Bioactivity of the putative apelin proprotein expands the repertoire of apelin receptor ligands

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Abstract

Background—Apelin is a peptide ligand for a class A G-protein coupled receptor called the apelin receptor (AR or APJ) that regulates angiogenesis, the adipoinular axis, and cardiovascular functions. Apelin has been shown to be bioactive as 13, 17, and 36 amino acid isoforms, C-terminal fragments of the putatively inactive 55-residue proprotein (proapelin or apelin-55). Although intracellular proprotein processing has been proposed, isolation of apelin-55 from colostrum demonstrates potential for secretion prior to processing and the possibility of proapelin-AR interaction.

Methods—Apelin isoform activity and potency were compared by an In-Cell WesternTM assay for ERK phosphorylation using a stably AR-transfected HEK293A cell line. Conformational comparison of apelin isoforms was carried out by circular dichroism and heteronuclear solution-state nuclear magnetic resonance spectroscopy.

Results—Apelin-55 is shown to activate the AR, with similar maximum ERK phosphorylation response and potency to the shorter isoforms except for apelin-13, which exhibited a greater potency. Correlating to this shared activity, highly similar conformations are exhibited in all apelin isoforms for the shared C-terminal region responsible for receptor binding and activation.

Conclusions—AR activation by all apelin isoforms likely hinges upon shared conformation and dynamics in the C-terminus, with apelin-55 providing an alternative bioactive isoform despite the addition of 19 N-terminal residues relative to apelin-36.

General significance—Beyond providing novel insight into the physiology of this system, re-annotation of proapelin to the bioactive apelin-55 isoform adds to the molecular toolkit for dissection of apelin-AR interactions and expands the repertoire of therapeutic targets for the apelinergic system.

Graphical Abstract

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Keywords

apelin (APLN); apelin receptor (APJ, APLNR); proprotein; In-Cell Western assay; NMR spectroscopy; CD spectropolarimetry

1. Introduction

Apelin is one of two cognate peptide hormone ligands for a single class A G-protein coupled receptor (GPCR) called the apelin receptor (AR, or APLNR, initially known as APJ) [1]. Apelin-AR binding and activation regulates multiple biological systems. These include the cardiovascular system, where apelin is one of the most potent physiological inotropic agents, and the central nervous system, where apelin regulates fluid homeostasis [1]. Apelin-AR binding also causes vasodilation or vasoconstriction in an eNOS-dependent manner [1]. This system also has links to various pathological states, including cardiovascular diseases, diabetes, and obesity [1, 2]. Taken together, all these factors make apelin-AR signalling of great interest for increased understanding of disease aetiology and potential treatment of many diseases.

The putative 55-residue apelin proprotein (proapelin or apelin-55) is processed from a 77-residue preproprotein through removal of a 22-residue signal peptide (Table 1). Apelin-55 is further processed into bioactive isoforms of 36, 17, or 13 residues, likely through protease-mediated mechanisms. To date, the only processing mechanism shown is conversion of the 55-residue proprotein directly and preferentially to apelin-13 by proprotein convertase subtilisin kexin subtype 3 (PCSK3, or furin) [3].

Apelin-55 was not detected when apelin was originally discovered in 1998 [4], leading to the attribution of this isoform as either being non-existent or an inactive proprotein [5]. Correspondingly, apelin processing was theorized to occur intracellularly [6], leading to a release of only the shorter 36 to 13 residue bioactive isoforms. In contrast to these hypotheses, intact apelin-55 was more recently detected in colostrum and milk [7], providing direct evidence for extracellularly localized proprotein. The presence of extracellular apelin-55 implies the potential that processing of apelin-55 to shorter isoforms may occur extracellularly, putting into question mechanisms relying exclusively on intracellular processing. This also leads to the question of whether apelin-55 can directly activate the AR or whether proteolytic processing is essential for bioactivity.

