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Variable CGRP family peptide signaling durations and the structural determinants thereof

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Abstract

Calcitonin gene-related peptides alpha and beta (αCGRP, βCGRP), adrenomedullin (AM), and adrenomedullin 2/intermedin (AM2/IMD) function in pain signaling, neuroimmune communication, and regulation of the cardiovascular and lymphatic systems by activating either of two class B GPCRs, CLR and CTR, in complex with a RAMP1, −2, or −3 modulatory subunit. Inspired by our recent discovery that AM2/IMD(1-47) activation of CLR-RAMP3 elicits long duration cAMP signaling, here we used a live-cell cAMP biosensor assay to characterize the signaling kinetics of the two CGRP peptides and several bioactive AM and AM2/IMD fragments with variable N-terminal extensions. Remarkably, AM2/IMD(8-47) and AM2/IMD-53 exhibited even longer duration signaling than the 1-47 fragment. AM2/IMD(8-47) was a striking 8-fold longer acting than AM(13-52) at CLR-RAMP3. In contrast, the N-terminal extension of AM had no effect on signaling duration. AM(1-52) and (13-52) were equally short-acting. Analysis of AM2/IMD-AM mid-region chimeras and AM2/IMD R23 and R33 point mutants showed the importance of these residues for long-duration signaling and identified AM2/IMD peptides that exhibited up to 17-fold diminished signaling duration at CLR-RAMP3, while retaining near wildtype signaling potencies. βCGRP was ~3-fold longer acting than αCGRP at the CGRP (CLR-RAMP1) and the amylin$_1$ (CTR-RAMP1) receptors. Chimeric CGRP peptides showed that the single residue difference near the N-terminus, and the two differences in the mid-region,

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equally contributed to the longer duration of βCGRP signaling. This work uncovers key temporal differences in cAMP signaling among the CGRP family peptides, elucidates the structural bases thereof, and provides pharmacological tools for studying long-duration AM2/IMD signaling.

Keywords

RAMP; GPCR; prolonged cAMP signaling; long-acting agonist; BRET biosensor; peptide hormone

1. Introduction

The calcitonin/CGRP family of peptides in humans includes calcitonin (CT), amylin, calcitonin gene-related peptides alpha and beta (αCGRP, βCGRP), adrenomedullin (AM), and adrenomedullin 2/intermedin (AM2/IMD) [1]. These have diverse functions including, but not limited to, regulation of calcium homeostasis and bone turnover (CT), regulation of blood glucose and food intake (amylin), pain signaling and neuroimmune communication (CGRP), and regulation of the cardiovascular and lymphatic systems (CGRP, AM, and AM2/IMD) [2-6]. At least 7 cell surface receptors mediate the actions of this peptide family. The receptors comprise either of two related class B G protein-coupled receptors (GPCRs), the calcitonin (CTR) and calcitonin-like (CLR) receptors, either alone (CTR only) or in heterodimeric complexes with any of three receptor activity modifying proteins (RAMP1-3) that determine their peptide ligand selectivity [1, 7]. It is crucial to obtain a thorough understanding of the signaling mechanisms of the CGRP family peptides and their receptors because they control many fundamental physiological processes, and they are important drug targets. Antagonists of CGRP signaling are used to treat migraine headache [5] and an amylin receptor agonist showed promise in clinical trials for treating obesity [8]. There is also promise in targeting AM and AM2/IMD signaling for cardiovascular and other diseases [9-11], although no such therapeutics have reached the market to date.

The CGRP family peptide receptors efficiently couple to Gs to activate cAMP signaling [12-14]. The nomenclature of the receptors is thus largely based on our understanding of their molecular pharmacology obtained from end-point concentration-response cAMP accumulation assays performed in the presence of phosphodiesterase (PDE) inhibitor [1]. CLR-RAMP1 forms the CGRP receptor that is potently activated by CGRP and with lower potency by AM. CLR-RAMP2 and −3 are the AM_1 and AM_2 receptors, respectively, that are both potently activated by AM and weakly activated by CGRP. CTR is the CT receptor and its complexes with RAMP1, −2, or −3 form three amylin receptor subtypes, AMY_1R, AMY_2R, and AMY_3R, respectively. CGRP is also a potent agonist of the AMY_1R, but the functions of this pairing remain poorly understood [1, 15]. The α and β forms of CGRP differ by only 3 amino acids and they are thought to have very similar cAMP signaling pharmacology [1, 16]. AM2/IMD is the most recently discovered and least understood of the CGRP family peptides. It was thought to be a relatively non-selective agonist of the three CLR-RAMP complexes [1, 17, 18], however, by using a live-cell, real-time cAMP signaling assay in the absence of PDE inhibitor, we recently discovered that it elicits long duration signaling at the AM_2R [19]. We showed that the prolonged signaling resulted from a slow

receptor off-rate and hence long receptor residence time. Thus, AM2/IMD is kinetically selective for the AM_2R. The unique temporal aspects of the AM2/IMD-AM_2R cognate pair have significant implications for AM2/IMD biology and for drug development targeting the AM receptors.

Our prior study of the temporal aspects of cAMP signaling at the CLR-RAMP complexes focused on the AM2/IMD(1-47) peptide. We did not compare the two CGRP peptides nor the various bioactive forms of AM and AM2/IMD that have variable N-terminal extensions. The latter includes AM(13-52) and the (1-52) version bearing an N-terminal extension, and two additional AM2/IMD peptides that may arise from differential proteolytic processing of the AM2/IMD propeptide: AM2/IMD(8-47) with a deleted N-terminal extension, and AM2/IMD-53 with an additional 6-residue N-terminal extension on the 1-47 fragment [10, 17]. These extensions appear to have little if any effect on the pharmacology of AM2/IMD at cloned receptors *in vitro*, but there are hints in the literature that they may alter its pharmacology *in vivo* [10, 17, 20, 21]. Here, we asked if any of these other CGRP family peptides have unique temporal features of their cAMP signaling that have been missed in end-point concentration-response cAMP accumulation assays. We report that the N-terminal extensions of AM2/IMD altered its signaling kinetics resulting in changes in signaling duration, and that βCGRP exhibited longer duration signaling than αCGRP. We defined the elements of βCGRP responsible for its longer signaling and identified AM2/IMD variants deficient in long-duration signaling. This work reveals important temporal differences in cAMP signaling among the CGRP family peptides, elucidates the structural bases thereof, and provides pharmacological tools for future studies seeking to define the functions of long-duration AM2/IMD signaling.

2. Materials and Methods

2.1 Cell Culture

COS-7 cells (CRL 1651) were from American Type Culture Collection (Manassas, VA, USA). COS-7 cells were used because they do not endogenously express CTR, CLR, or RAMPs. Previous work showed that the AM2/IMD(1-47) cAMP signaling duration properties were similar in COS-7 and HEK293 cell lines [19]. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM with 4.5 g/L glucose and L-glutamine and 110 mg/L sodium pyruvate) from Gibco (11995-081) (Thermo Fisher Scientific; Waltham, MA, USA) with 10 % v/v fetal bovine serum (Gibco 16000-044). Cells were grown at 37°C, 5% CO_2 in a humidified incubator and passaged twice per week with cells utilized between passages 5 and 30 for assays.

2.2 Plasmid constructs

Mammalian expression plasmids encoding human CLR, $CTR_{(a)}$, or RAMP1-3 in the pcDNA3.1(+) vector were from cDNA resource center. The CAMYEL cAMP biosensor plasmid was previously described [19, 22]. The HiBiT-CLR plasmid (pKB009) used to compare cell-surface expression levels of the three CLR-RAMP complexes was constructed in the pcDNA3.1(+) vector using traditional cloning techniques and included the HiBiT sequence (Promega; Madison, WI, USA) followed by a GSSGGSSG linker inserted after the

signal peptide. Plasmids for transfections were prepared using the Machery-Nagel midi-prep kit (Machery-Nagel; Allentown PA) according to the manufacturer's directions.

2.3 Synthetic peptides

All peptides were the human sequences unless specified otherwise. αCGRP(1-37), βCGRP(1-37), AM(13-52), AM(1-52), AM2/IMD(1-47), and rat Amylin(1-37) were purchased from Bachem (Bubendorf, Switzerland). AM2/IMD-53 was purchased from Phoenix Pharmaceuticals (Burlingame, CA). The αCGRP(8-37) [N31D/S34P/K35W/A36S] and AM(22-52) [S48G/Q50W] antagonists, and the AM-AM2-AM and AM2-AM-AM2 mid-region chimeras were previously described [19, 23]. Peptides custom synthesized and HPLC-purified by RS Synthesis (Louisville, KY) or Biosynth (Gardner, MA) included the following: AM2/IMD(8-47), AM2/IMD(8-47) [R23K], AM2/IMD(8-47) [R33K], AM2/IMD(8-47) [R23K/R33K], AM2/IMD(8-47) [R23Q/R33K], α-βCGRP(1-37) chimera, β-αCGRP(1-37) chimera, and the AC187 antagonist. The lyophilized powders were resuspended at ~10 mg/mL in sterile ultrapure water and the concentrations were determined by UV absorbance at 280 nm with dilutions in 10 mM Tris-HCl, 1 mM EDTA at pH 8.0. Extinction coefficients were calculated from Tyr, Trp, and cystine content. The concentrations of the CGRP peptides lacking Tyr and Trp residues were determined based on the peptide content reported from Bachem or an assumed 80% peptide content for custom-synthesized peptides. Resuspended peptides were stored at −80°C as multiple small aliquots to limit the number of freeze-thaws.

