOVA followed by a Dunnett's post-test relative to either sCT or hCT.

values (prestimulation) for each well. Data were normalized to the maximum response of sCT for each receptor and each G protein to allow pooling of results.

Trafficking. COS-1 cells were PEI-transfected with 6 ng per well of human CTR_{aleu}-Rluc8; 6 ng of human RAMP1, RAMP3, or pcDNA; and 24 ng ng/well of targeted BRET biosensor. Cells were plated at 35,000 cells per well into 96-well Grenier CELLSTAR white-walled plates, and assays were performed 48 hours later. Cells were washed once to remove phenol red and HBSS with 10 mM HEPES, and 0.1% (w/v) ovalbumin was added. Cells were incubated at 37°C for 30 minutes before being assayed. At 10 minutes before the assay, prolume purple coelentrastazine was added to give a final assay concentration of 1.3 μ M. BRET measurements were performed on a PHERAstar using 410/80-nm/515/30-nm filters, with baseline measurements taken for 4 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 then baseline-corrected by subtracting to the mean BRET ratio of baseline values for each well and vehicle-corrected by subtracting for a vehicle-treated well from the same transfection ratio for the ligand-treated well.

Quantification and Statistical Analysis. All experimental data were analyzed using Prism 8 software (GraphPad Software Inc., San Diego, CA). Concentration-response signaling data were analyzed 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, and the top was shared.

For membrane kinetic competition binding, each experiment was individually fitted using the kinetics of competitive binding according to the model of Motulsky and Mahan (1984), and the parameters of the probe ligand sCT8-32:AF568 from association/dissociation experiments (Furness et al., 2016) were used as the constraints for the model (Kon = 19514165 minute⁻¹ and Koff = 0.1043 minute⁻¹). Kd was calculated as Koff/Kon and then converted to log, and *t*_{1/2} was calculated as ln (2)/koff and then converted to antilog. Data are represented as means $\pm$ S.E.M. or S.D. and were compared using ANOVA, followed by Dunnett's or Tukey's post-test to assess statistical significance. The null hypothesis was rejected at *P* < 0.05.

Results

AMYRs are formed through the heterodimerization of CTR and RAMPs; however, it has not been possible to identify in vitro expression conditions in which AMYRs can be formed exclusively. Consequently, interrogation of AMYR pharmacology is done by inference, relative to responses at CTR expressed alone, and to reference agonists that have the greatest relative selectivity for CTR or AMYRs, typically hCT and rAmy, respectively. Because of the previously reported weak phenotype for AMY₂R (Hay et al., 2015), the current study was restricted to CTR, AMY₁R, and AMY₃R, which are the best-characterized AMYRs. This study was performed on the CTR_{aleu} form of CTR, which lacks a 16-amino-acid insert in intracellular loop 1, and has a leucine at position 447 in the C-terminal tail (Gorn et al., 1992; Kuestner et al., 1994; Dal Maso et al., 2018).

Second Messenger Production

cAMP Accumulation. Like other class B1 GPCRs, the CT receptor family is canonically coupled to Gs-mediated cAMP production, and measurement of cAMP accumulation has been the primary assay used to determine peptide selectivity and potency. CTR or individual AMYRs were transiently expressed in COS-1 cells, and peptide-induced cAMP

TABLE 1
Peptide potency for cAMP accumulation at CTR, AMY₁R, and AMY₃R

Data were normalized to the maximal peptide response and were fit to a biphasic equation, which was determined as the best fit based on an *F* test, with Hill slopes of 1; the top was shared. pEC₅₀ is the negative logarithm of the concentration of agonist that produces half the maximal response. All values are means $\pm$ S.E.M. from the curve fit of three to four experiments conducted in triplicate. Statistical comparisons were performed using two-way ANOVA with Dunnett's post-test for differences between the control peptides hCT (**P* < 0.05), rAmy (^Δ*P* < 0.05), and other peptides, or between CTR (^Δ*P* < 0.05) and AMYRs.

	CTR						AMY ₁ R						AMY ₃ R					
	pEC ₅₀ (high)			pEC ₅₀ (low)			pEC ₅₀ (high)			pEC ₅₀ (low)			pEC ₅₀ (high)			pEC ₅₀ (low)		
	Mean	S.E.M.	n	Mean	S.E.M.	n	Mean	S.E.M.	n	Mean	S.E.M.	n	Mean	S.E.M.	n	Mean	S.E.M.	n
	Fraction (high)	Fraction (high)	Fraction (high)	Fraction (high)	Fraction (high)	Fraction (high)	Fraction (high)	Fraction (high)	Fraction (high)	Fraction (high)	Fraction (high)	Fraction (high)	Fraction (high)	Fraction (high)	Fraction (high)	Fraction (high)	Fraction (high)	Fraction (high)
AM833	9.71 ^Δ	0.21	4	7.78	0.16	4	9.81	0.21	4	8.18	0.23	4	10.14	0.18	4	8.19	0.15	4
Pramlintide	9.26*	0.20	3	7.50	0.22	3	9.67	0.23	3	8.04	0.33	3	10.14 ^Δ	0.17	3	8.26	0.19	3
sCT	10.19 ^Δ	0.21	4	8.15	0.16	4	9.91	0.12	4	7.75	0.26	4	10.08	0.13	4	8.06	0.20	4
hCT	10.36 ^Δ	0.18	4	8.04	0.18	4	10.32 ^Δ	0.20	4	8.49	0.28	4	10.58 ^Δ	0.17	4	8.25	0.12	4
rAmy	8.64*	0.24	4	7.37	0.43	4	9.44 ^Δ	0.16	4	7.76	0.41	4	9.47 ^Δ	0.15	4	7.95	0.43	4
AM1213	8.97*	0.38	4	7.60	0.20	4	9.54*	0.17	4	7.67	0.23	4	9.78 ^Δ	0.16	4	8.12	0.27	4
AM1784	10.16 ^Δ	0.28	4	8.27	0.15	4	10.28 ^Δ	0.19	4	8.25	0.18	4	10.10	0.18	4	8.45	0.20	4

accumulation was measured for 30 minutes. For all receptors, the data were best fit with biphasic concentration-response curves, as has been previously noted in similar studies with CTR (Furness et al., 2016; Dal Maso et al., 2018). There were no significant differences in log EC₅₀ values between peptides or receptors for the low-potency site or in the fraction of receptors in high or low sites. However, the expected induction of rAmy phenotype was seen for the initial high-potency phase of the response at the AMY₁R and AMY₃R, relative to CTR alone. The AMYR selective peptide AM1213 exhibited a similar trend that was significant for AMY₃R, and there was also a significant increase in pramlintide potency at the AMY₃R compared with CTR (Fig. 1, A–C; Table 1); however, the potency for hCT, sCT, AM833, and AM1784 was similar at each of the receptors. These differences were also reflected in the relative potency of peptides to the reference agonists hCT or rAmy at each of the receptors (Table 1). These data confirmed the induction of the expected AMY₁R and AMY₃R phenotypes and that AM833, like sCT, was a nonselective agonist of CTR and AMYRs in the prototypical assay of CT family receptor function. We subsequently sought to explore the relative pharmacology of AM833 in a broad series of assays of receptor activation and trafficking.

Intracellular Calcium Mobilization. Calcium is a critical second messenger for multiple cellular functions, and intracellular calcium is mobilized in response to activation of CT family receptors and has previously been shown to depend on multiple upstream signaling pathways (Morris et al., 2008; Dal Maso et al., 2018). Robust responses were observed for all receptor phenotypes, with sCT equal to or the most potent peptide for all receptors. There was only a limited effect of RAMP1 (AMY₁R) or RAMP3 (AMY₃R) cotransfection on peptide response, whether measured at the peak response (Fig. 1, D–F), area under the curve (Supplemental Fig. 2, A–C), or in the time to peak (Supplemental Fig. 2D). Nonetheless, sCT was significantly more potent than AM833 and rAmy when measuring the peak response at AMY₃R (Fig. 1, F and G; Table 2), although this did not achieve significance when measured as AUC, whereas the difference in potency between rAmy and sCT and hCT at the CTR was significantly different when calculated as AUC (Fig. 1, D and H; Supplemental Fig. 2A; Table 2). No other differences in peptide responses were observed.

Peptide Residence Time. The half-life of peptide binding to CT family receptors, or residence time, has been a key feature that has been exploited to extend the duration of action. To determine the dissociation rates of peptides from their receptors, cell plasma membrane was harvested from CTR or CTR plus RAMP1 (AMY₁R) or RAMP3 (AMY₃R) transiently transfected into Freestyle HEK-293 cells. These cells enable very high expression of the receptors, which is required for robust signal to noise in fluorescence polarization-based competition binding assays. Competition for the nonselective antagonist probe peptide sCT(8-32):AF568 was performed with increasing concentrations of agonist peptide, with fluorescence binding of the probe monitored in real time (Fig. 2, A–F; Supplemental Fig. 3, A–E). Kinetic traces were analyzed by the method of Motulsky and Mahan (1984) to estimate off-rates/residency time of individual peptides (Fig. 2G; Table 3). AM833 exhibited rapid dissociation with a residence time (~3–6 minutes) that was not different between receptor subtypes (Fig. 2G;

TABLE 2

Peptide potency for iCa²⁺ mobilization at peak and AUC at CTR, AMY₁R, and AMY₃R. Data were normalized to 100% of the 10 μM ATP response and 0% vehicle response, and the concentration-response data of the peptides of time to peak and AUC of 40 seconds poststimulation were fit to a three-parameter curve. pEC₅₀ is the negative logarithm of the concentration of agonist that produces half the maximal response. All values are means ± S.E.M. of three to four experiments conducted in duplicate. Statistical comparisons were performed using two-way ANOVA with Dunnett's post-test for differences between the control peptides sCT (*P < 0.05), hCT (*P < 0.05), and other peptides.