The C-terminal residues of apelin are required for receptor binding [8]. Apelin-55 is therefore theoretically capable of binding to the AR and activating downstream signalling pathways. Interestingly, studies have indicated that apelin isoforms differ in their binding

affinities for the AR, where shorter isoforms (e.g., apelin-13) bind weakly and longer isoforms (e.g., apelin-36) more tightly [9]. These differing affinities, in turn, may explain the observed isoform-dependent differences in potencies [4], rates of receptor recycling [10], and signalling biases [11]. The other ligand of the AR, apela (also known as ELABELA [12] or Toddler [13]), also exhibits isoform-dependent differences in signalling bias and potencies [14]. Much less is known about the apela family of peptides, or even which isoform(s) are present *in vivo*. In terms of apelinergic system function, the majority of published work to date has focused on the shortest of the apelin ligands: apelin-13. The physiological roles of longer isoforms remain relatively little studied, but may behave profoundly differently.

Here, we report a biophysical and pharmacological comparison of all endogenously produced apelin isoforms (apelin-55, -36, -17 and -13). In particular, atomic-level characterization of the conformation and dynamics of each of the apelin isoforms provides a structural basis for receptor binding and activation. In combination, we identify regions of structure convergence and variation in apelin isoforms that are likely to be responsible for regulating the apelin-AR interaction and signalling.

2. Materials and Methods

2.1 Apelin isoform production and purification

Human proapelin/apelin-55 (C-terminal 55-residues of pre(pro)apelin with N-terminal serine from TEV cleavage; total 56 residues) was expressed in *Escherichia coli* C41(DE3) and purified using Ni-NTA affinity and cation exchange chromatography, as outlined previously [3]. The precipitated protein was re-dissolved in deionized H₂O and further purified using reverse phase (RP) high performance liquid chromatography (HPLC) using a ProStar HPLC (Varian Canada Inc., Mississauga, ON), a preparative C₁₈ column (20 mm I.D. × 250 mm, Cosmosil, Nacalai USA Inc. San Diego, CA), with a binary solvent system (A: H₂O with 0.1% trifluoroacetic acid (TFA) and B: acetonitrile with 0.1% TFA; flow rate 8 mL/min). Gradients are as follows: i) 2 to 20% solvent B in 5 min, ii) 20 to 45% solvent B in 25 min, iii) 45% to 2% solvent B in 1 min. Eluent peaks were collected, lyophilized, and analyzed by electrospray ionization mass spectrometry (ESI-MS; Mass Spectrometry Laboratory, Department of Chemistry, Dalhousie University, Halifax, NS). The purity of apelin-55 was analyzed by analytical RP-HPLC (C₁₈ column, 5 µm, 5 mm × 250 mm, AAPPTec, Louisville, KY), with the same binary system as for preparative column purification (2 to 40% solvent B in 30 min, followed by 40% to 2% in 5 min).

The C-terminal phenylalanine of apelin-55 was mutated to alanine through site-directed mutagenesis using the Phusion® High-Fidelity DNA Polymerase (NEB, Whitby, ON), as confirmed by sequencing (Bio Basic Inc., Markham, ON). The vector expressing the mutant gene was electroporated into the *E. coli* C41 (DE3) strain, F55A-apelin-55 protein expression was induced, and the resulting mutant protein purified using the same protocol as the wildtype with identity confirmed by ESI-MS.

An open reading frame coding for N-terminally hexa-histidine SUMO (His-SUMO)-tagged human apelin-36 (bacterial codon optimized) was cloned into the pET-HN vector (gift from

Dr. Xiang-Qin Liu, Dalhousie University) and transformed into the BL21 (DE3) strain of *E. coli*. Protein expression was induced through addition of 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) once cells reached logarithmic growth phase (optical density=0.6) and protein expression was carried out for 4 hours. The cells were lysed through French Press in buffer containing 50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8. Soluble proteins were applied to a column packed with Ni-NTA beads (Roche Diagnostics, Indianapolis, IN). The column was washed with a buffer containing higher imidazole content (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8) and any bound proteins were subsequently eluted using buffer with 250 mM imidazole (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8). The resulting solution was dialyzed and then diluted until the total protein concentration reached 15.0 μ M (identified through A_{280} and $\epsilon_{280} = 6980$ for fusion protein calculated using the method outlined by Gill and von Hippel [15]). SUMO protease (produced in-house; reaction in 50 mM Tris-HCl, 150 mM NaCl, pH 8) was added to reach ~1:100 molar ratio to its substrate. SUMO proteolysis was allowed to occur overnight and monitored by SDS-PAGE. The resulting reaction mixture was dialyzed and lyophilized. Apelin-36 was then purified by RP-HPLC (Varian ProStar) using the same gradient method, preparative column, and solvents as for apelin-55. Purity and peptide identity were confirmed identically to apelin-55 by analytical RP-HPLC and ESI-MS, respectively.