2.4 Real-time BRET cAMP signaling kinetics biosensor assay with antagonist challenge

This assay for the three CLR-RAMP complexes transiently expressed in COS-7 cells was performed at room temperature as previously described using 100 nM agonist stimulation followed by 10 μM antagonist challenge [19]. The antagonists used were αCGRP(8-37) [N31D/S34P/K35W/A36S] for CLR-RAMP1 and AM(22-52) [S48G/Q50W] for CLR-RAMP2/3 [23]. For the CTR-RAMP1 (AMY_1R) experiments, the COS-7 cells were transiently transfected with 25 ng CTR, 25 ng RAMP1, 125 ng CAMYEL biosensor, and 75 ng empty pcDNA3.1(+) using PEI as previously described [19]. The cells were stimulated with 10 μM coelenterazine h (NanoLight; Pinetop, Arizona) and 1 nM of the indicated agonists for 15 min followed by challenge with 10 μM AC187 antagonist or buffer control and additional readings for ~60 min. The 475/535 BRET ratio was used to plot the data over time with buffer control subtracted from each curve. The decay phase after antagonist addition was fit by nonlinear regression to a one-phase exponential decay model in GraphPad Prism v9.4.1 (GraphPad Software). The Y_0 value was constrained to be equal to the last point on a linear regression line fit to the data for the five minutes prior to antagonist addition. For the AMY_1R data, the curves were also constrained to plateau at 0 because of the very slow decay rates observed at this receptor.

2.5 End-point concentration-response BRET cAMP biosensor assay

COS-7 cells were seeded at a density of 20,000 cells/well with 100 μL/well in a 96-well white plate and incubated for 24 hrs at 37°C and 5% CO_2. On day two, the cells were transiently transfected with 250 ng total DNA and 375 ng of branched polyethylenimine (PEI) per well. 25 ng receptor, 25 ng RAMP, 125 ng CAMYEL biosensor, and 75 ng empty

pcDNA3.1(+) was used. 48 hrs after transfection, the cells were washed with PBS and then incubated for 30 min in 80 μL of sterile filtered assay buffer of 25 mM NaHEPES pH 7.4, 104 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 1 mg/mL fatty-acid-free bovine serum albumin, and 5 mM glucose. PEI and general chemicals were from MilliporeSigma (St. Louis MO). This was followed by addition of 10 μL Coelenterazine h at 5 μM and 10 μL 10x peptide serial dilutions. The stimulated cells were then incubated for 30 min and the emissions at 475 and 535 nm were read in a PolarSTAR Omega plate reader (BMG Labtech, Ortenberg, Germany) using a dual luminescence optic. Each of the incubations was performed at 37°C and the plate reader was warmed to 37°C. The peptide concentration was plotted against the 475/535 BRET ratio and the buffer control was subtracted from the data set (depicted as V on the x-axis). The curves were fit by nonlinear regression to a three-parameter (for CLR) or a four-parameter (for CTR) log concentration versus response equation in GraphPad Prism v9.4.1 (GraphPad Software; La Jolla CA).

2.6 Real-time BRET cAMP signaling kinetics biosensor assay with agonist washout

COS-7 cells were seeded and transfected as above. 48 hours after transfection the cells were washed with 1X PBS and incubated with 40 μL of assay buffer of 25 mM NaHEPES pH 7.4, 104 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 1 mg/mL fatty-acid-free bovine serum albumin, and 5 mM glucose at room temperature for 30 min. The cells were then stimulated with 10 nM agonist in the presence of 10 μM coelenterazine h for 20 min in 50 μL total volume. 475 and 535 nm luminescence was read in a PheraStar FS platereader (BMG labtech) for the final 10 min to establish the agonist-stimulated baseline. The agonist-containing solution was then manually aspirated from the cells followed by two washes with 100 μL of buffer containing substrate. 50 μL of buffer with substrate was added back and the reading was continued for ~ 50 min. The BRET ratio 475/535 was plotted over time in GraphPad Prism with the buffer control (no agonist) subtracted.

2.7 Cell surface expression of CLR-RAMP complexes

Cell surface expression of the three HiBiT-CLR-RAMP complexes was compared using the LgBiT reagent (Promega), which upon binding HiBiT reconstitutes nanoluciferase. COS-7 cells were seeded at 20,000 cells/well in a white 96-well plate. 24 hours after seeding, the cells were transfected with 25 ng HiBiT-CLR, 25 ng RAMPs, and 200 ng empty vector. On day 4, the cells were washed with PBS and incubated with 1/200 LgBiT reagent and 1X furimazine per Promega's instructions for 30 min. 460 nm luminescence was read on a PheraStar FS platereader (BMG Labtech).

2.8 Statistical Analysis

For each assay, duplicate technical replicates were used and data points are shown as mean ± SD in the representative plots. All experiments were performed with three independent replicates on different days and the parameters of interest (e.g. pEC_{50}, $t_{1/2}$) were reported as mean ± SEM. Statistical analysis was performed using GraphPad Prism on the log form of the values with one-way ANOVA and Tukey's post hoc test with a confidence interval of 95% reaching statistical significance of $p<0.05$ comparing the mean of the three independent

replicates. Only the most important comparisons were shown in figures. Pearson correlation analyses were performed in GraphPad Prism.

3. Results

3.1 N-terminal extensions of AM2/IMD alter its cAMP signaling kinetics

We tested the hypothesis that the AM2/IMD N-terminal extensions (Fig. 1A) modulate its cAMP signaling kinetics. cAMP was measured in real-time using the BRET cAMP biosensor CAMYEL for the CGRPR, AM_1R, and AM_2R transiently expressed in COS-7 cells. The receptor-expressing cells were stimulated with the three different AM2/IMD peptides for 15 min, followed by challenge with an excess of high-affinity, competitive peptide antagonist. The cAMP signal decay after antagonist addition provides a measure of signal duration and may be a proxy for agonist dissociation [19]. The decay phase was fit to a one-phase exponential decay model as previously described, to determine the decay rate and half-life for each agonist [19]. The shortest AM2/IMD fragment, AM2/IMD(8-47), exhibited ~ 2-3-fold longer signaling duration at all three receptor complexes than the 1-47 fragment (Fig. 1B-E; Table 1). The 8-47 duration surpassed even that of αCGRP(1-37) at CLR-RAMP1 and AM(13-52) at CLR-RAMP2, and its decay half-life at CLR-RAMP3 ($t_{1/2}$ ~ 20 min) was 8-fold longer than that of AM ($t_{1/2}$ ~ 2.5 min). Interestingly, AM2/IMD-53 with the longest N-terminal extension also had a signaling duration longer than 1-47 at each receptor, but slightly shorter than 8-47 (Fig. 1B-E; Table 1). These results indicated that the N-terminal extensions of AM2/IMD modulated its cAMP signaling kinetics with the rank order of signaling duration: AM2/IMD(8-47) > AM2/IMD-53 > AM2/IMD(1-47).

Next, we compared the cAMP signaling potencies of the three AM2/IMD fragments in end-point concentration-response assays using the CAMYEL cAMP biosensor. They exhibited similar potencies at the AM_1 and AM_2 receptors, but differed at the CGRP receptor where the potency rank order was AM2/IMD(8-47) > AM2/IMD-53 > AM2/IMD(1-47) and the 8-47 fragment was ~10-fold more potent than 1-47 (Fig. 1F-I; Table 1). Notably, despite their unique signaling kinetics, the AM2/IMD peptides were not more potent than CGRP at CLR-RAMP1 or AM at CLR-RAMP2/3.

We compared the cell surface expression levels of the three CLR-RAMP complexes in the transiently transfected COS-7 cells using a HiBiT-tagged CLR construct to take advantage of the LgBiT reagent (Promega) that allows detection of cell surface receptor by a simple luminescence reading. The three complexes were expressed at levels that differed from each other with statistical significance, however, their expression levels were within ~2-fold or less variation of each other (Fig. 2). These data strongly suggested that the differences in signaling duration we observed were not a result of drastically different receptor expression levels.

3.2 Structural determinants of AM2/IMD long-duration signaling

Using AM and AM2/IMD chimeras, we previously showed that the mid-region of AM2/IMD, residues 23-33, was necessary for its long-duration cAMP signaling at AM_2R (CLR-RAMP3) [19]. Structural analyses and molecular dynamics simulations suggested that

AM2/IMD R23 and R33 at either end of this segment formed salt-bridge and hydrogen-bond interactions, respectively, that were important for the slow off-rate and long duration signaling. Here, we directly tested this hypothesis through mutagenesis of R23 and R33 to define the structural basis of the long-duration signaling.

First, we tested R23Q/R33K and R23K/R33K double mutants in the AM2/IMD(8-47) backbone. The former changed the Arg residues to those found at the equivalent positions in the AM peptide, and the latter was designed to maintain a positively charged side chain at both positions, while altering the hydrogen-bonding potential. Both double mutants had significant decreases in signaling duration as reflected by their decreased half-lives at each CLR-RAMP complex in the real-time signaling assay, most dramatically at CLR-RAMP3, where the half-lives were less than or equal to that of AM(13-52) (Fig. 3A-D; Table 2). The double mutants were also tested in the concentration-response assay to assess their potencies. The mutants displayed decreased potencies at each receptor complex, with the R23Q/R33K variant being more negatively impacted than R23K/R33K (Fig. 3 E-G; Table 2). The R23K/R33K double mutant was ~10-fold less potent than wildtype at the CGRP and AM_1 receptors, but it had only ~3-fold decreased potency at the AM_2 receptor.

Next, we tested R23K and R33K single mutants. In the real-time cAMP signaling assay, both single mutants had diminished signaling duration at each receptor complex, albeit less diminished than the double mutants (Fig. 3I-L; Table 2). R33K trended towards being more negatively affected than R23K at the AM_2R, but this did not reach statistical significance. The R33K mutant did not completely lose long-duration signaling at the AM_2R, but its signal decay half-life was diminished 3.4-fold compared to wildtype. Notably, the single mutants retained near wildtype signaling potencies at the CGRP and AM_1 receptors and wildtype potency at the AM_2 receptor (Fig. 3M-P; Table 2). These mutagenesis results prove the critical roles of R23 and R33 in the mid-region of AM2/IMD for long-duration cAMP signaling at AM_2R.