	CTR			AMY ₁ R			AMY ₃ R		
	pEC ₅₀ (peak)		n	pEC ₅₀ (AUC)		n	pEC ₅₀ (peak)		n
	Mean	S.D.		Mean	S.D.		Mean	S.D.	
AM833	7.36	0.47	4	7.10	0.47	4	6.94*	0.28	4
Pramlintide	7.74	0.17	4	7.59	0.36	3	7.44	0.08	4
sCT	7.83	0.25	4	7.54	0.60	4	7.71	0.38	3
hCT	7.85	0.15	3	7.21	0.13	3	7.33	0.26	3
rAmy	7.15	0.28	4	7.13	0.22	4	6.80*	0.07	3
AM1213	7.26	0.40	4	7.22	0.57	4	7.18	0.47	4
AM1784	7.47	0.35	4	7.56	0.76	3	7.42	0.36	4

Fig. 2. Residence time of peptide ligands at CTR, AMY₁R, and AMY₃Rs, measured through fluorescence polarisation (FP). (A–F) Grouped kinetic traces for inhibition of sCT8-32:AF568 binding to HEK FreeStyle cells transiently transfected with CTR (A and B), AMY₁R (C and D), or AMY₃R (E and F) by AM833 (A, C, and E) or sCT (B, D, and F). (G) Quantitative analysis of residence time of peptide ligands expressed as log $t_{1/2}$. Values are means $\pm$ S.D. from four independent experiments. Statistical comparisons were performed using two-way ANOVA followed by Dunnett's post-test. Significant differences from sCT are reported. ** $P < 0.01$; *** $P < 0.001$.

Table 3). Pramlintide, hCT, AM1213, and rAmy had qualitatively similar residence times of ~1–3 minutes at all receptors (Fig. 2G). In contrast, sCT and AM1784 had very slow rates of dissociation from all receptors (Fig. 2G; Supplemental Fig. 3E; Table 3).

Duration of Action. In addition to long residency at CTRs, sCT has extended duration of action in assays of cellular cAMP (Michelangeli et al., 1983; Andreassen et al., 2014b). To understand whether this was primarily a property of binding off-rates, we investigated cAMP production in cells

TABLE 3

Peptide residence times at CTR, AMY₁R, and AMY₃R

Quantitative analysis of the residence time of peptide ligands, expressed as log *t*_{1/2} (minute) and *t*_{1/2} (minute). Values are means ± S.D. calculated from four independent experiments. Statistical comparisons were performed using two-way ANOVA, followed by Dunnett's post-test. Significant differences from sCT are reported. **P* < 0.05.

	CTR				AMY ₁ R				AMY ₃ R			
	Log <i>t</i> _{1/2}		<i>t</i> _{1/2}	<i>n</i>	Log <i>t</i> _{1/2}		<i>t</i> _{1/2}	<i>n</i>	Log <i>t</i> _{1/2}		<i>t</i> _{1/2}	<i>n</i>
	Mean	S.D.			Mean	S.D.			Mean	S.D.		
AM833	0.50*	0.11	3.22	4	0.69*	0.24	5.40	4	0.55*	0.10	3.64	4
Pramlintide	0.03*	0.36	1.37	4	0.19*	0.07	1.56	4	0.01*	0.65	2.02	4
sCT	1.60	0.26	47	4	1.60	0.260	45	4	1.80	0.17	63	4
hCT	0.20*	0.23	1.71	4	0.15*	0.17	1.15	4	0.26*	0.21	1.96	4
rAmy	−0.05*	0.39	1.13	4	−0.01*	0.51	1.39	4	0.31*	0.43	2.80	3
AM1213	0.17*	0.14	1.55	4	0.07*	0.31	1.40	4	0.38*	0.27	2.74	4
AM1784	2.10	0.37	173	4	2.10	0.61	291	4	2.40	0.66	652	4

over 24 hours. Because of the impact of the extended culture on transiently transfected cells, these experiments were restricted to COS-7 cells that stably expressed the CTR.

Duration-of-action studies were performed at saturating (1 μM) or subsaturating (10 nM; 100 nM AM1213) concentrations of peptide with either continuous stimulation for 24 hours or with cells washed after 1 hour of stimulation. Under all conditions, sCT induced the largest response, which peaked at 1 hour, and this was sustained for at least 8 hours

(Fig. 3, A–D; Table 4). Interestingly, under continuous stimulation at saturating concentrations of peptide, both hCT and AM1784 responses paralleled those of sCT (Fig. 3A), although there was a more rapid decline of the hCT, but not AM1784, following the 1-hour buffer wash, albeit this did not achieve statistical significance (Fig. 3B; Table 4). Despite saturating concentrations of peptide, lower responses were observed for AM833, pramlintide, AM1213, and rAmy, although the pattern was equivalent for all peptides, with the

Fig. 3. Duration of action of cAMP signaling of peptides in COS-7 cells stably transfected with CTR. Time courses of cAMP production over 24 hours by CTR after stimulation with a saturating concentration (1 μM) (A and B) or 10 nM (100 nM, AM1213) of peptide (C and D). In the continuous-stimulation treatments (A and C), the cells were exposed to the peptide for the indicated times, whereas for the ligand-washout treatment (B and D), the peptide was washed out after 1 hour of stimulation. Values are normalized to the response to the 10 μM forskolin (Fsk) at 10 minutes and are means ± S.E.M. from four experiments performed in quadruplicate.

Fig. 4. Kinetics of AM833- and sCT-induced G protein activation at CTR, AMY₁R, and AMY₃R in COS-1 cells. The kinetic profiles of Gs (A), Gi1 (B), G11 (C), and G12 (D) activation by AM833 and sCT at CTR, AMY₁R, and AMY₃R. The BRET ratio was measured for three baseline readings before the cells were stimulated with peptide and then read for a further 20 minutes. The data were normalized to the maximum (max) response to sCT, and values are means ± S.E.M. from four to five independent experiments.

peak response at 1 hour, and there was progressive reduction in signal that returned to baseline by 24 hours (Fig. 3A). The minor exception to this was rAmy, for which the peak response occurred at 10 minutes. For these non-CT peptides, after buffer wash at 1 hour, the cAMP response was not significantly different to baseline by 4 hours (Fig. 3B).

In contrast to results with saturating peptide concentrations, experiments with lower concentrations of peptides revealed unexpected differences in peptide responses. Despite similar levels of cAMP production measured at 10 minutes for all peptides, there were marked differences in cAMP levels at 1 hour, with sCT having the largest response, which (with the

Fig. 5. G protein activation at CTR, AMY_1R , and AMY_3R in COS-1 cells. Concentration-response curves of peptide-induced activation of G_s (A), G_{i1} (B), G_{11} (C), and G_{12} (D) at CTR, AMY_1R , and AMY_3R . Data were the ligand-induced BRET normalized to the maximum response to sCT and were fit with either a three-parameter curve or a biphasic equation with Hill slopes of 1, which was determined as the best fit based on an F test. Values are means $\pm$ S.E.M. from four to five independent experiments.

exception of hCT) was higher than all other peptides at this time point, including AM1784, which had the slowest off-rate in kinetic binding experiments (Fig. 3C). However, although the hCT response decayed rapidly by 4 hours, the AM1784 response was sustained for at least 8 hours. Likewise, the sCT response was sustained for at least 8 hours, with very little decay from the peak response over this time frame. All other

peptides had lower responses that gradually returned to baseline by 24 hours (Fig. 3C; Table 4).

G Protein Activation. To further interrogate the pharmacology of the peptides, we investigated the proximal activation of members of each major family of heterotrimeric G proteins, the primary transducers of GPCR signaling. The TruPath assay is a real-time assay designed principally to

TABLE 5

Peptide potency and E_{max} values of the AUC of G protein activation with CTR, AMY₁R, AMY₃R

Data were the ligand-induced BRET normalized to the maximum response to sCT and were fit with either a three-parameter curve or a biphasic equation, with Hill slopes fixed to 1, which was determined as the best fit based on an *F* test. Values are means ± S.E.M. from four to five independent experiments. Statistical comparisons were performed using one-way ANOVA, followed by Dunnett's post-test. When only one site was present in a subset of data, comparisons were made only to the site that was present in all data sets. Significant differences are reported from sCT (**P* < 0.05).