Apelin-17 and apelin-13 were chemically synthesized and purified as detailed previously [16]. Pyroglutamate-modified apelin-13 (Pyr-apelin-13) was purchased from AnaSpec (Fremont, CA). Apelin-17 and -13 purity were determined using the same analytical RP-HPLC protocol as for apelin-55.

The concentration (c) of each apelin peptide was determined using the Beer-Lambert law ($c = A \cdot \epsilon^{-1} \cdot l^{-1}$, where A is absorbance at a given wavelength, ϵ is molar absorptivity at that wavelength, and l is pathlength). For apelin-36 and -55, absorbance at 280 nm was employed (ϵ of 5,500 M⁻¹cm⁻¹); for apelin-13 and -17, absorbance at 214 nm was employed (ϵ of 30,893 M⁻¹cm⁻¹ for apelin-13 and 40,030 M⁻¹cm⁻¹ for apelin-17), as estimated by the method described by Kuipers and Gruppen [17]).

2.2 Isotopically labeled peptide production

For ¹⁵N and ¹³C-enriched apelin-55 and apelin-36 production, *E. coli* cells were grown in LB medium to optical density of 0.6–0.8. Pelleted cells were re-suspended in a 1/2-equivalent volume of minimal medium (100 mM NaH₂PO₄, 40 mM K₂HPO₄, 4 mM MgSO₄, 1.8 μ M FeSO₄, 2 g/L ¹³C₆ D-glucose, 1 g/L ¹⁵NH₄Cl and 100 mg/L ampicillin, titrated with NaOH to pH 7.3 [18]) and allowed to express isotopically enriched proteins for 4 hours at 37 °C. Proteins were purified as detailed above and the incorporation of NMR-active isotopes was confirmed by ESI-MS. The mass gain of the major protein species was used to estimate isotope labeling efficiency.

2.3 pERK assay for transiently transfected HEK293A

Human Embryonic Kidney (HEK) 293A cells were cultured in 10 cm dishes and grown in DMEM (High Glucose, Sigma Aldrich, Oakville, ON) containing penicillin and

streptomycin (500 units/L, Sigma Aldrich) supplemented with 10% Fetal Bovine Serum (FBS, Sigma Aldrich). At ~70% confluency, cells were transfected by addition of serum-free DMEM (High Glucose, Sigma Aldrich) containing 10 µg of the AR-coding pcDNA 3.1 plasmid (cDNA Resource Center, Bloomsburg, PA) and 100 µg of polyethylenimine (PEI, Sigma Aldrich). Cells were incubated with the transfection mixture for 24 hours, at which time the medium was aspirated and refreshed with serum-free DMEM. After an additional 24 hours, plates of cells were stimulated with Pyr-apelin-13 or apelin-55 or non-stimulated (DMEM, no apelin), respectively, for 5 minutes. Immediately after stimulation, the medium was aspirated and cells were harvested with cold PBS, treated with DNase I (10 mg/mL) then resuspended in radioimmuno-precipitation assay (RIPA) buffer with added protease inhibitor cocktail (50 mM Tris/HCl, pH 7.5, 10 mM MgCl₂, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, Complete Protease inhibitors (Roche)). To aid in separation of a protein fraction free from cellular debris, 30 µL of a suspension of Protein A Sepharose beads (100 mg/mL, Sigma Aldrich) in RIPA containing bovine serum albumin (BSA) were added to each sample. The samples were vortexed and nutated for 20 minutes at 4 °C. Finally, lysates were centrifuged (13 000g, 15 min, 4 °C) and the supernatant protein fractions were collected and added to Laemmli sample buffer for SDS-PAGE loading.