3.3. Identification of AM2/IMD variants that may be useful pharmacological tools

The results with the R23 and R33 point mutants indicated that some AM2/IMD variants can exhibit diminished signaling duration while retaining near normal signaling potency. We thus sought to compare the signaling durations and potencies of a series of AM2/IMD variants to assess which might be useful pharmacological tools for studying the biological effects of long-duration signaling. To have a larger number of variants for a correlation analysis, we first re-visited the two AM2/IMD-AM mid-region chimeras for which we previously reported their cAMP signaling durations, but did not determine their signaling potencies [19]. These are the AM2-AM-AM2 chimera, which is AM2/IMD(8-47) with residues 23-33 replaced with the equivalent mid-region of AM, and the AM-AM2-AM chimera, which is AM(13-52) with its mid-region replaced with residues 23-33 of AM2/IMD. Here, we examined their potencies in the end-point concentration-response cAMP biosensor assay (Fig. 4A-D; Table 3). The two chimeras were equipotent at the CGRPR, but were ~8-fold less potent than AM2/IMD(8-47). At the AM_1R, AM-AM2-AM was equipotent to AM2/IMD(8-47) and AM2-AM-AM2 exhibited more diminished potency. The two chimeras exhibited similar potencies at the AM_2R that were only slightly weaker

than that of AM2/IMD(8-47). To facilitate comparisons among the variants, the signaling duration half-lives of the chimeras determined in our prior study are reproduced in Table 3. AM2-AM-AM2 exhibited a dramatic loss-of-function in signaling duration at AM_2R, whereas AM-AM2-AM had a gain-of-function at both AM receptors [19].

A Pearson correlation analysis was performed for each CLR-RAMP complex to compare the signaling durations and potencies of the AM2/IMD peptides from this study, including the two mid-region chimeras. In addition, αCGRP was included for the CGRPR and AM(13-52) for the two AM receptors. This revealed a positive correlation between agonist cAMP signaling duration and potency at the CGRPR with r =0.67 (Fig. 5A). In contrast, relatively poor correlations were observed at the two AM receptors with r= 0.36 for AM_1R (Fig. 5B) and r=0.23 for AM_2R (Fig. 5C). Variants away from the linear regression trendline are those for which signaling duration and potency may be decoupled. Most notable in this regard were AM2/IMD(8-47) R23K/R33K and the AM2-AM-AM2 chimera at the AM_2R, which had signaling potencies within ~3-fold or less change from the wildtype AM2/IMD peptides, but had signaling durations that were ~8-fold and ~17-fold diminished, respectively, as compared to AM2/IMD(8-47) or AM2/IMD-53 (Fig. 5C). Also notable was the AM-AM2-AM chimera, which had >10-fold longer signaling duration at both AM receptors as compared to AM(13-52) despite having >10-fold weaker signaling potency (Fig. 5B, C).

3.4 The N-terminal extension of AM has no effect on its cAMP signaling kinetics

The two AM agonists commonly in use, the 1-52 peptide and the truncated 13-52 fragment, have equal potencies in concentration-response cAMP accumulation assays [24]. Given that the N-terminal extensions of AM2/IMD altered its cAMP signaling kinetics, we asked if the N-terminal extension of AM could similarly alter its cAMP signaling duration. In stark contrast to the results with AM2/IMD, we observed no detectable difference in the cAMP signaling duration between AM(1-52) and AM(13-52) at each of the three CLR-RAMP complexes in the real-time signaling assay (Fig. 6A-D; Table 4). These data indicated that the N-terminal extension of AM has no effect on its cAMP signaling kinetics, further differentiating the structural bases of AM and AM2/IMD pharmacology.

3.5 βCGRP exhibits longer duration cAMP signaling than αCGRP at two CGRP receptors

The α and β CGRP peptides (Fig. 7A) are equipotent for cAMP signaling at the human CGRPR (CLR-RAMP1) [1, 16, 25]. In radioligand binding studies, they exhibited very similar equilibrium binding affinities, with βCGRP perhaps being slightly higher affinity than the α form [25-27]. To our knowledge, neither a receptor binding kinetics nor signaling kinetics comparison of α and βCGRP has been reported. In the real-time cAMP signaling assay, βCGRP exhibited an ~3-fold longer duration of cAMP signaling ($t_{1/2}$ ~ 5.1 min) than αCGRP ($t_{1/2}$ ~ 1.8 min) at the CGRP receptor (CLR-RAMP1) (Fig. 7B, E; Table 5). In contrast, the two CGRP peptides exhibited no detectable difference in signaling duration at the two AM receptors (Fig. 7C-E). We then sought to determine which region of βCGRP was responsible for the longer half-life at the CGRPR by testing the signaling duration of two chimeras. The α-βCGRP chimera had the N-terminal region (position 3 difference) from αCGRP and the mid-region (positions 22 and 25 differences) from βCGRP

and the β-αCGRP chimera had the opposite arrangement (Fig. 7A). The cAMP signaling duration for these chimeras was between that of αCGRP and βCGRP with similar half-life values of 2.4 min for α-βCGRP and 2.7 min for β-αCGRP (Fig. 7F, G; Table 5). These results indicated that the single amino acid difference in the N-terminal region and the two differences in the mid-region of βCGRP contributed approximately equally to its longer half-life at CLR-RAMP1.

CGRP is also a potent agonist of the amylin receptor AMY_1R (CTR-RAMP1), so we asked if the longer duration signaling of βCGRP also occurs at AMY_1R. Studying the pharmacology of the AMY_1R is challenging because unlike the CGRPR, co-expression of CTR and RAMP1 likely results in a mixed cell surface receptor population of free CTR and CTR-RAMP1 heterodimer and each of these species can contribute to downstream signals such as cAMP [1]. To minimize this complication and target primarily the CTR-RAMP1 complex, we sought to identify agonist concentrations for use in the signaling duration assay that would activate the AMY_1R, but not CTR. End-point concentration-response assays indicated that 1 nM of the two CGRP peptides robustly activated the AMY_1R, but not CTR alone (Fig. 8A). Similar results were observed for the control agonist rat amylin (rAmy), although 1 nM rAmy did activate CTR to a low level (Fig. 8B). The two CGRP peptides and rAmy were equipotent when tested with cells co-expressing CTR and RAMP1 (Fig. 8C).

In the real-time cAMP signaling duration assay using 1 nM agonist stimulation followed by challenge with the AMYR-selective peptide antagonist AC187 [15, 28], βCGRP exhibited a signaling duration ($t_{1/2}$ ~ 63 min) that was similar to rAmy and ~2.5-fold longer than αCGRP ($t_{1/2}$ ~ 25 min) at the AMY_1R (Fig. 8D and E; Table 5). The half-lives for the signaling decay of the two CGRP peptides at the AMY_1R (CTR-RAMP1) were an order of magnitude longer than at the CGRPR (CLR-RAMP1). The basis for the apparent longer signaling duration of the CTR-based RAMP1 complex is unclear. Regardless, these data indicated that βCGRP exhibits an approximately 3-fold longer signaling duration than αCGRP at the AMY_1R in addition to the CGRPR.

3.6 AM2/IMD and βCGRP are long-acting in a washout assay

Last, as an alternative way to measure AM2/IMD and βCGRP cAMP signaling duration, we examined their behavior in a real-time cAMP biosensor assay in which the decay phase was initiated by agonist washout rather than antagonist challenge. This was done to eliminate any possible confounding factors resulting from agonist/antagonist competition at the receptor and to mimic the *in vivo* situation in which the peptides would be diluted in the extracellular space or circulation upon receptor unbinding. Importantly, the washout assays confirmed that AM2/IMD(8-47) was longer-acting than AM(13-52) at the AM_2R (CLR-RAMP3) (Fig. 9A) and that βCGRP was longer acting than αCGRP at the CGRPR (CLR-RAMP1) (Fig. 9B).

4. Discussion

Inspired by our recent discovery that AM2/IMD(1-47) elicits long-duration cAMP signaling via the AM_2R (CLR-RAMP3) [19], here we performed an analysis of the temporal aspects of cAMP signaling for several other CGRP family peptides. This resulted in novel findings

including that the N-terminal extensions of AM2/IMD modulated its signaling kinetics, whereas that of AM had no effect. In addition, βCGRP was found to exhibit longer duration signaling than αCGRP. Using amino acid-substituted variants or chimeras, the structural determinants of cAMP signaling duration were identified. Our results revealed considerable variability in the signaling duration capabilities of αCGRP, βCGRP, AM, and AM2/IMD and their structural determinants of signaling duration as summarized in Fig. 10. At their cognate CLR-RAMP complexes the three AM2/IMD peptides and βCGRP are long-acting to varying degrees, and the two AM peptides and αCGRP are short-acting.

The AM2/IMD(1-47) peptide is produced in human brain, kidney, and plasma [29]. Differential proteolytic processing of the AM2/IMD propeptide may give rise to the AM2/IMD(8-47) and AM2/IMD-53 peptides [10, 17, 30], but to our knowledge it remains unclear if these exist *in vivo.* Here, we discovered that the shorter AM2/IMD(8-47) and longer AM2/IMD-53 peptides both elicited longer duration cAMP signaling than the long-acting AM2/IMD(1-47) fragment (Fig. 1B-E, 10A). The three peptides had similar potencies at the two AM receptors, but the 8-47 fragment was ~ 10-fold more potent than 1-47 at the CGRP receptor (Fig. 1F-I). These findings are generally consistent with prior work comparing the potencies of the 1-47 and 8-47 peptides, although disparate results have been reported for their activities at the CGRP receptor (CLR-RAMP1). Like our results, Roh et al. reported that 8-47 was ~10-fold more potent than 1-47 using the SK-N-MC cell line, which endogenously expresses the CGRP receptor [18]. In contrast, Musa et al. found that they were equipotent at the CGRP receptor transiently expressed in COS-7 cells [31]. The basis for this discrepancy is unclear, but it could be due to the use of different cAMP assay methods or perhaps different receptor expression levels. Overall, these results support that the three AM2/IMD peptides are not equivalent in their cAMP signaling pharmacology. All three peptides are routinely employed in studies, but they are rarely compared in the same study and many studies fail to clearly state which version was used. Unfortunately, this makes it hard to determine if their cAMP signaling duration differences correlate with different effects *in vivo.*

Our prior study showed that the mid-region of AM2/IMD, residues R23 to R33, was a critical contributor to its long duration signaling [19]. MD simulations indicated that this region formed more stable interactions with CLR than the mid-region of AM. AM2/IMD R23 formed an intermolecular salt-bridge with D96 in the CLR extracellular domain (ECD) and AM2/IMD R33 formed an intermolecular hydrogen-bond with the CLR ECD D90 main-chain and intramolecular hydrogen-bonds to the end of the α-helical segment of the peptide. These were proposed to be important for the slow AM_2R off-rate and long-duration signaling of AM2/IMD, but experimental validation was lacking. Our results here showing that single and double Lys substitutions of these Arg residues severely diminished signaling duration (Fig. 3, 10A), provided proof of their important roles in long-duration signaling, and validated the prior MD simulations. The Gln and Lys residues at the equivalent positions in the AM mid-region are likely significant contributors to its short-acting phenotype (Fig. 10B). While the structural basis for the AM2/IMD mid-region effect on signaling duration is now clearer, it is not obvious how the N-terminal extensions modulate duration nor why the N-terminal extension of AM has no effect on its signaling duration (Fig. 6, 10B). The extensions are typically not visible in experimentally determined structures suggesting that

they are flexible and largely disordered. Elucidation of the unbinding pathways of these peptides may be required to understand the structural basis of the effects of the N-terminal extensions on signaling duration, but this is not a trivial task.