	CTR										AMY ₁ R										AMY ₃ R									
	Log EC ₅₀ (high)					Log EC ₅₀ (low)					Log EC ₅₀ (high)					Log EC ₅₀ (low)					Log EC ₅₀ (high)					Log EC ₅₀ (low)				
	Mean	S.E.M.	Mean	S.E.M.	n	Mean	S.E.M.	Mean	S.E.M.	n	Mean	S.E.M.	Mean	S.E.M.	n	Mean	S.E.M.	Mean	S.E.M.	n	Mean	S.E.M.	Mean	S.E.M.	n	Mean	S.E.M.	Mean	S.E.M.	n
G _q	AM833	7.92*	0.05		2	5	8.09*	0.14		5	85	75*	5	4	7.71*	0.10			5	4	106				4					4
	Pranlintide	7.46*	0.07		3	5	8.04*	0.10		3	100	100	2	5	8.16	0.13			2	5	88				5					5
	sCT	8.79	0.03		1	5	8.73	0.05		1	86*	86*	2	5	8.54	0.10			2	5	103				4					4
	bCT	8.63	0.06		2	5	8.35	0.07		2	89*	89*	2	5	7.90*	0.10			2	5	100				4					4
	rAmy	6.76*	0.06		3	5	7.43*	0.15		3	104	104	4	4	7.51*	0.21			4	5	84*				5					4
G ₁₁	AM1213	6.82*	0.06		4	4	7.51*	0.21		4	98	98	3	4	8.20	0.09			3	4	93				7					4
	AM1784	8.44*	0.05		2	4	8.49	0.08		2	71*	71*	5	5	11.28	0.82			5	5	116				4					4
	AM833	10.37	0.91		5	5	11.28	0.82		5	68*	68*	4	5	9.94	0.52			4	5	84				6					4
	Pranlintide	10.56	0.40		6	5	9.99	0.44		6	86	86	7	5	9.87	0.26			7	5	88				6					4
	sCT	11.23	0.53		3	5	12.05	0.55		3	101	101	3	5	10.68	0.37			3	5	104				4					5
G ₁₂	bCT	10.53	0.60		5	5	8.22	0.18		5	92	92	6	5	10.31	0.50			6	5	98				4					4
	rAmy	12.28	0.78		5	5	10.87	0.58		5	54*	54*	5	5	10.87	0.25			5	5	65				8					4
	AM1213	9.62	0.70		7	4	9.24	0.66		7	71*	71*	10	4	9.61	0.67			10	4	79				45					4
	AM1784	10.79	0.40		3	4	8.17	0.15		3	96	96	5	4	8.20	0.23			5	4	87				7					4
	AM833	12.52	0.81		4	4	11.34	0.52		4	91	91	3	4	10.98	0.52			3	4	85				5					4
AMY ₁ R	Pranlintide	10.75	0.58		7	4	7.60	0.20		7	97	97	8	4	11.11	0.59			8	4	106				13					4
	sCT	12.01	0.47		3	4	8.50	0.10		3	100	100	3	4	10.94	0.39			3	4	104				4					4
	bCT	11.00	0.62		4	4	7.98	0.14		4	102	102	5	4	10.94	0.39			5	4	84				4					4
	rAmy				7	4	7.07	0.28		7	66*	66*	9	4	10.33	0.35			9	4	86				6					4
	AM1213				6	4	10.96	0.48		6	96	96	8	4	9.03*	0.40			8	4	84				8					4
AMY ₃ R	AM1784	11.23	0.49		5	4	8.16	0.13		5	105	105	5	4	10.78	0.58			5	4	101				9					4
	AM833	9.72*	0.42		3	5	10.03	0.28		3	83*	83*	2	5	9.95	0.49			2	5	81*				4					4
	Pranlintide	9.57*	0.32		3	5	10.62	0.43		3	86*	86*	3	5	10.68	0.37			3	5	81*				3					4
	sCT	11.07	0.15		1	5	10.69	0.30		1	102	102	2	5	10.96	0.15			2	5	103				1					4
	bCT	10.45	0.21		8	5	10.45	0.49		8	86*	86*	3	5	10.97	0.21			3	5	86*				1					4
AMY ₃ R	rAmy	8.86*	0.25		5	5	9.22*	0.25		5	79*	79*	6	4	9.56*	0.23			6	4	76*				5					4
	AM1213	8.73*	0.40		6	4	8.94*	0.22		6	89	89	8	4	9.08*	0.28			8	4	89*				5					4
	AM1784	10.68	0.59		4	4	10.30	0.33		4	94	94	2	4	10.23	0.32			2	4	96				3					4
	AM833				4	4	8.07	0.11		4	100	100	2	4	10.23	0.32			2	4	96				3					4
	Pranlintide				4	4	8.07	0.11		4	100	100	2	4	10.23	0.32			2	4	96				3					4

detect G protein activation through loss of BRET signal when the $G\alpha$ and $G\beta\gamma$ subunits dissociate after agonist addition (Olsen et al., 2020). However, changes in G protein conformation that alter the relative position of the donor and acceptor prior to subunit dissociation may also contribute to the observed signal, either as an increase or decrease in BRET. Kinetic traces over the 20 minutes poststimulation for all G protein, receptor, and peptide combinations (Fig. 4, A–D; Supplemental Figs. 4 and 5) were analyzed for total response (AUC; Fig. 5, A–D; Table 5), as well as at 2, 4, 10, and 20 minutes to assess potential differences in rates of activation (Fig. 6; Supplemental Figs. 6 and 7; Table 6).

For the G_s sensor, an increase in BRET signal was observed for all peptides at low concentrations prior to the loss of signal over time, which is indicative of subunit dissociation (Fig. 4A; Supplemental Fig. 4). This effect is not captured in the AUC data analysis, which only records the negative change from vehicle control, although it contributes to the curve fitting for the response data measured at 2, 4, 10, and 20 minutes (Supplemental Fig. 6).

Assessment of the cumulative response (AUC) over the 20 minutes of stimulation revealed differences in the complexity of response for assay of different G protein sensors. For all receptors, G_s concentration-response data were best fit to a three-parameter logistic equation, whereas responses to the other G proteins were best fit to a biphasic response equation, with minor exception (and this is likely due to data variance rather than distinct pharmacology in the cases of exception). The mechanistic basis for the biphasic response is not clear, but it may (at least in part) be due to initial conformation changes prior to G protein subunit dissociation; this would be consistent with the absence of a second phase in the G_s response data where only the negative BRET data were quantified.

G_s Activation. In contrast to cAMP accumulation, AM833 had lower potency at the CTR than sCT, hCT, and AM1784 in the cumulative AUC for G_s activation (Fig. 5A; Table 5), although it was more potent than rAmy, AM1213, and pramlintide, which was broadly consistent with the cAMP accumulation observations. Potencies for AM833, sCT, and AM1784 were similar across all three receptors (Fig. 5A; Fig. 6A; Table 5). Potencies of rAmy and pramlintide were increased at both AMY_1R and AMY_3R , whereas AM1213 potency was selectively increased at the AMY_1R , and potency of hCT was selectively decreased at the AMY_3R (Fig. 5A; Table 5). rAmy also had a lower E_{max} at the $AMYRs$, and this was significantly lower than AM833 for both these receptors (Table 5).

Intriguingly, time-resolved analysis of the CTR data revealed that the lower-potency ligands had distinct activation kinetics compared with the other peptides, with constant potency over time—in contrast to AM833, sCT, hCT, and AM1784, which increased potency over time. By comparison, all peptides displayed increased potencies over time at both $AMYRs$, albeit with peptide-specific differences in the magnitude of this effect (Fig. 6A; Supplemental Fig. 4, A–C; Table 6). However, rAmy, pramlintide, and AM1213 had a lower E_{max} relative to sCT at later time points at the AMY_1R , and rAmy and pramlintide also have lower E_{max} at the AMY_3R (Supplemental Fig. 6, A–C; Table 6). These data illustrate that there are differences in the kinetics of G protein activation even when the summative response may be similar,

as illustrated in both the biosensor AUC data, and response downstream of G_s activation, as illustrated by the cAMP accumulation data (Fig. 1; Table 1).

G_{i1} Activation. The cumulative G_{i1} AUC response to peptides was best fit to a biphasic response curve for most peptides, with the exception of hCT at the AMY_1R and AM1784 at the AMY_1R and AMY_3R (Fig. 5B; Table 5). For all peptides, there was little difference in potency for activation of G_{i1} between receptors. AM1213, pramlintide, and rAmy tended to have the lowest potency for the more robust second phase of response, and sCT, hCT, and AM1784 had more-potent responses, albeit the differences were only significant between AM1213 and the more-potent peptides at the AMY_3R (Fig. 5B; Table 5). AM833 generally exhibited an intermediate potency between the two peptide clusters. There were also differences in the maximal cumulative response that was most evident for rAmy, which was significantly lower than sCT at the CTR and AMY_1R (Fig. 5B; Table 5).

Time-resolved analysis of the data was consistent with the peptide clustering noted for the cumulative data, although it provided evidence for qualitative phenotypic distinction between peptide behavior between receptor subtypes (Fig. 6B; Supplemental Fig. 4, D–F; Supplemental Fig. 6, D–F; Table 6). sCT and hCT both exhibited time-dependent increases in potency across all three receptors, whereas AM1784 potency increased over time at the CTR and AMY_1R but showed no change at the AMY_3R . rAmy and AM1213 tended to decrease potency at CTR and AMY_1R but differed in pattern of response at the AMY_3R , where either no change (rAmy) or a trend toward increased potency (AM1213) was observed. AM833 potency was not altered over time at any of the receptors, whereas pramlintide exhibited varied patterns of response across the receptors, with no change at CTR, decreased potency at AMY_1R , and a potential biphasic response at the AMY_3R (Fig. 6B; Table 6). Interestingly, differences in the magnitude of the change in BRET signal between peptides were evident even at the 2-minute time point. At all time points and receptors, sCT had the largest effect and rAmy had the lowest, with the other peptides displaying a range of effect that varied in a receptor- and time-dependent manner (Supplemental Fig. 6, D–F; Table 6).