Immunoblots were probed with primary antibodies for extracellular signal-regulated kinase (ERK; 1:1000 rabbit polyclonal ERK2; Santa Cruz Biotech, sc-154) and pERK (1:500 mouse monoclonal pERK; Santa Cruz Biotech, sc-7383) and fluorophore-conjugated secondary antibodies (Dylight 549, donkey anti-rabbit, 1:2000; and Dylight 649, donkey anti-mouse, 1:1000; Rockland, Limerick, PA). The membranes were then imaged by fluorescent excitation and detection using a VersaDoc imaging system (Bio-Rad, Mississauga, ON). Digital images were analyzed using Bio-Rad's Image Lab software. Apparent molecular weights were inferred from the migration distance of a given band relative to the BLUEye prestained protein ladder (Froggabo, North York, ON). Ratios between pERK and ERK values were determined, averaged between datasets, and statistically significant differences between the resulting values were determined using unpaired Student's t-tests.

2.4 Development of stably-AR transfected HEK293A cell line

Untransfected HEK293A cells were seeded into a 10 cm dish. Upon reaching 80% confluency, cells were transfected (treated with a transfection mixture containing 20 µg plasmid DNA and 60 µL PEI for 24 hours) with a pIRES2-EGFP plasmid (gift from Dr. Younes Anini, Dalhousie University, Halifax, NS) containing the WT AR gene (Clontech, Mountain View, CA), which was first linearized using the AflIII restriction enzyme (NEB). This plasmid encodes both the protein of interest and a second separate open reading frame on the same mRNA transcript, allowing for verification of successful transfection without fluorescent modification of the protein of interest. After 24 hours, the antibiotic G418 (Sigma-Aldrich) was added to the growth medium at a concentration of 400 mg/mL, and maintained at this concentration during subsequent passages. After 3–5 passages, an individual colony of green cells was isolated and seeded onto a new 10 cm dish.

2.5 In-Cell Western™ assay

HEK293A cells stably expressing the WT-AR gene were seeded into 96-well microplates in the growth media (High Glucose DMEM, 10% FBS and 5% penicillin-streptomycin, 100 μ L/well). After 24 hours, the growth medium was removed and replaced with serum-free DMEM (50 μ L/well). After an additional 24 hours, cells were stimulated: i) for 5 minutes at 37 °C with a range of doses of apelin (Pyr-13, -17, -36, -55; 10^{-11} M to 10^{-5} M); ii) with 1 μ M dose of apelin isoforms for various times (0 to 30 min); or, iii) with a 50% FBS positive control. In each case, 50 μ L of stimulation solution was added to the 50 μ L already in each well. Subsequently, the medium was removed and replaced with 100 μ L/well of fixation solution (4% paraformaldehyde in PBS), for 10 minutes. Next, the cells were washed with buffer (0.01% Tween-20 in PBS, 100 μ L/well) and then treated with blocking buffer (20% Odyssey Blocking Buffer (LI-COR, Lincoln, NE) in PBS with 0.4% Triton X-100, 40 μ L/well) for 1 hour on a shaker at RT. Blocking buffer was removed and replaced with primary antibody solution (1:500 mouse monoclonal pERK (Santa Cruz Biotech, sc-7383) and 1:500 rabbit polyclonal ERK2 (Santa Cruz Biotech, sc-154) in blocking buffer), and cells were incubated overnight on a shaker at 4 °C. The next day, cells were again washed for 5×3 min on a shaker at RT. Wash buffer was removed and replaced with secondary antibody solution (1:500 Goat anti-Mouse IgG Dylight-680, Cross Adsorbed (Thermo Fisher, Burlington, ON, CAT#35519) and 1:500 Donkey anti-Rabbit IgG Dylight-800, Cross Adsorbed (Thermo Fisher, CAT#SA5-10044) in blocking buffer), and cells were incubated for 1 hour on a shaker at RT, in the dark. Finally, cells were again washed (5×3 min on a shaker at RT), in the dark, and subsequently imaged using the LI-COR Odyssey scanner (LI-COR). The signal from the 700 nm (pERK) and 800 nm (ERK) channels was quantified using the LI-COR Odyssey scanning software. Dose-response data were collected as pERK/ERK ratios, normalizing against total ERK. The averaged ratios were then plotted as percentages of the maximum response induced by the positive control FBS stimulation. For each individual microplate, a non-linear regression was performed, yielding EC_{50} (dose at which a half-maximal pERK response was reached) and E_{max} (maximum achievable response) values. The determined values were then averaged across at least 3 separate experiments. Statistically significant differences between the resulting values were determined using unpaired Student's t-tests.