The AM2/IMD Arg to Lys mutants and the AM2/IMD-AM mid-region chimeras may be useful pharmacological tools for deciphering the effects of long-duration signaling at the AM receptors, particularly in cell culture models where the CLR-RAMP complex of interest is known. AM2/IMD R23K/R33K and AM2-AM-AM2 both had severely diminished signaling duration at the AM_2R while having only a minor decrease in potency (Fig 5C). When compared to wildtype AM2/IMD at this receptor, different downstream outcomes may be attributable to their different signaling durations. However, these tools should be used with care because there is evidence that the CGRP and AM peptides can exhibit signaling bias [32] and this could complicate the interpretation of results with mutated peptides. We cannot rule out the possibility that our variants have altered bias profiles. In this respect, the R23K/R33K variant may have an advantage in being more minimally and conservatively altered than AM2-AM-AM2, although the latter has the advantage of a shorter signaling duration. The AM-AM2-AM chimera had a substantially longer signaling duration than AM at both AM receptors and was even longer-acting than AM2/IMD at AM_2R (Fig. 5B, C). This peptide may be useful to explore the effects of gain-of-function signaling duration at the AM_1R or AM_2R. These tools may also be useful in more complex situations such as cell culture systems expressing multiple receptors or animal models, but even more care will be needed because the altered pharmacology of the variants is not limited to one receptor. Nonetheless, they may still be useful in these situations when combined with the use of receptor selective antagonists, where available.

αCGRP is more well-studied than βCGRP, perhaps in part due to the fact they only differ by 3 amino acids and were thought to be pharmacologically near equivalent. The two CGRP peptides differ in their expression profiles, at least in rodents where βCGRP is more prevalent in enteric neurons [33]. One reason for two CGRP peptides could be to support different functions based on their different locations. Indeed, a recent study showed that βCGRP, rather than αCGRP, played a key role in enteric neurogenic inflammation caused by *C. difficile* infection [34]. The different signaling duration capabilities of the two CGRP peptides at the CGRP receptor (Fig. 7, 10C) provides another rationale for the two versions. βCGRP may be needed to support certain functions that require a longer duration signal. The longer duration signaling of βCGRP also carried over to the AMY_1 receptor (Fig. 8), at which it was equivalent to the agonist rat amylin. Structurally, this may reflect, in part, the fact that these peptides share an Asn residue at position 3 because the CGRP chimera results indicated that this residue contributed to the longer duration βCGRP signaling. The significance of different durations of the two CGRP peptides at AMY_1R is unclear because the physiological relevance of these ligand-receptor pairings is unknown.

The long signaling durations of the three AM2/IMD peptides at the AM_2R are likely the result of slow receptor off-rates that result in extended receptor occupancy and hence prolonged signaling. This is supported by our previous study of AM2/IMD(1-47) where the receptor off-rate measured in direct binding experiments appeared to be in reasonable agreement with the cAMP signal decay rate observed in the real-time signaling assay [19].

The different signaling durations of the two CGRP peptides at the CGRP (CLR-RAMP1) and AMY_1R (CTR-RAMP1) receptors probably also reflect differences in their receptor off-rates, but it is notable that the cAMP signaling duration of the CTR-based complex was significantly longer than the CLR-based RAMP complexes (compare Fig. 7B and 8D). It is possible that for the CTR-RAMP1 complex the cAMP signal decay observed upon antagonist challenge is not a good proxy for agonist dissociation. Indeed, a recent study showed that some AMYR agonist peptides elicited hours long cAMP signaling durations despite having only minutes long receptor residence times [14]. This apparent disconnect may warrant future study.

The previously unappreciated differences in signaling duration of the CGRP family peptides revealed here have significant implications for their functions *in vivo* and their potential therapeutic uses. Clearly, there must be a need for different signaling durations of the CLR-RAMP complexes to support certain physiological functions, particularly via the AM2/IMD agonist where up to three versions may exist *in vivo* that differ in the extent of their long-duration signaling capabilities. It will be important in the future to determine the biological functions that are dependent on long duration signaling. An interesting question is whether the temporal differences in signaling translate to spatial differences in signaling throughout the cell. Notably, even the “short-acting” αCGRP has been shown to promote persistent pain signaling from internalized CGRP receptor in endosomes [35], so why a longer-acting βCGRP is necessary is not obvious. Agonist receptor residence times and signaling durations are also an important consideration in drug development for GPCRs [36]. Given the significant clinical potential of agonist versions of CGRP, AM, and AM2/IMD as drugs for various diseases [5, 9, 10], the structural determinants of their signaling durations revealed here may inform the development of analogs with signaling durations optimized as desired for particular therapeutic uses.

In conclusion, the CGRP family peptides α/βCGRP, AM, and AM2/IMD exhibit considerable variability in the duration of cAMP signaling they elicit by activation of their respective CLR-RAMP and CTR-RAMP complexes. βCGRP is longer-acting than αCGRP and AM2/IMD is longer-acting than AM. The three AM2/IMD peptides differ in the extent of their long-duration signaling. It is important for researchers to clearly state which AM2/IMD peptide they use in their studies because the 3 fragments are not equivalent. Determining the structural basis for the signaling durations of each peptide revealed variability in their determinants of duration of action and allowed us to identify peptides that should be useful tools for studying the roles of long-duration signaling. Notable among these include AM2/IMD R23K/R33K and AM2-AM-AM2 with significantly diminished signaling duration at AM_2R, and AM-AM2-AM with gain-of-function long-duration signaling at the two AM receptors. Overall, our results emphasize the importance of not limiting our thinking about the pharmacology of this important peptide family to receptor affinities and signaling potencies derived from end-point equilibrium assays, which have obscured temporal differences in their receptor binding and signaling mechanisms that almost certainly have important physiological consequences.

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Data availability:

Data will be made available upon request.

Abbreviations:

AM	adrenomedullin
AM_1R	CLR-RAMP2
AM2/IMD	adrenomedullin 2/intermedin
AM_2R	CLR-RAMP3
AMY	amylin
AMY_1R	CTR-RAMP1
AMY_2R	CTR-RAMP2
AMY_3R	CTR-RAMP3
BRET	Bioluminescent resonance energy transfer
CAMYEL	cAMP sensor using Yellow fluorescent protein-Epac-Renilla luciferase
CGRP	Calcitonin gene-related peptide
CGRPR	CGRP receptor (CLR-RAMP1)
CLR	calcitonin-like receptor
CT	calcitonin
CTR	calcitonin receptor
ECD	extracellular domain
GPCR	G protein-coupled receptors
Gs	Gαs subunit of heterotrimeric G protein
HPLC	high-pressure liquid chromatography
PDE	phosphodiesterase
PEI	polyethylenimine
RAMP	receptor activity modifying protein

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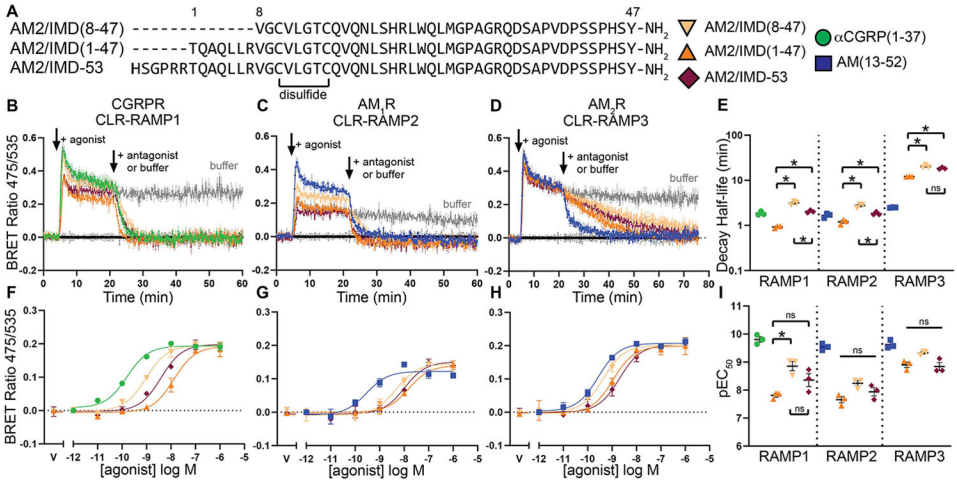

Figure 1: cAMP signaling kinetics and potencies for AM2/IMD peptides with variable N-terminal extensions at the CGRP and AM receptors:

A) Amino acid sequence alignment of the three AM2/IMD peptides. **B-D)** CAMYEL cAMP signaling kinetics at the indicated receptors with 100 nM agonist αCGRP(1-37) (green circle), AM(13-52) (blue square), AM2/IMD(8-47) (peach down triangle), AM2/IMD(1-47) (orange up triangle), and AM2/IMD-53 (maroon diamond) followed by 10 μM antagonist challenge or buffer addition (gray). The buffer addition control shown in the plots used the agonist AM2/IMD(1-47). **E)** Scatter plot of decay half-lives obtained from B-D for the decay phase after antagonist addition. **F-H)** CAMYEL cAMP end-point agonist concentration-response assays at the indicated receptors. **I)** Scatter plot of pEC_{50} values obtained from F-H. All plots show a single representative of three independent experiments with standard deviation for duplicate technical replicates. cAMP signaling decay kinetics and pEC_{50} values are summarized in Table 1. Statistical significance was determined with a one-way ANOVA using Tukey's post hoc test.