G_{i1} Activation. The cumulative G_{i1} AUC data for CTR were best fit to a biphasic response curve for sCT, hCT, AM833, AM1784, and pramlintide, although there was only a small response for the high-potency phase. For rAmy and AM1213, only a single low-potency phase could be robustly fit. At the CTR, pramlintide, rAmy, and AM1213 had a lower potency than the other peptides for the second phase of response; however, this was only significant for sCT versus pramlintide (Fig. 5C; Table 5). At the AMY_1R , only AM833 and AM1213 had a resolvable high-potency phase. At this receptor, rAmy and AM1213 had lower potency than the other peptides, and this was significant relative to sCT for both peptides and relative to AM1784 for rAmy (Fig. 5C; Table 5). At the AMY_3R , a robust biphasic fit was observed for all peptides except for hCT, and AM1213 had a significantly lower potency than the other peptides. For the second response phase, rAmy, AM1213, and pramlintide had the lowest potencies and were significantly different from hCT, and rAmy and AM1213 were also significantly different from sCT (Fig. 5C; Table 5).

Fig. 6. Peptide potency for G protein activation at CTR, AMY₁R, and AMY₃R in COS-1 cells at various time points post-peptide stimulation. Log EC₅₀ values (mean ± S.E.M.) of peptides from the concentration-response curves at 2, 4, 10, and 20 minutes for Gs (A), Gi1 (B), G11 (C), and G12 (D). Arrows indicate the direction of the shift in potencies over time, and lines represent no change.

Time-resolved analysis of the data revealed interesting further differences in the pattern of peptide responses (Fig. 6C; Supplemental Figs. 4–7; Table 6), particularly with respect to the effect of time on peptide potency. Unlike the cumulative data, the concentration-response data at individual time points could be fit to a simple three-parameter logistic equation, and there was a differential effect of increasing time on the potency of peptide response. These could be broadly separated into two groups: 1) rAmy, AM1213, and pramlintide

displayed a reduction in potency over time at the CTR and AMY₁R, and for AM1213 at the AMY₃R, whereas rAmy and pramlintide displayed an apparent biphasic pattern of response with an initial decrease in potency at the earliest time points that was reversed at the later time point; 2) sCT, hCT, and AM1784 had increased or unchanged potency over time in a receptor- and peptide-dependent manner, whereas AM833 was an exception for which potency was not altered at the CTR and AMY₃R but was progressively reduced over time at the

TABLE 6

Peptide potency and $E_{\max}$ values for G protein activation at 2, 4, 10, and 20 minutes with CTR, AMY₁R, AMY₃R

Data were the ligand-induced BRET normalized to the maximum AUC response of sCT and were fit with a three-parameter logistic curve. Values are means $\pm$ S.E.M. from four to five independent experiments. Statistical comparisons were performed using two-way ANOVA, followed by Dunnett's post-test. Significant differences are reported from sCT (* $P < 0.05$) and the 2-minute time point (* $P < 0.05$).

	2 min				4 min				10 min				20 min							
	pEC ₅₀		E _{max}		n	pEC ₅₀		E _{max}		n	pEC ₅₀		E _{max}		n					
	Mean	S.E.M.	Mean	S.E.M.		Mean	S.E.M.	Mean	S.E.M.		Mean	S.E.M.	Mean	S.E.M.						
CTR Gs activation																				
AM833	7.56*	0.09	-73.1	3.1	5	7.71*	0.10	-76.4	3.7	5	8.01*	0.10	-86.3	4.0	5	8.30**	0.12	-85.8	4.0	5
Pramlintide	7.60*	0.15	-74.6	5.3	5	7.61*	0.14	-77.5	5.4	5	7.57*	0.12	-83.4	5.2	5	7.57*	0.14	-79.0	5.9	5
sCT	8.38	0.15	-78.8	4.4	5	8.61	0.08	-82.0	2.5	5	8.95*	0.07	-91.6	2.4	5	9.15*	0.07	-98.8	2.3	5
hCT	8.32	0.09	-71.1	2.3	5	8.52	0.11	-71.6	3.0	5	8.84*	0.10	-79.7	3.0	5	8.92*	0.12	-79.2	3.5	5
rAmy	6.81*	0.15	-71.1	7.4	5	6.82*	0.16	-77.8	8.7	5	6.77*	0.15	-84.3	9.4	5	6.75*	0.18	-82.2	10.7	5
AM1213	6.87*	0.13	-80.4	6.8	4	6.88*	0.16	-86.9	9.1	4	6.89*	0.15	-92.7	9.9	4	6.97*	0.17	-87.8	10.8	4
AM1784	8.11	0.08	-78.4	2.6	4	8.22	0.09	-86.9	3.4	4	8.61*	0.09	-95.4	3.4	4	8.85*	0.08	-103.0	3.0	4
AMY ₁ R Gs activation																				
AM833	7.73*	0.11	-60.3	3.1	4	7.87*	0.12	-65.9	3.7	4	8.27**	0.11	-80.0	3.7	4	8.50**	0.10	-88.7	3.4	4
Pramlintide	8.02	0.10	-57.8	2.5	4	8.03*	0.12	-61.5	3.4	4	8.32*	0.11	-70.9*	2.9	4	8.63**	0.15	-65.3*	3.6	4
sCT	8.22	0.09	-66.8	2.3	5	8.47	0.11	-72.7	3.1	5	8.91*	0.09	-90.3	2.9	5	9.07*	0.07	-97.5	2.5	5
hCT	8.18	0.09	-62.4	2.3	5	8.31	0.11	-69.2	3.3	5	8.72*	0.08	-82.3	2.5	5	8.78*	0.09	-82.6*	2.9	5
rAmy	7.46*	0.13	-50.3*	3.3	5	7.53*	0.14	-55.3*	3.9	5	7.92**	0.12	-56.7*	3.5	5	7.96**	0.14	-58.1*	3.7	5
AM1213	7.22*	0.14	-58.1	4.4	4	7.36*	0.15	-62.2	5.1	4	7.80**	0.12	-69.2*	4.1	4	8.03**	0.13	-72.8*	4.2	4
AM1784	8.20	0.11	-62.5	2.8	4	8.29	0.12	-68.2	3.4	4	8.70*	0.09	-85.7	2.9	4	8.94*	0.09	-97.8	3.2	4
AMY ₃ R Gs activation																				
AM833	7.34*	0.12	-80.9	4.8	4	7.46*	0.15	-82.9	6.4	4	7.81*	0.15	-90.5	6.8	4	8.09**	0.14	-92.6	6.3	4
Pramlintide	7.88	0.15	-70.0	4.8	5	8.03	0.17	-71.9	6.1	5	8.34	0.16	-77.6	5.7	5	8.49*	0.19	-71.7*	6.3	5
sCT	8.08	0.09	-80.1	2.9	5	8.28	0.11	-81.7	3.8	5	8.72*	0.09	-93.3	3.3	5	8.99*	0.10	-94.4	3.9	5
hCT	7.66	0.10	-78.0	3.5	4	7.86	0.10	-75.2	3.5	4	8.02*	0.11	-83.1	4.2	4	8.08*	0.14	-83.0	5.2	4
rAmy	7.41*	0.17	-69.7	6.4	4	7.51*	0.21	-69.4	7.9	4	7.77*	0.18	-68.3*	7.1	4	7.94*	0.20	-66.4*	7.4	4
AM1213	6.99*	0.17	-79.3	8.0	4	7.08	0.21	-80.3	10.8	4	7.28*	0.22	-85.0	11.2	4	7.56**	0.27	-82.1	12.2	4
AM1784	7.82	0.09	-82.6	3.2	4	7.98	0.09	-87.7	3.5	4	8.29	0.10	-100.9	4.1	4	8.64*	0.09	-102.4	3.7	4
CTR Gi1 activation																				
AM833	7.57	0.18	-60.7*	4.0	5	7.72	0.27	-59.3*	6.1	5	7.90	0.25	-53.5*	4.9	5	7.63*	0.28	-62.0*	6.6	5
Pramlintide	7.36	0.31	-66.6*	8.7	5	7.60	0.23	-63.1*	5.9	5	7.12*	0.22	-69.3	7.9	5	7.31*	0.33	-70.5	11.6	5
sCT	7.88	0.14	-96.0	4.7	5	8.16	0.12	-90.1	3.7	5	8.54	0.11	-84.5	2.9	5	8.52	0.18	-88.7	4.6	5
hCT	7.84	0.22	-80.6	6.0	5	8.09	0.20	-75.8	5.1	5	8.20	0.16	-65.7	3.6	5	8.36	0.20	-64.0*	4.2	5
rAmy	7.50	0.25	-53.7*	4.5	5	8.09	0.28	-45.4*	4.1	5	7.21*	0.27	-40.7*	5.0	5	7.19*	0.57	-43.8*	11.4	5
AM1213	7.39	0.21	-61.5*	4.9	4	7.72	0.28	-55.7*	5.8	4	7.43*	0.33	-39.7*	5.6	4	7.15	0.33	-56.3*	9.7	4
AM1784	7.78	0.19	-71.1*	4.7	4	7.81	0.18	-71.5	4.6	4	8.04	0.17	-70.4	4.1	4	8.26	0.19	-68.9	4.5	4
AMY ₁ R Gi1 activation																				
AM833	8.13	0.20	-67.8*	4.4	4	7.76	0.22	-52.6*	4.3	4	7.96	0.25	-52.8*	5.2	4	7.85	0.20	-65.3	4.5	4
Pramlintide	7.87	0.25	-78.9	6.2	4	7.73	0.23	-58.5*	4.9	4	7.47*	0.19	-56.2*	4.6	4	7.12*	0.18	-59.0	6.3	4
sCT	7.91	0.13	-94.3	4.6	5	8.09	0.12	-87.0	3.6	5	8.48	0.11	-81.6	2.7	5	8.54	0.16	-79.8	4.4	5
hCT	7.91	0.19	-92.7	6.1	5	7.93	0.22	-73.2	5.0	5	8.04	0.17	-73.1	4.7	5	8.48	0.30	-68.9	6.9	5
rAmy	8.04	0.21	-58.5*	3.9	5	7.99	0.18	-43.8*	2.9	5	7.36*	0.29	-36.9*	4.8	5	7.17**	0.42	-30.1*	6.4	5
AM1213	7.99	0.37	-63.1*	8.0	4	7.93	0.36	-49.9*	7.0	4	6.98**	0.44	-51.3*	12.1	4	6.93**	0.21	-56.1*	6.9	4
AM1784	7.74	0.15	-102.3	5.1	4	7.97	0.18	-83.6	5.1	4	8.41	0.15	-76.7	3.8	4	8.29	0.32	-89.7	9.2	4
AMY ₃ R Gi1 activation																				
AM833	8.12	0.19	-59.1*	3.9	4	7.88	0.19	-72.0	4.8	4	7.84	0.20	-61.6	4.5	4	8.50	0.29	-55.6	5.2	4
Pramlintide	8.04	0.24	-67.4*	4.8	5	9.02	0.18	-58.0*	3.1	5	7.49	0.28	-60.1	6.8	5	7.37	0.47	-59.0	12.5	5
sCT	7.85	0.14	-94.8	4.5	5	8.08	0.16	-91.4	4.5	5	8.26	0.15	-76.9	4.0	5	8.34	0.20	-68.2	5.2	5
hCT	7.74	0.17	-92.6	5.1	4	7.96	0.18	-83.4	4.9	4	8.10	0.19	-78.6	5.6	4	8.04	0.15	60.5	3.8	4
rAmy	8.17	0.38	-64.8*	6.2	4	8.52	0.27	-55.9*	3.7	4	8.26	0.38	-41.2*	4.3	4	8.26	1.03	-41.0*	11.4	4
AM1213	8.21	0.22	-57.8*	4.1	4	8.83	0.23	-54.3*	3.5	4	9.18	0.37	-38.3*	4.4	4	8.98	0.87	-32.2*	9.0	4
AM1784	8.06	0.20	-72.7*	4.9	4	7.93	0.23	-73.9	5.7	4	8.14	0.22	-65.4	5.2	4	8.12	0.25	-63.1	5.3	4