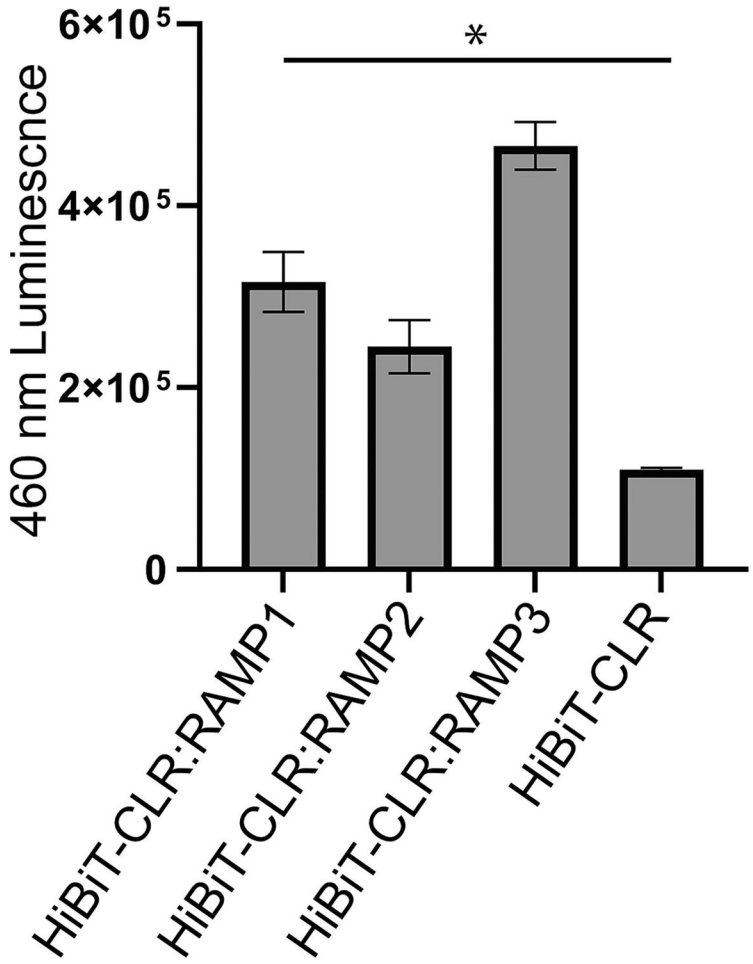

Figure 2: Cell surface expression of the three CLR-RAMP complexes in COS-7 cells.

Data shown are the mean ± SEM from three independent replicates. Statistical significance was determined with a one-way ANOVA using Tukey's post hoc test with each data set statistically significant from one another.

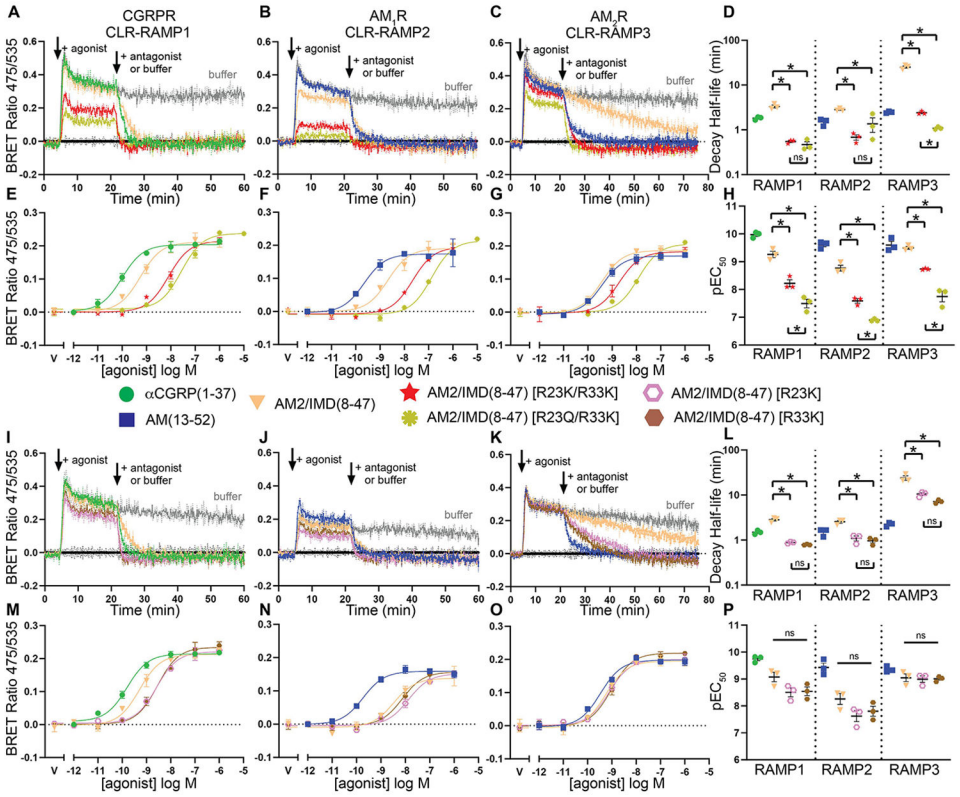

Figure 3: cAMP signaling kinetics and potencies for the R23- and R33-substituted AM2/IMD variants at the CGRP and AM receptors:

A-C) CAMYEL cAMP signaling kinetics at the indicated receptors with 100 nM agonist αCGRP(1-37) (green circle), AM(13-52) (blue square), AM2/IMD(8-47) (peach down triangle), AM2/IMD(8-47) [R23K/R33K] (red star), and AM2/IMD(8-47) [R23Q/R33K] (yellow star) followed by 10 μM antagonist challenge or buffer addition (gray). The buffer addition control shown in the plots used the agonist AM2/IMD(8-47). **D)** Scatter plot of decay half-life values obtained from A-C after antagonist addition. **E-G)** CAMYEL cAMP end-point agonist concentration response assays at indicated receptors with the same agonists used in A-C. **H)** Scatter plot of pEC_{50} values obtained from E-G. **I-K)** As in A-C for CAMYEL cAMP signaling kinetics with 100 nM agonist AM2/IMD(8-47) [R23K] (pink open hexagon) and AM2/IMD(8-47) [R33K] (brown hexagon). **L)** Scatter plot of decay half-lives from I-K after antagonist addition. M-O) As in E-G for CAMYEL cAMP end-point agonist concentration response with the indicated AM2/IMD single mutants. **P)** Scatter plot of pEC_{50} values obtained from M-O. All plots show a single representative of three independent experiments with standard deviation for duplicate technical replicates. cAMP signaling decay kinetics and pEC_{50} values are summarized in Table 2. Statistical significance was determined with a one-way ANOVA using Tukey's post hoc test.

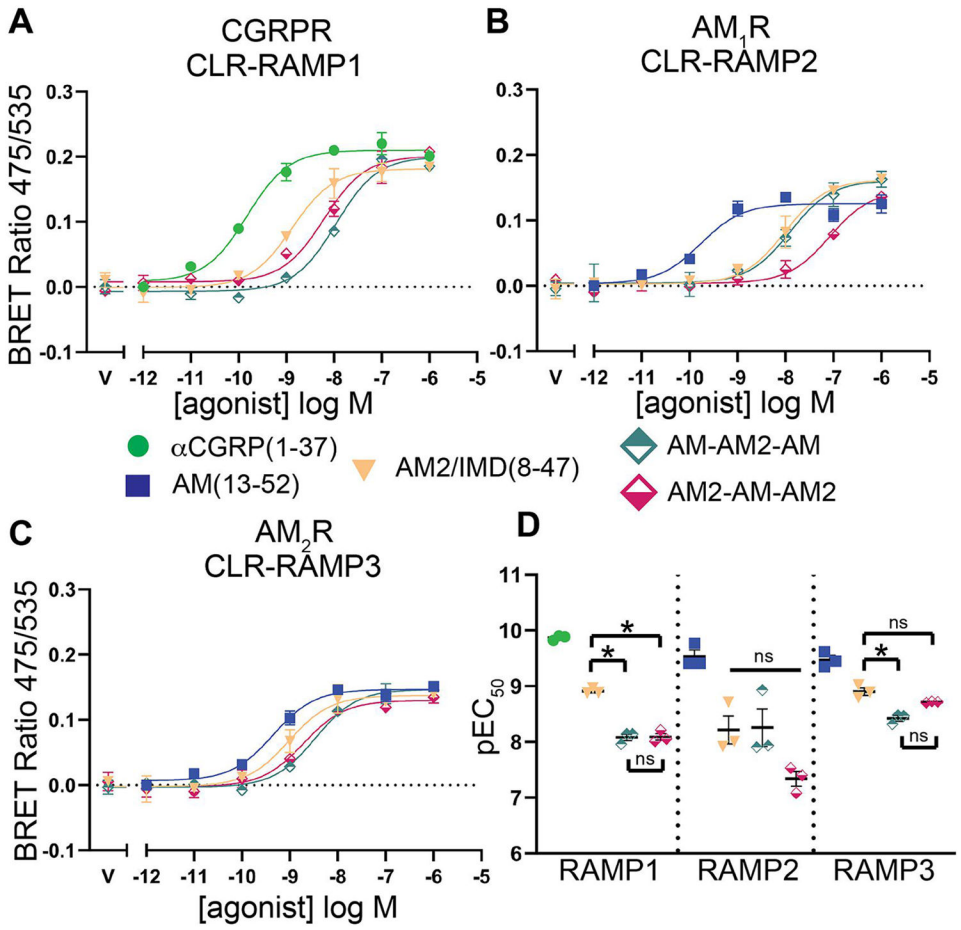

Figure 4: cAMP potencies for the AM2/IMD-AM mid-region chimeras at the CGRP and AM receptors:

A-C) CAMYEL cAMP end-point agonist concentration response assays at the indicated receptors with αCGRP(1-37) (green circle), AM(13-52) (blue square), AM2/IMD(8-47) (peach down triangle), AM-AM2-AM (teal diamond), and AM2-AM-AM2 (pink diamond). **D)** Scatter plot of pEC_{50} values obtained from A-C. These values are summarized in Table 3. All plots show a single representative of three independent experiments with standard deviation for duplicate technical replicates. Statistical significance was determined with a one-way ANOVA using Tukey's post hoc test.

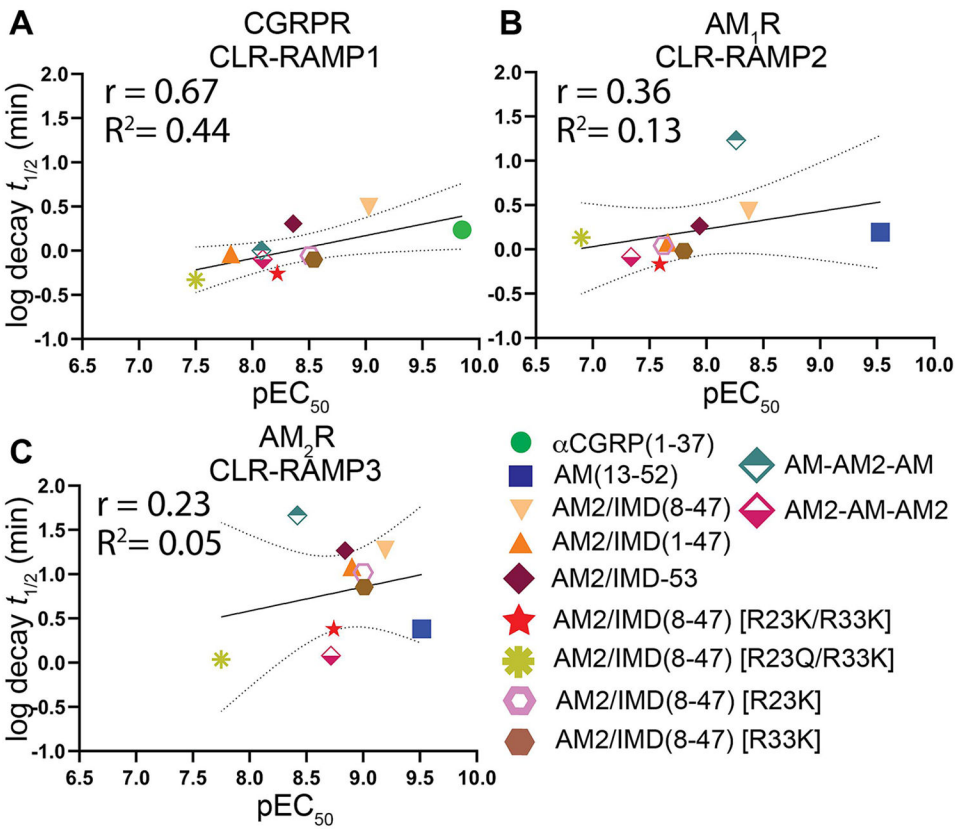

Figure 5: Pearson correlation plots for AM2/IMD variants at CGRP and AM receptors:
Correlation of cAMP signaling potencies and durations for the indicated peptides at the CGRPR **(A)**, AM_1R **(B)**, or AM_2R **(C)**. The symbols for each plot are as follows: αCGRP(1-37) (green circle), AM(13-52) (blue square), AM2/IMD(8-47) (peach down triangle), AM2/IMD(1-47) (orange up triangle), AM2/IMD-52 (maroon diamond), AM2/IMD(8-47) [R23K/R33K] (red star), AM2/IMD(8-47) [R23Q/R33K] (yellow star), AM2/IMD(8-47) [R23K] (pink open hexagon), AM2/IMD(8-47) [R33K] (brown hexagon], AM-AM2-AM chimera (teal diamond), and AM2-AM-AM2 chimera (pink diamond). pEC_{50} values for αCGRP(1-37), AM(13-52), and AM2/IMD(8-47) were averaged data from Figures 1, 3, and 4 and half-life values were averaged from Figures 1 and 3. Half-life values for the mid-region chimeras were from ref. 19 as reproduced in Table 3.

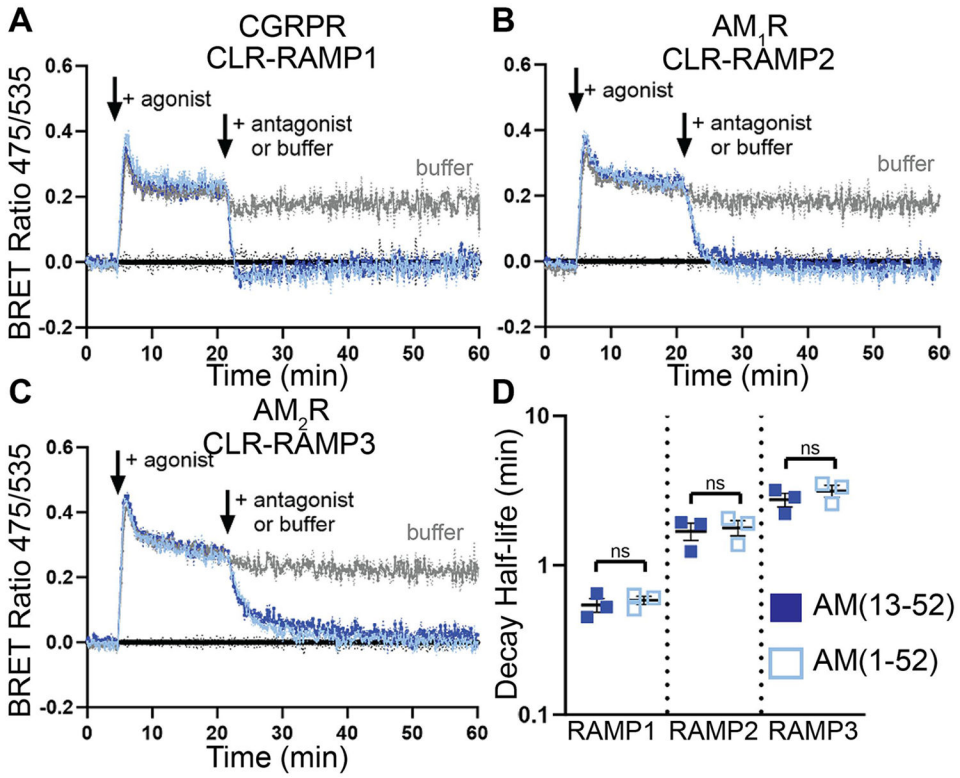

Figure 6: cAMP signaling kinetics of AM(1-52) and (13-52) at the CGRP and AM receptors: **A-C)** CAMYEL cAMP signaling kinetics at indicated receptors with 100 nM agonist AM(13-52) (blue square) and AM(1-52) (light blue open square) followed by addition of 10 μM antagonist or buffer (gray). The buffer addition control shown in the plots used the agonist AM(13-52). **D)** Scatter plot of decay half-lives obtained from A-C. All plots show a single representative of three independent experiments with standard deviation for duplicate technical replicates. cAMP signaling decay kinetic values are summarized in Table 4. Statistical significance was determined with a one-way ANOVA using Tukey's post hoc test.

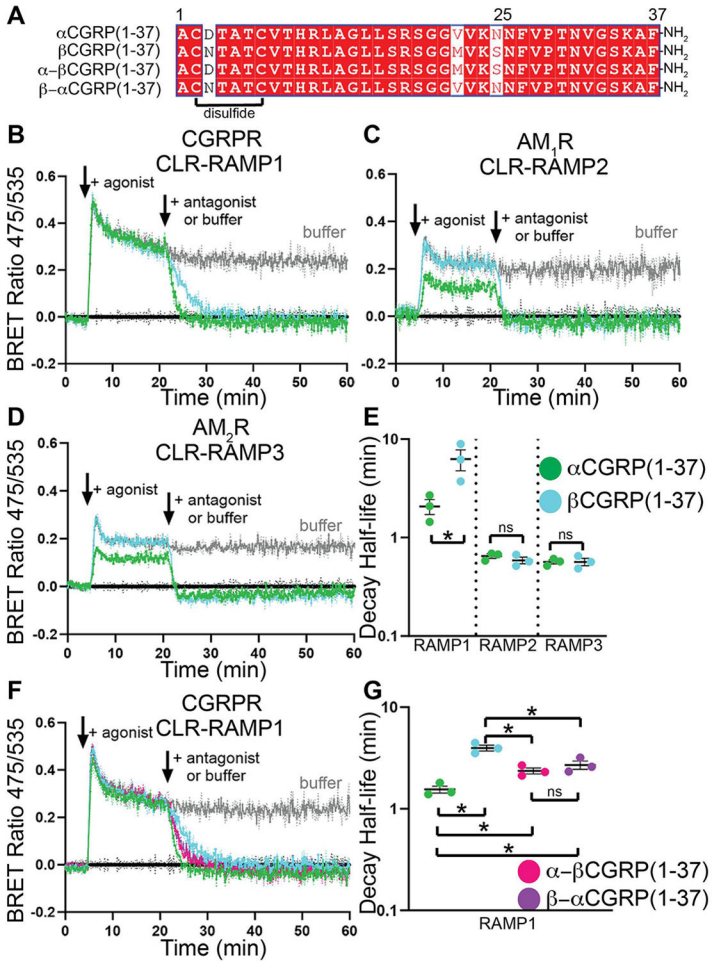

Figure 7: cAMP signaling kinetics of αCGRP and βCGRP at the CGRP and AM receptors:

A) Amino acid sequence alignment of αCGRP, βCGRP, and their chimeras. **B-D)** CAMYEL cAMP signaling kinetics of αCGRP(1-37) (green circle) and βCGRP(1-37) (cyan circle) at indicated receptors with 100 nM agonist followed by addition of 10 μM antagonist or buffer (gray). The buffer addition control shown in the plots used the agonist βCGRP(1-37), **E)** Scatter plot of decay half-lives obtained from B-D after antagonist addition. **F)** CAMYEL cAMP signaling kinetics of α-βCGRP(1-37) chimera (pink circle) and β-αCGRP(1-37) chimera (purple circle) at CGRPR. **G)** Scatter plot of decay half-lives obtained from F after antagonist addition. All plots show a single representative of three independent experiments with standard deviation for duplicate technical replicates. cAMP signaling decay kinetic values are summarized in Table 5. Statistical significance was determined with a one-way ANOVA using Tukey's post hoc test.