(continued)

TABLE 6—Continued

	2 min						4 min						10 min						20 min					
	pEC ₅₀			E _{max}			pEC ₅₀			E _{max}			pEC ₅₀			E _{max}			pEC ₅₀			E _{max}		
	Mean	S.E.M.	n	Mean	S.E.M.	n	Mean	S.E.M.	n	Mean	S.E.M.	n	Mean	S.E.M.	n	Mean	S.E.M.	n	Mean	S.E.M.	n	Mean	S.E.M.	n
CTR G11 activation																								
AM833	8.29	0.26	4	-60.5	3.90	4	8.39	0.24	4	-63.1*	3.7	4	8.15	0.23	4	-68.2	5.1	4	8.12	0.26	4	-71.2*	7.2	4
Pramlintide	7.77	0.22	4	-63.3	3.60	4	8.24	0.23	4	-62.0*	4.2	4	7.53*	0.26	4	-68.7	6.6	4	7.22*	0.30	4	-70.6*	9.30	4
sCT	8.14	0.37	4	-63.6	6.60	4	8.20	0.16	4	-84.8	4.1	4	8.56	0.09	4	-84.2	2.2	4	8.65	0.10	4	-91.9	2.8	4
hCT	8.56	0.25	4	-62.2	4.10	4	8.63	0.20	4	-71.2	3.8	4	8.71	0.26	4	-65.5	5.3	4	8.52	0.20	4	-71.6*	4.40	4
rAmy	8.04	0.26	4	-60.1	4.70	4	8.03	0.23	4	-55.7*	4.3	4	7.74	0.30	4	-54.8*	5.6	4	6.94*	0.25	4	-69.6*	9.9	4
AM1213	8.09	0.31	4	-52.4	4.60	4	8.25	0.36	4	-51.6*	5.4	4	7.32*	0.21	4	-57.3*	5.1	4	7.47*	0.39	4	-51.4*	9.70	4
AM1784	8.07	0.25	4	-64.2	4.60	4	8.04	0.18	4	-72.7	4	4	8.03	0.19	4	-85.0	5.2	4	8.40	0.15	4	-87.3	4.1	4
AMY ₂ R G11 activation																								
AM833	8.61	0.28	4	-66.0	4.8	4	8.14	0.24	4	-64.0	5.0	4	7.96	0.20	4	-60.6	4.5	4	8.09	0.28	4	-85.0	8.1	4
Pramlintide	9.12	0.33	4	-80.3	5.8	4	8.66	0.36	4	-72.0	6.6	4	8.83	0.30	4	-66.2	5.9	4	8.21	0.31	4	-83.8	9.6	4
sCT	7.88	0.19	4	-85.2	5.3	4	8.18	0.15	4	-77.8	4.0	4	8.39	0.15	4	-91.6	4.6	4	8.78	0.19	4	-82.7	5.9	4
hCT	8.01	0.33	4	-82.7	9.1	4	7.69	0.23	4	-76.6	6.6	4	7.96	0.23	4	-92.1	7.3	4	8.14	0.26	4	-84.0	8.4	4
rAmy	8.17*	0.29	4	-51.7	5.0	4	7.87	0.46	4	-49.1	8.0	4	7.06*	0.38	4	-49.7	10.7	4	7.52*	0.39	4	-39.6*	8.1	4
AM1213	8.36	0.24	4	-65.3	4.5	4	7.23*	0.27	4	-61.8	6.6	4	7.22*	0.24	4	-66.7	6.6	4	6.87*	0.24	4	-88.6	11.5	4
AM1784	8.05	0.22	4	-75.1	5.9	4	7.96	0.29	4	-78.0	8.0	4	8.09	0.15	4	-92.0	5.1	4	8.32	0.15	4	-103.6	5.7	4
AMY ₂ R G11 activation																								
AM833	7.72	0.27	4	-60.0*	5.1	4	7.97	0.24	4	-66.8	4.6	4	7.89	0.26	4	-61.5	5.7	4	7.91	0.29	4	-69.4	7.3	4
Pramlintide	8.00	0.32	4	-75.9	7.1	4	8.30	0.26	4	-76.1	5.3	4	7.94	0.30	4	-71.0	7.2	4	7.19*	0.41	4	-80.2	14.9	4
sCT	7.87	0.19	4	-85.1	4.8	4	8.15	0.20	4	-85.6	5.2	4	8.29	0.19	4	-79.2	5.2	4	8.72	0.16	4	-87.0	4.0	4
hCT	8.63	0.21	4	-58.2*	3.3	4	8.19	0.21	4	-69.0	4.7	4	8.53	0.23	4	-61.7	4.6	4	8.79	0.23	4	-64.1*	4.9	4
rAmy	8.12	0.20	4	-67.2	3.7	4	8.67	0.20	4	-58.8*	3.4	4	8.95	0.30	4	-46.3*	4.4	4	7.76*	0.29	4	-54.5*	6.2	4
AM1213	8.92*	0.31	4	-50.3*	4.2	4	8.32	0.25	4	-56.1*	4.7	4	8.15	0.21	4	-51.1*	4.1	4	7.22*	0.19	4	-61.6*	6.0	4
AM1784	7.89	0.32	4	-69.2	6.4	4	8.89*	0.34	4	-61.1*	5.1	4	8.00	0.17	4	-70.1	4.3	4	8.04	0.29	4	-76.1	7.6	4
CTR G12 activation																								
AM833	7.98	0.11	5	-57.0*	2.4	5	8.00	0.11	5	-75.2*	3.0	5	8.19*	0.11	5	-73.3*	3.1	5	8.11*	0.11	5	-74.8*	3.2	5
Pramlintide	8.02	0.14	5	-59.5*	3.2	5	7.94*	0.12	5	-72.9*	3.2	5	7.80*	0.12	5	-71.3*	3.3	5	7.39*	0.16	5	-73.5*	5.4	5
sCT	8.10	0.11	5	-82.9	3.0	5	8.42	0.11	5	-91.0	3.0	5	8.73*	0.10	5	-91.8	2.5	5	8.93*	0.07	5	-90.5	1.8	5
hCT	8.22	0.13	5	-65.7*	3.0	5	8.44	0.12	5	-74.2*	3.9	5	8.59	0.09	5	-76.3*	2.2	5	8.66*	0.09	5	-77.5*	2.2	5
rAmy	7.72	0.11	5	-51.8*	2.3	5	7.70*	0.15	5	-57.7*	3.4	5	7.29*	0.16	5	-60.8*	4.6	5	6.97*	0.15	5	-57.8*	5.2	5
AM1213	7.71	0.13	5	-51.4*	2.7	4	7.43*	0.13	5	-71.6*	3.9	4	7.11*	0.15	5	-78.9	6.5	4	6.90*	0.15	5	-79.1*	6.7	4
AM1784	7.99	0.13	4	-65.7*	3.4	4	8.26	0.16	4	-79.2	4.3	4	8.44	0.13	4	-83.5	3.5	4	8.61*	0.15	4	-87.4	4.0	4
AMY ₂ R G12 activation																								
AM833	8.16	0.13	4	-54.7*	2.7	4	8.12	0.11	4	-63.8*	2.6	4	8.37	0.12	4	-69.3*	2.7	4	8.58	0.10	4	-70.1*	2.4	4
Pramlintide	8.26	0.13	4	-62.6	2.8	4	8.06	0.14	4	-70.5	3.6	4	8.32	0.13	4	-70.1*	2.9	4	8.51	0.13	4	-70.5*	2.9	4
sCT	7.91	0.07	5	-73.0	2.1	5	8.18	0.10	5	-82.0	3.0	5	8.65*	0.09	5	-88.1	2.3	5	8.93*	0.08	5	-93.2	2.4	5
hCT	8.21	0.16	5	-61.4*	3.2	5	8.38	0.13	5	-65.6*	2.9	5	8.67*	0.14	5	-76.7*	3.3	5	8.74*	0.14	5	-79.4*	3.5	5
rAmy	8.01	0.15	5	-44.1*	2.5	5	8.10	0.15	5	-52.2*	2.8	5	8.22	0.16	5	-53.3*	3.0	5	8.31*	0.17	5	-56.4*	3.3	5
AM1213	7.81	0.15	4	-58.5*	3.6	4	7.93	0.16	4	-63.7*	3.9	4	7.94*	0.14	4	-68.2*	3.9	4	8.15*	0.13	4	-74.8*	3.8	4
AM1784	7.86	0.15	4	-65.4	3.8	4	8.02	0.15	4	-79.3	4.5	4	8.40*	0.10	4	-86.2	2.6	4	8.66*	0.12	4	-96.2	3.6	4
AMY ₂ R G12 activation																								
AM833	7.83	0.15	4	-54.7*	3.3	4	8.16	0.14	4	-72.2*	3.5	4	8.27	0.19	4	-73.3*	4.8	4	8.47*	0.20	4	-72.3*	4.8	4
Pramlintide	8.12	0.22	4	-55.5*	4.2	4	8.18	0.15	4	-67.2*	3.6	4	8.44	0.17	4	-62.2*	3.5	4	8.26	0.18	4	-59.4*	3.6	4
sCT	8.23	0.12	4	-75.1	3.1	4	8.50	0.12	4	-87.3	3.2	4	8.66	0.10	4	-96.4	2.8	4	8.83*	0.08	4	-92.8	2.3	4
hCT	8.19	0.12	4	-64.7	2.7	4	8.31	0.12	4	-76.5	2.8	4	8.56	0.10	4	-74.9*	2.2	4	8.70*	0.10	4	-74.8*	2.4	4
rAmy	8.06	0.10	4	-54.5*	2.1	4	8.23	0.15	4	-65.4*	3.4	4	8.06*	0.16	4	-60.3*	3.8	4	8.01*	0.16	4	-59.6*	3.8	4
AM1213	7.85	0.16	4	-50.5*	3.2	4	7.97	0.12	4	-67.8*	3.3	4	7.90*	0.13	4	-74.8*	3.7	4	7.60*	0.14	4	-82.1	4.6	4
AM1784	8.07	0.14	4	-65.4	3.5	4	8.31	0.15	4	-83.2	4.1	4	8.46	0.13	4	-87.8	3.7	4	8.60*	0.13	4	-90.8	3.5	4

Fig. 7. Trafficking of CTR-Rluc8 cotransfected with pcDNA (CTR), RAMP1 (AMY₁R), and RAMP3 (AMY₃R) in COS-1 cells. (A) Diagram of the localization of the subcellular BRET sensors. Concentration-response curves of BRET between CTR-Rluc8 and rGFP-CAAX (B), rGFP-FYVE (C), rGFP-Rab11 (D), and rGFP-Rab4 (E). Data were fit to a three-parameter logistic curve, except for AM1784 at Rab11, which was fit to a biphasic equation with the Hill slopes fixed to 1, which was determined as the best fit based on an *F* test. Values are means + S.E.M. from four to six independent experiments.

Fig. 8. Peptide potency and $E_{\max}$ values of CTR-Rluc8 intracellular trafficking upon cotransfection with pcDNA (CTR), RAMP1 (AMY₁R), and RAMP3 (AMY₃R) in COS-1 cells. Comparison of the log EC_{50} and $E_{\max}$ values of CTR-Rluc8 trafficking as part of CTR, AMY₁R, and AMY₃R as determined by BRET with the reporter rGFP-CAAX (A), rGFP-FYVE (B), and trGFP-Rab11 (C). Values were derived from the concentration-response data in Fig. 7, which were fit to a three-parameter logistic curve, except for AM1784 at Rab11, which was fit to a biphasic equation. The $E_{\max}$ value for AM1784 is based on the high-affinity site. Values are means $\pm$ S.E.M. from four to six independent experiments. Statistical comparisons were performed using two-way ANOVA, followed by Dunnett's post-test. Significant differences are reported from sCT (* P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001).

AMY₁R (Fig. 6C; Supplemental Fig. 7, A–C; Table 6). Moreover, although the magnitude of the change in BRET signal was similar at 2 minutes, differences in the maximal responses became evident at the later time points (Table 6).

G12 Activation. Of all the G proteins, peptide activation of G12 provided the clearest separation of the data into two phases of response, and this allowed for a more refined quantitative analysis of the distinction in peptide response at each of the individual receptors (Fig. 5D; Table 5). In the cumulative AUC response, only rAmy and pramlintide exhibited a change in

potency, with increased potency at AMY₁R and AMY₃R for both the high-potency and low-potency phases of response, although this was only significant for pramlintide at the AMY₃R for both phases and both peptides for AMY₁R for the lower-potency phase. Overall, sCT, hCT, and AM1784 had the most potent response for both phases and across the three receptors, whereas rAmy and AM1213 had the lowest potencies. AM833 was intermediate in potency between these peptide clusters, whereas pramlintide was closest to rAmy and AM1213 at the CTR and to AM833 at the AMYRs (Fig. 5D; Table 5).

TABLE 7

Peptide potency and $E_{\max}$ for the trafficking of CTR-Rluc8 alone (CTR) and upon cotransfection with RAMP1 (AMY₁R) or RAMP3 (AMY₃R)

Data were ligand-induced BRET fit to a three-parameter curve (except for AM1784 at Rab11, which was fit to a biphasic equation) with Hill slopes of 1, which was determined as the best fit based on an F test, and the high-affinity site is included in analyses. Values are means $\pm$ S.E.M. from four to six independent experiments. Statistical comparisons were performed using two-way ANOVA, followed by Dunnett's post-test. Significant differences are reported from sCT (* P < 0.05) or CTR ($\wedge P$ < 0.05).

	CTR					AMY ₁ R					AMY ₃ R				
	Log EC ₅₀		E _{max}		<i>n</i>	Log EC ₅₀		E _{max}		<i>n</i>	Log EC ₅₀		E _{max}		<i>n</i>
	Mean	S.E.M.	Mean	S.E.M.		Mean	S.E.M.	Mean	S.E.M.		Mean	S.E.M.	Mean	S.E.M.	
rGFP-CAAX															
AM833	-8.21	0.39	0.97**	0.12	6	-7.76	0.22	1.32	0.11	6	-7.85	0.24	0.83	0.08	6
Pramlintide	-7.32*	0.29	1.71**	0.21	6	-7.95	0.30	1.34	0.15	6	-7.90	0.26	1.39*	0.12	6
sCT	-8.07	0.30	1.69	0.18	6	-8.02	0.20	1.75	0.13	6	-7.83	0.23	1.37*	0.11	6
hCT	-7.96	0.22	1.77**	0.14	6	-7.93	0.23	1.53	0.13	6	-7.77	0.34	1.10 $\wedge$	0.13	6
rAmy	-6.70*	0.35	1.32	0.27	6	-7.42*	0.47	1.25	0.19	6	-7.93	0.37	0.96	0.11	6
AM1213	-7.00*	0.34	1.35	0.22	6	-7.48*	0.19	1.02	0.08	6	-7.41	0.40	0.96	0.15	6
AM1784	-8.51	0.33	1.31	0.13	6	-7.92	0.20	1.20	0.09	6	-8.20	0.23	0.99	0.08	6
rGFP-FYVE															
AM833	-9.00*	0.26	0.38	0.03	4	-9.12	0.41	0.39	0.04	4	-9.61	0.62	0.55	0.08	4
Pramlintide	-8.49*	0.26	0.37	0.03	4	-8.95	0.36	0.38	0.04	4	-8.96*	0.48	0.53	0.07	4
sCT	-9.77	0.35	0.45	0.04	4	-9.36	0.33	0.44	0.04	4	-10.04	0.48	0.53	0.06	4
hCT	-9.68	0.47	0.50	0.06	4	-9.05	0.40	0.51	0.06	4	-8.91 $\wedge$	0.43	0.60	0.08	4
rAmy	-8.71*	0.45	0.45	0.06	4	-9.87 $\wedge$	0.41	0.31	0.03	4	-8.98*	0.50	0.50	0.07	4
AM1213	-7.79*	0.31	0.48	0.06	4	-9.05 $\wedge$	0.40	0.38	0.04	4	-9.91 $\wedge$	0.48	0.49	0.06	4
AM1784	-8.94*	0.20	0.42	0.03	4	-8.93	0.36	0.44	0.05	4	-9.06*	0.44	0.44	0.06	4
trGFP-Rab11															
AM833	-10.74	0.30	0.81	0.07	6	-10.81	0.36	0.58	0.06	6	-10.35	0.31	0.94	0.08	6
Pramlintide	-9.75*	0.34	0.93	0.10	6	-10.72 $\wedge$	0.47	0.53 $\wedge$	0.07	6	-10.77 $\wedge$	0.43	0.66	0.08	6
sCT	-10.83	0.24	0.91	0.06	6	-10.88	0.44	0.52 $\wedge$	0.06	6	-10.82	0.49	0.90	0.13	6
hCT	-10.55	0.33	1.00	0.09	6	-10.27	0.33	0.78	0.08	6	-9.55 $\wedge$	0.39	1.07	0.13	6
rAmy	-9.13*	0.29	0.91	0.09	6	-9.77 $\wedge$	0.55	0.40 $\wedge$	0.07	6	-9.85 $\wedge$	0.46	0.65	0.10	6
AM1213	-9.04*	0.26	0.81	0.09	6	-9.82 $\wedge$	0.42	0.60	0.08	6	-9.95 $\wedge$	0.34	0.85	0.09	6
AM1784	-10.88	0.44	1.03	0.09	6	-11.12	0.74	0.73 $\wedge$	0.09	6	-10.67	0.51	1.03	0.11	6