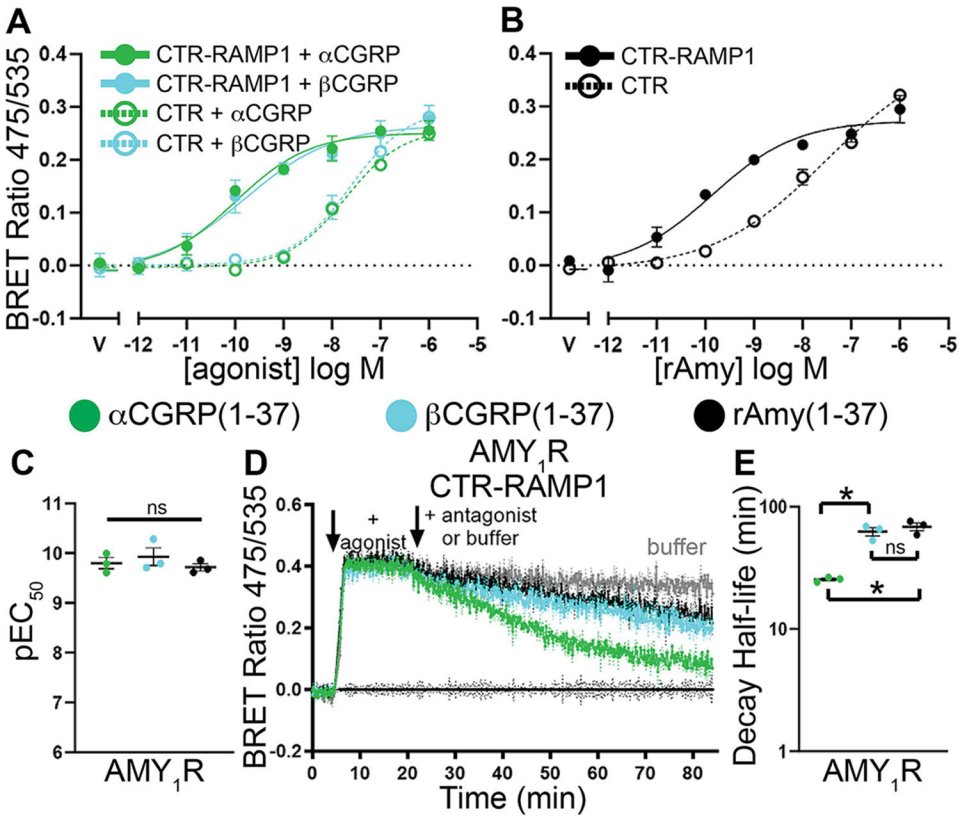

Figure 8: cAMP signaling kinetics of αCGRP and βCGRP at the Amylin$_1$ receptor:

A) CAMYEL cAMP end-point agonist concentration response assay for αCGRP(1-37) (green) and βCGRP(1-37) (cyan) at CTR (open circle) and AMY$_1$R (closed circle). The pEC$_{50}$ values of αCGRP at CTR and AMY$_1$R are 7.67 ± 0.05 and 9.80 ± 0.11, respectively, and the pEC$_{50}$ values for βCGRP at CTR and AMY$_1$R are 7.79 ± 0.08 and 9.93 ± 0.18, respectively. **B)** As in A, but for rAmy(1-37) (black) at CTR (open circle) and AMY$_1$R (closed circle). The pEC$_{50}$ values or rAmy at CTR and AMY$_1$R are 7.63 ± 0.24 and 9.72 ± 0.07, respectively. **C)** Scatter plot of pEC$_{50}$ values of αCGRP(1-37), βCGRP(1-37), and rAmy(1-37) at AMY$_1$R **D)** CAMYEL cAMP signaling kinetics with 1 nM agonist αCGRP(1-37), βCGRP(1-37), or rAmy(1-37) at AMY$_1$R followed by addition of 10 μM antagonist challenge or buffer (gray). The buffer addition control shown in the plot used the agonist αCGRP(1-37). **E)** Scatter plot showing decay half-lives of αCGRP(1-37), βCGRP(1-37), and rAmy(1-37) at AMY$_1$R obtained from D after antagonist addition. All plots show a single representative of three independent experiments with standard deviation for duplicate technical replicates. cAMP signaling decay kinetic values are summarized in Table 5. Statistical significance was determined with a one-way ANOVA using Tukey's post hoc test.

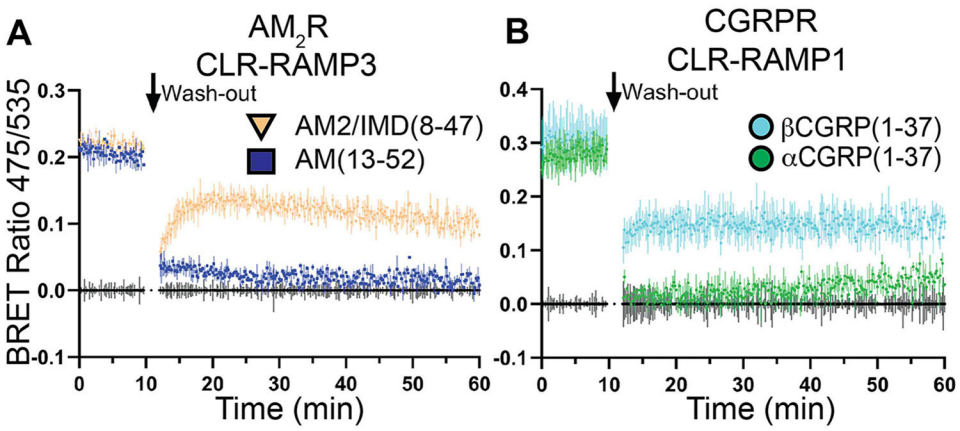

Figure 9: cAMP signaling duration after washout:

A) CAMYEL cAMP signaling at AM_2R stimulated with 10 nM AM(13-52) (blue square) and AM2/IMD(8-47) (peach down triangle) followed by washout at the indicated time. **B)** CAMYEL cAMP signaling at CGRPR stimulated with 10 nM αCGRP(1-37) (green circle) and βCGRP(1-37) (cyan circle) followed by washout at the indicated time. All plots show a representative of three independent experiments with standard deviation for duplicate technical replicates.

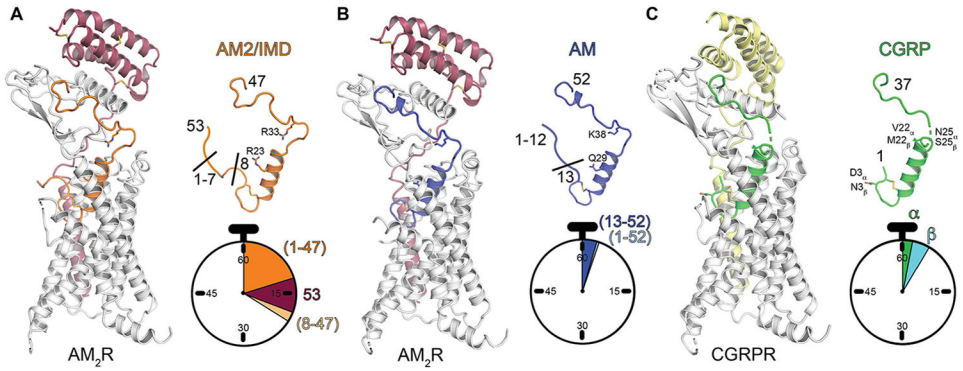

Figure 10: Summary of the cAMP signaling durations of α/βCGRP, AM, and AM2/IMD peptides at the CLR-RAMP complexes and the structural determinants thereof:

A) Left, Cryo-EM structure of CLR (white), RAMP3 (maroon), and AM2/IMD (orange). Right, Structural determinants affecting AM2/IMD signaling duration with decay phase half-life values represented in the stopwatch (in minutes) for the different forms of AM2/IMD at AM_2R. **B)** Left, Cryo-EM structure of CLR (white), RAMP3 (maroon), and AM (blue). Right, structural determinants affecting AM signaling duration with decay phase half-life values represented in the stopwatch for the different forms of AM at AM_2R. **C)** Left, Cryo-EM structure of CLR (white), RAMP1 (yellow), and αCGRP (green). Right, structural determinants affecting CGRP signaling duration with decay phase half-life values represented in the stopwatch for the two forms of CGRP at CGRPR. The structures in A and B are from [19, 37] and the structure in C is PDB code 6E3Y [38].