Time-resolved analysis of the data revealed distinct patterns of effect on peptide potency over time that were also receptor-dependent. At the CTR, there were progressive increases in the potency of sCT, hCT, and AM1784 and decreases in the potencies of rAmy, pramlintide, and AM1213, whereas AM833 exhibited minimal change over time (Fig. 6D; Supplemental Fig. 7, D–F; Table 6). In contrast, at the AMY₁R, all peptides exhibited increased potency with time, although the changes were largest for sCT and AM1784 and smallest with rAmy and AM1213 (Fig. 6D; Table 6). The patterns of response at the AMY₃R were intermediate between that of the AMY₁R and CTR, with either minimal change or a small decrease in potency for rAmy and AM1213 but increased potency over time for the other peptides (Fig. 6D; Table 6). Although sCT elicited the largest, or equally largest, effect at all receptors, differences were observed in the magnitude of change in BRET signal with individual peptides that could be observed at 2 minutes but which was amplified or reduced at later time points in a peptide- and receptor-dependent manner (Supplemental Fig. 7, D–F; Table 6), reflecting additional differences in the nature of G12 activation.

Receptor Trafficking. The effect of the peptides on the trafficking of the CTR, AMY₁R, and AMY₃R was assessed by monitoring the interaction of the labeled CTR component over time with markers of different cellular compartments (Fig. 7A). Movement within the membrane and between compartments is a dynamic process and reflects the rates with which the receptors move, as well as the total number of receptors that are trafficking in response to different concentrations of peptide agonists.

In studies interrogating peptide-induced changes in the bystander BRET interaction between the plasma membrane

localized CAAX reporter and CTR, AM833, sCT, hCT, and AM1784 were significantly more potent than pramlintide, rAmy, and AM1213 in altering the BRET signal (Fig. 7B; Fig. 8A; Supplemental Fig. 8, A–C; Table 7), whereas pramlintide was more potent than rAmy, and AM1784 was more potent than hCT, within these major groupings. However, although AM833 had a similar potency to hCT and sCT, it exhibited a lower maximal response (Fig. 7B; Fig. 8A; Table 7). Potencies of AM833, sCT, hCT, and AM1784 were broadly similar between the CTR and AMYRs, although there was a small decrease in AM1784 potency at the AMY₁R, whereas pramlintide, rAmy, and AM1213 exhibited higher potencies at both AMYRs relative to CTR. However, only rAmy exhibited distinct potencies at the AMY₁R and AMY₃R, with highest potency at the latter receptor (Fig. 7B; Fig. 8A; Table 7). The magnitude of effect of individual peptides at each receptor was generally similar (Fig. 7B; Fig. 8A; Table 7).

Interestingly, the magnitude of response seen when the endosomal marker FYVE was used as the reporter was equivalent for all peptides, suggesting that either the level of receptor internalization is similar or that the equilibrium for receptors moving into and away from early endosomes is equivalent when stimulated by each of the peptides (Fig. 7C; Fig. 8B; Supplemental Fig. 9, A–C; Table 7). In contrast to the magnitude of response, the potencies of peptides to drive individual receptors into FYVE endosomes varied substantially in a peptide- and receptor-dependent manner (Fig. 7C; Fig. 8B; Table 7). sCT was the most potent peptide at the CTR, and also the AMY₃R, but exhibited a small decrease in potency at the AMY₁R, although this did not achieve significance. hCT was equipotent to sCT at the CTR but had lower potency at both AMYRs. Unlike the observed potency with the CAAX reporter, AM1784 had lower potency than sCT and hCT at the

A

CTR

B

AMY₁R

C

AMY₃R

Fig. 9. Potency web plots for CTR (A), AMY₁R (B), and AMY₃R (C). The potency of peptide response for each of the assays, expressed as pEC₅₀, is plotted on a log scale. pEC₅₀s for iCa²⁺ are displayed as the peak (P), or for data fit biphasically the high (H) and low (L) values are both plotted. The webs for AM833, sCT, and pramlintide are connected by solid lines. All other peptide webs are connected by a dashed line.

CTR, but its potency was not altered between receptor subtypes. AM833 had a similar potency to AM1784 at the CTR and AMY₁R but exhibited a small increase in potency at the AMY₃R. Pramlintide was less potent than AM833 and AM1784 at the CTR but displayed only small increases in potency at the AMYRs that did not achieve significance. rAmy demonstrated a selective increase in potency at the AMY₁R and was the most potent peptide in eliciting movement of this receptor into FYVE endosomes. The most dramatic differences in peptide response were observed with AM1213, which was the lowest-potency ligand at the CTR but exhibited increased potency at the AMY₁R that was further increased at the AMY₃R, where it had similar potency to sCT and AM833 (Fig. 7C; Fig. 8B; Table 7).

Monitoring of movement through recycling endosomes using the Rab11 marker revealed a second phase to the response for AM1784 only (Fig. 7D; Supplemental Fig. 10, A–C). Comparisons to AM1784 were to the first phase of this response only. For many peptides, the lowest peptide concentration (10^{-12} M) was often below the vehicle control (Supplemental Fig. 10, A–C), as such peptide effects were measured as AUC from the 10^{-12} M response. sCT and AM1784 elicited a similar potency and maximal response for all three receptors, whereas the responses to the other peptides varied between receptor subtypes (Fig. 7D; Fig. 8C; Table 7). AM833 had similar potencies at CTR and AMY₁R that were also equivalent to those of sCT and AM1784; however, it had reduced potency at the AMY₃R. rAmy and AM1213 had increased potencies at both AMYRs, relative to CTR, but similar potencies to each other at individual receptors. Pramlintide had an intermediate potency between sCT and rAmy at the CTR but had increased potency at the AMYRs, where it was equal in potency to sCT. The difference in potency between pramlintide and rAmy/AM1213 was maintained at all three receptors (Fig. 7D; Fig. 8C; Table 7). Interestingly, examination of the kinetic traces revealed that pramlintide had a distinct change in BRET relative to the vehicle when compared with other peptides (Supplemental Fig. 10, A–C). The mechanism behind this observation is not clear; however, one possibility may be a different equilibrium of receptor trafficking to and from this compartment for pramlintide relative to the other peptides.

The BRET signal for movement of receptors through sorting endosomes as monitored by the marker Rab4 was relatively weak and the potency and magnitude of responses too low to robustly quantify concentration-response data to the peptides (Fig. 7E; Supplemental Fig. 11, A–C). Nonetheless, the responses to AM833 and sCT were equivalent, and this contrasted with the response to pramlintide and rAmy that was lower at all three receptors and to AM1784, which had higher magnitude of response at the CTR and AMY₃R (Fig. 7E).

Discussion

AM833 or cagrilintide (the proposed International Non-proprietary Name) is an investigational new drug that recently successfully completed a phase 2 clinical trial for the treatment of obesity (<http://www.globenewswire.com/news-release/2020/06/18/2050266/0/en/Novo-Nordisk-successfully-completes-AM833-phase-2-trial-and-phase-1-combination-trial-with-AM833-and-semaglutide-in-obesity.html>; <https://>

<https://www.planion.com/Web/User/AbstractDet?ACCOUNT=TOS&ABSID=23842&CONF=OW2020&ssoOverride=OFF&CKEY=>). AM833 is closest in sequence similarity to pramlintide, with only three amino acid substitutions (N14E, V17R, and P37Y) and lipidation (C20-diacid- γ Glu) of the N-terminal lysine. Of these modifications, only the Pro37-NH₂ is a feature of CT peptides that terminate with Pro32-NH₂ (Supplemental Fig. 1). As a result of these changes, AM833 was reported as a nonselective agonist of CT and AMY receptors in canonical cAMP accumulation assays (<http://novonordisksciencehub.com/obesityweek2020/Amylin/Kruse#home>). These observations were replicated in the current study, although in this study, pramlintide was also a potent agonist at all three receptors and only displayed higher potency at the AMY₃R.

AMYRs are heterodimers of CTR and one of three RAMPs, and RAMPs have diverse effects on receptor function, including altering peptide binding affinity, signaling, and trafficking of receptors (Hay et al., 2005; Morfis et al., 2008; Gingell et al., 2014, 2020). The current study explores the pharmacology of AM833 in a broad range of assays, including binding, second messenger signaling, proximal G protein activation, and receptor trafficking, revealing that AM833 has a unique pattern of receptor responses compared with other peptides, which can be displayed visually as web plots of ligand potency for the pharmacological endpoints (Fig. 9, A–C).

The biphasic responses in cAMP accumulation observed in the present study are consistent with previous results with CTR, although this has not been reported with the AMYRs (Furness et al., 2016; Dal Maso et al., 2018). With the more complex fits, the differences between CTR and AMYRs are less pronounced; however, an induction of the expected AMY₁R and AMY₃R phenotypes could be observed with amylin peptides. The results of intracellular calcium mobilization are also consistent with previous studies in which hCT, rAmy, and sCT were shown to have similar potencies across CTR and AMYRs (Morfis et al., 2008; Dal Maso et al., 2018). However, despite the similarities in second messenger signaling, greater peptide- and receptor-dependent differences were observed with the measurement of more proximal G protein activation, which has not previously been examined for these receptors.

In the current study, using localized BRET sensors, peptide-induced internalization and intracellular trafficking of CTR-Rluc8 were observed with and without cotransfection with RAMPs. CTR has previously been shown to internalize, although studies with fluorescently labeled pramlintide and amylin indicate more limited internalization of the AMY₁R (Hilton et al., 2000; Dal Maso et al., 2018; Gingell et al., 2020; Zakariassen et al., 2020b). Therefore, future studies that follow the movement of RAMP1 would be valuable in discerning the specific contribution of AMYRs to the signal. Another factor that may complicate this analysis and affect assay sensitivity is the ability of CTR to constitutively internalize (Dal Maso et al., 2018). Nonetheless, the trafficking assays revealed interesting differences in the relative $E_{\max}$ of peptides. For example, sCT has a higher $E_{\max}$ than AM833 for the CAAX trafficking reporter at CTR and AMY₃R, which was not observed for the FYVE reporter. This may indicate differences in the ability of peptides to promote receptor redistribution in the membrane before internalization or in the total number of receptors that were internalized.

A confounding factor in the pharmacological characterization of AMYRs has been the inability to engineer a cellular system that comprises exclusively AMYRs without background expression of the GPCR component of the heterodimer, the CTR. Although selectivity of receptors has been demonstrated using selective radioligands such as [125 I]hCT or [125 I]rAmy (Hay et al., 2005; Morfis et al., 2008), determining the functional impact of RAMPs has been more difficult. In the current study, we have used real-time kinetic assays, and this has provided greater texture in analysis of responses. This has revealed patterns in changes to potency over time that are otherwise masked in summative or cumulative measures, particularly in the analysis of G protein activation. This provided greater separation of effects between receptors and peptides that allowed us to identify relatively subtle distinctions in the pharmacology of peptides, such as those between rAmy and AM1213 (Supplemental Fig. 12, E and F) or between AM833 and the clinically used peptides sCT and pramlintide (Supplemental Fig. 13, A–C).

Overall, AM833 was a nonselective agonist of CT and AMYRs across multiple assays of proximal and distal receptor signaling and receptor trafficking, albeit there were subtle differences in the kinetic response for activation of select G proteins (Supplemental Fig. 12A). sCT was the least selective peptide (Supplemental Fig. 12B), and AM1784 also had only minor differences in responses between receptor subtypes (Supplemental Fig. 12G). Despite the background CTR expression in all systems, there were significant reductions in hCT potency in the $i\text{Ca}^{2+}$ mobilization and FYVE and Rab11 responses at both AMYRs and selective reductions in potency with RAMP3 coexpression (AMY₃R) for Gs activation and with RAMP1 coexpression (AMY₁R) for G11 activation (Supplemental Fig. 12D). Although pramlintide was often more potent, pramlintide, rAmy, and AM1213 were all selective agonists of AMYRs, although peptide- and receptor-dependent differences could be observed in the G protein activation and trafficking assays (Supplemental Fig. 12, C, E, and F). These data collectively illustrate both the complexity of the pharmacology of CT family receptors and that each of the peptide agonists studied has a unique pharmacology.

One of the key features of the pharmacology of sCT is its slow off-rate from CT family receptors, which results in a long residency time and prolonged duration of action in vitro and in vivo (Michelangeli et al., 1983; Lutz et al., 2000; Andreassen et al., 2014b; Furness et al., 2016). Because of this property, analogs based on the sCT backbone have been developed with the aim of providing extended in vivo duration of action at CT and AMYRs (Mack et al., 2010; Gydesen et al., 2017b; Larsen et al., 2019). However, despite no causal cellular mechanism having been identified, salmon calcitonin-containing compounds have been associated with tumor growth. Indeed, sCT is restricted from long-term clinical use because of an unfavorable risk-benefit profile that is based on a weak link to cancer risk in meta-analyses of patients treated with sCT and the lack of a robust effect on osteoporosis (Overman et al., 2013). As such, we were interested in the residence time of AM833 and sCT on CTR and AMYRs.

AM833 exhibited a short residence time at CT family receptors that was similar to that of pramlintide. As has been previously reported, hCT and rAmy also had short residency times (Hilton et al., 2000; Furness et al., 2016), and this was also true of the amylin analog AM1213. In contrast, AM1784 had a very slow off-rate and long receptor residency time that

was at least as pronounced as that of sCT. We have previously used chimeras of sCT and hCT to identify the residues that were the key drivers for the slow off-rate of sCT. This revealed that the triplet of amino acid residues 11–13 (KLS in sCT; TYT in hCT) were sufficient to engender the major differences in off-rate kinetics (Hilton et al., 2000; Furness et al., 2016). In the amylin-like analogs, the triplet sequence is RLA, whereas in AM1784, the sequence is RLS, suggesting that the Ser¹³ could be required for formation of H-bonding that can contribute to the slow off-rate observed.

Interestingly, studies of the duration of action of the peptides in cAMP production revealed that, although there was some correlation with peptide off-rate, the binding kinetics of the peptides was not the sole contributor to persistence of response. These studies also revealed significant differences between peptide responses, despite similarities in potency in acute cAMP accumulation assays. The largest response was to saturating concentrations of sCT, AM1784, and hCT, the peptides most potent in the Gs activation assay at CTR (Fig. 3, A and B; Fig. 9A). In the ligand-washout studies, both the sCT and AM1784, with the longest residence times, had an extended duration of action that was not evident for the other peptides. The ability of sCT to stimulate higher cAMP production than hCT over extended periods of time is consistent with previous studies (Andreassen et al., 2014). However, the peak response to AM1784 was lower than that of hCT for both continuous stimulation and washout studies. This was potentially due to the small difference in AM1784 and hCT potency for Gs activation, but it may also be due to the substantial differences in potency of hCT and AM1784 in receptor trafficking assays. Although assays examining the potential of cAMP production from endosomal receptors have not been performed for CT family receptors, this is commonly seen for other class B1 GPCRs, including the glucose-dependent insulinotropic receptor and the parathyroid hormone receptor-1, and is likely to also be important in signaling from this pathway for CTRs (Ferrandon et al., 2009; Feinstein et al., 2011; Ismail et al., 2016).

Many analogs have been designed to engineer a longer residency time, but how this relates to therapeutic value in obesity treatment is unclear. Davalintide, an sCT-based nonselective agonist that binds almost irreversibly to AMYRs, entered phase 2 trials for obesity but showed no benefit over pramlintide, and development was discontinued (Mack et al., 2010; Williams et al., 2020). There is, nonetheless, preclinical evidence that dual activation of both CTR and AMYRs may be superior in reducing body weight, supporting the development of nonselective agonists (Andreassen et al., 2014; Gydesen et al., 2016; Larsen et al., 2020a). The nonselective agonists AM1784 and AM833 fit this profile of potential improved obesity therapeutics, irrespective of residency time. Another consideration is the effect of lipidation modification of these peptides, as little is known about long-acting nonselective CTR and AMYR agonists and how that may prolong/enhance the response. Currently, AM833 is the only one of these ligands that has been tested in humans. Initial results released from a phase 2 study of AM833 are promising, with AM833 promoting a 10.8% weight loss over 26 weeks of once-weekly dosing, compared with 3.0% with placebo (<https://tos.planion.com/Web/User/AbstractDet?ACCOUNT=TOS&ABSID=23842&CONF=OW2020&ssoOverride=OFF&CKEY=>). Also promising are the results of a recent

phase 1 combination trial of AM833 and semaglutide, in which after 20 weeks participants receiving the highest dose lost an average of 17.1% body weight (<http://www.globenewswire.com/news-release/2020/06/18/2050266/0/en/Novo-Nordisk-successfully-completes-AM833-phase-2-trial-and-phase-1-combination-trial-with-AM833-and-semaglutide-in-obesity.html>). The results of this phase 1 trial provide human evidence of the benefit of combining CTR/AMYR and GLP-1R agonists and indicates this may be a way forward for development of more efficacious therapeutics.

In summary, our data reveal complex pharmacology for the investigated series of AMYR and CTR peptide agonists. Each of the peptides has a unique profile of engagement with these receptors that is revealed by broad investigation of the binding, signaling, and regulatory properties of the ligands. AM833 is a promising investigational drug that has demonstrated robust efficacy in a phase 2 clinical trial for treatment of obesity, with enhanced benefit when coadministered with the GLP-1R agonist semaglutide in a recent phase 1 study (<http://www.globenewswire.com/news-release/2020/06/18/2050266/0/en/Novo-Nordisk-successfully-completes-AM833-phase-2-trial-and-phase-1-combination-trial-with-AM833-and-semaglutide-in-obesity.html>). These robust clinical effects indicate that the slow off-rate kinetics of sCT-like peptides is not required for efficacy in weight loss. The differences in signaling and regulation of target receptors, induced by AM833 relative to other peptides, provide important insight for optimization of the pharmacology of future drugs that target CT family receptors.

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