Table 1:

Summary of cAMP decay kinetics and pEC_{50} values of AM2/IMD with different N-terminal extensions at CGRPR, AM_1R. and AM_2R.*

		CGRPR	AM_1R	AM_2R
AM2/IMD(1-47)	Rate (min^{-1} ± SEM)	0.75 ± 0.03	0.58 ± 0.04	0.057 ± 0.0003
	Half-life† (min ± SEM)	0.92 ± 0.03	1.20 ± 0.08	12.14 ± 0.07
	Time constant‡ (min ± SEM)	1.33 ± 0.05	1.73 ± 0.12	17.51 ± 0.10
	pEC_{50} (± SEM)	7.81 ± 0.06	7.66 ± 0.11	8.90 ± 0.09
AM2/IMD(8-47)	Rate (min^{-1} ± SEM)	0.22 ± 0.01	0.25 ± 0.02	0.034 ± 0.001
	Half-life (min ± SEM)	3.15 ± 0.17	2.75 ± 0.16	20.32 ± 0.88
	Time constant (min ± SEM)	4.54 ± 0.25	3.98 ± 0.22	29.32 ± 1.27
	pEC_{50} (± SEM)	8.86 ± 0.17	8.24 ± 0.10	9.33 ± 0.04
AM2/IMD-53	Rate (min^{-1} ± SEM)	0.34 ± 0.01	0.38 ± 0.02	0.038 ± 0.001
	Half-life (min ± SEM)	2.03 ± 0.08	1.84 ± 0.09	18.47 ± 0.53
	Time constant (min ± SEM)	2.93 ± 0.11	2.65 ± 0.14	26.65 ± 0.76
	pEC_{50} (± SEM)	8.36 ± 0.23	7.94 ± 0.15	8.84 ± 0.14
αCGRP(1-37)	Rate (min^{-1} ± SEM)	0.38 ± 0.02	ND§	ND
	Half-life (min ± SEM)	1.85 ± 0.11	ND	ND
	Time constant (min ± SEM)	2.67 ± 0.15	ND	ND
	pEC_{50} (± SEM)	9.81 ± 0.10	ND	ND
AM(13-52)	Rate (min^{-1} ± SEM)	ND	0.42 ± 0.03	0.28 ± 0.003
	Half-life (min ± SEM)	ND	1.67 ± 0.11	2.49 ± 0.03
	Time constant (min ± SEM)	ND	2.40 ± 0.16	3.60 ± 0.04
	pEC_{50} (± SEM)	ND	9.54 ± 0.07	9.63 ± 0.08

*Values derived from Figure 1 experiments

†Half-life calculated as ln 2 divided by decay rate.

‡Time constant calculated as the inverse of the decay rate.

§ND = not done

Table 2:

Summary of cAMP signaling decay kinetics and pEC_{50} values of AM2/IMD variants at CGRPR, AM_1R, and AM_2R.*

		CGRPR	AM_1R	AM_2R
AM2/IMD(8-47)†	Rate (min^{-1} ± SEM)	0.23 ± 0.01	0.26 ± 0.01	0.029 ± 0.002
	Half-life‡ (min ± SEM)	3.08 ± 0.18	2.68 ± 0.11	24.6 ± 1.40
	Time constant§ (min ± SEM)	4.44 ± 0.26	3.87 ± 0.16	35.49 ± 2.02
	pEC_{50} (± SEM)	9.17 ± 0.10	8.51 ± 0.15	9.27 ± 0.12
AM2/IMD(8-47) R23K/R33K	Rate (min^{-1} ± SEM)	1.27 ± 0.07	1.06 ± 0.16	0.29 ± 0.01
	Half-life (min ± SEM)	0.55 ± 0.03	0.68 ± 0.09	2.40 ± 0.11
	Time constant (min ± SEM)	0.79 ± 0.04	0.98 ± 0.14	3.46 ± 0.16
	pEC_{50} (± SEM)	8.22 ± 0.13	7.59 ± 0.08	8.74 ± 0.02
AM2/IMD(8-47) R23Q/R33K	Rate (min^{-1} ± SEM)	1.56 ± 0.22	0.67 ± 0.25	0.64 ± 0.02
	Half-life (min ± SEM)	0.47 ± 0.08	1.36 ± 0.49	1.09 ± 0.04
	Time constant (min ± SEM)	0.67 ± 0.11	1.96 ± 0.71	1.57 ± 0.06
	pEC_{50} (± SEM)	7.50 ± 0.15	6.90 ± 0.02	7.75 ± 0.19
AM2/IMD(8-47) R23K	Rate (min^{-1} ± SEM)	0.79 ± 0.02	0.66 ± 0.10	0.067 ± 0.005
	Half-life (min ± SEM)	0.88 ± 0.02	1.10 ± 0.14	10.47 ± 0.78
	Time constant (min ± SEM)	1.27 ± 0.03	1.58 ± 0.20	15.11 ± 1.12
	pEC_{50} (± SEM)	8.51 ± 0.16	7.62 ± 0.21	9.00 ± 0.12
AM2/IMD(8-47) R33K	Rate (min^{-1} ± SEM)	0.88 ± 0.01	0.74 ± 0.08	0.097 ± 0.005
	Half-life (min ± SEM)	0.79 ± 0.01	0.96 ± 0.10	7.15 ± 0.38
	Time constant (min ± SEM)	1.14 ± 0.02	1.39 ± 0.14	10.32 ± 0.54
	pEC_{50} (± SEM)	8.54 ± 0.16	7.80 ± 0.19	9.01 ± 0.05
αCGRP(1-37)†	Rate (min^{-1} ± SEM)	0.43 ± 0.02	ND**	ND
	Half-life (min ± SEM)	1.66 ± 0.09	ND	ND
	Time constant (min ± SEM)	2.39 ± 0.13	ND	ND
	pEC_{50} (± SEM)	9.86 ± 0.06	ND	ND
AM(13-52)†	Rate (min^{-1} ± SEM)	ND	0.47 ± 0.03	0.30 ± 0.01
	Half-life (min ± SEM)	ND	1.51 ± 0.10	2.36 ± 0.09
	Time constant (min ± SEM)	ND	2.17 ± 0.14	3.40 ± 0.12
	pEC_{50} (± SEM)	ND	9.52 ± 0.09	9.48 ± 0.09

*Values derived from Figure 3 experiments

†Values derived from 6 independent experiments combining Figure 3A-C and I-K for cAMP signaling kinetics and Figure 3E-G and M-O for pEC_{50} values

‡Half-life values calculated as ln 2 divided by decay rate.

§Time constant calculated as the inverse of the decay rate.

**ND = not done

Table 3:

Summary of cAMP decay kinetics and pEC_{50} values for AM2/IMD-AM mid-region chimeras.

		CGRPR	AM_1R	AM_2R
AM2(8-22)-AM(29-38)-AM2(34-47) (“AM2-AM-AM2”)	Half-life * (min ± SEM)	0.80 ± 0.07	0.82 ± 0.06	1.2 ± 0.099
	pEC_{50} † (± SEM)	8.09 ± 0.06	7.34 ± 0.13	8.72 ± 0.01
AM(13-38)-AM2(23-33)-AM(39-52) (“AM-AM2-AM”)	Rate (min^{-1} ± SEM)	1.0 ± 0.05	17 ± 2.1	46 ± 4.2
	PEC_{50} (± SEM)	8.08 ± 0.06	8.26 ± 0.34	8.42 ± 0.05

* Half-life values reproduced from ref. 19 for comparison purposes.

† pEC_{50} values derived from Figure 4.

Table 4:

Summary of cAMP decay kinetics of AM(1-52) and (13-52) at CGRPR, AM_1R, and AM_2R.*

		CGRPR	AM_1R	AM_2R
AM(13-52)	Rate (min^{-1} ± SEM)	1.3 ± 0.13	0.43 ± 0.07	0.26 ± 0.03
	Half-life† (min ± SEM)	0.54 ± 0.06	1.69 ± 0.23	2.75 ± 0.29
	Time constant‡ (min ± SEM)	0.78 ± 0.08	2.44 ± 0.33	3.97 ± 0.42
AM(1-52)	Rate (min^{-1} ± SEM)	1.2 ± 0.08	0.40 ± 0.05	0.22 ± 0.02
	Half-life (min ± SEM)	0.58 ± 0.04	1.78 ± 0.21	3.15 ± 0.29
	Time constant (min ± SEM)	0.84 ± 0.05	2.57 ± 0.30	4.54 ± 0.41

*Values derived from Figure 6 experiments

†Half-life calculated as ln 2 divided by decay rate.

‡Time constant calculated as the inverse of decay rate.

Table 5:

Summary of cAMP decay kinetics of α/βCGRP, their chimeras, and rAmy at CGRPR, AM_1R, AM_2R, and AMY_1R.[*]

		CGRPR	AM_1R	AM_2R	AMY_1R
αCGRP(1-37)[†]	Rate (min^{-1} ± SEM)	0.40 ± 0.04	1.07 ± 0.06	1.23 ± 0.06	0.03 ± 0.001
	Half-life[‡] (min ± SEM)	1.81 ± 0.21	0.65 ± 0.03	0.57 ± 0.03	25.5 ± 0.64
	Time constant[§] (min ± SEM)	2.61 ± 0.30	0.94 ± 0.05	0.82 ± 0.04	36.8 ± 0.93
βCGRP(1-37)[‡]	Rate (min^{-1} ± SEM)	0.15 ± 0.02	1.20 ± 0.09	1.24 ± 0.11	0.01 ± 0.001
	Half-life (min ± SEM)	5.12 ± 0.85	0.59 ± 0.04	0.57 ± 0.05	62.6 ± 4.91
	Time constant (min ± SEM)	7.39 ± 1.23	0.85 ± 0.06	0.82 ± 0.07	90.4 ± 7.08
α-βCGRP(1-37)	Rate (min^{-1} ± SEM)	0.30 ± 0.02	ND[**]	ND	ND
	Half-life (min ± SEM)	2.37 ± 0.16	ND	ND	ND
	Time constant (min ± SEM)	3.41 ± 0.23	ND	ND	ND
β-αCGRP(1-37)	Rate (min^{-1} ± SEM)	0.26 ± 0.02	ND	ND	ND
	Half-life (min ± SEM)	2.70 ± 0.25	ND	ND	ND
	Time constant (min ± SEM)	3.90 ± 0.36	ND	ND	ND
rAmy(1-37)	Rate (min^{-1} ± SEM)	ND	ND	ND	0.01 ± 0.001
	Half-life (min ± SEM)	ND	ND	ND	68.6 ± 5.00
	Time constant (min ± SEM)	ND	ND	ND	99.0 ± 7.22

[*] Values derived from Figures 7 and 8 experiments

[†] n=6 for CGRPR combining data from Figure 7B and F, and n=3 for all other receptors.

[‡] Half-lives calculated as ln 2 divided by decay rate.

[§] Time constant calculated as the inverse of the decay rate.

[**] ND = not done