2.6 Far-UV CD spectropolarimetry

Each apelin isoform was re-suspended in an appropriate volume of CD buffer (1 mM NaN_3 , 1 mM $CaCl_2$, 25 mM Na_2HPO_4/NaH_2PO_4 buffer, pH 6.00 ± 0.05) to reach a final peptide concentration of 250 μ M. Far-UV CD spectra were acquired (J-810 spectropolarimeter, Jasco, Easton, MD) at 37 °C with a data pitch of 0.1 nm from 260 to 190 nm at 100 nm/min. Cuvettes of 0.1 mm path length (Hellma, Vaughan, ON) were employed. All spectra were acquired in triplicate, and experiments were carried out in duplicates using independently prepared samples. All data acquired were averaged, blank subtracted, reported as mean residue ellipticity (MRE). Finally, MRE values were subjected to a sliding window average over 3 nm stretches.

2.7 Apelin-55 stability

The stability of apelin-55 was assessed in NaCH₃COO (pH 5), 2-(N-morpholino) ethanesulfonic acid (MES; pH 6), NaH₂PO₄/Na₂HPO₄ (pH 6 and 7), Bis Tris (pH 6 and 7), and HEPES (pH 7) buffers. Solutions contained 25 mM buffer salt, 1 mM CaCl₂, and 1 mM NaN₃. Apelin-55 samples were prepared in the appropriate volume to reach a final concentration of 0.2 mM. Samples were incubated at 5 °C, room temperature (~23°C), or 37 °C for 1 week, and analyzed by SDS-PAGE and Coomassie staining.

2.8 NMR spectroscopy

Each apelin isoform was re-suspended in appropriate volume of NMR buffer (1 mM DSS, 1 mM NaN₃, 25 mM Na₂HPO₄/NaH₂PO₄ buffer, pH 6.00±0.05, 90%/10% H₂O/D₂O) to reach a final protein concentration of 0.2 mM for isotope-enriched apelin-55 and -36, 2.1 mM for unlabeled apelin-17, and 2.5 mM for unlabeled apelin-13. Sensitivity enhanced ¹H-¹⁵N heteronuclear single quantum spectroscopy (HSQC) experiments were acquired at 37 °C for each apelin isoform; for isotopically labeled apelin-36, an Avance 500 MHz spectrometer equipped with a room temperature 5 mm broadband fluorine observe (BBFO) SmartProbe with a z-axis gradient (Bruker Canada, Milton, ON; NMR³ facility, Dalhousie University) was employed; for all other apelin isoforms an Avance-III 700 MHz spectrometer with 5 mm triple resonance inverse (TCI) cryoprobe with a z-axis gradient (Bruker Canada; Biomolecular Magnetic Resonance Facility, National Research Council (NRC), Halifax, NS) was employed. Chemical shifts for the C', C_α, C_β, H_N, and N nuclei of apelin-55 were assigned through the main chain-directed approach [19] at 37 °C and at 5 °C using HNCA; HN(CO)CA; HN(CA)CO; HNCO; CBCANH (37 °C) or HNCACB (5 °C); and, CBCA(CO)NH (37 °C) triple-resonance experiments employing an Avance-III 700 MHz spectrometer (NRC)). Side chain ¹H chemical shifts were assigned using a ¹⁵N-edited total correlation spectroscopy (TOCSY)-HSQC experiment. At 5 °C, ¹⁵N-edited nuclear Overhauser effect (NOE) spectroscopy (NOESY; mixing time: 150 ms) experiment was also acquired to both assist in side chain ¹H assignment and to validate sequential assignment. Finally, the signal enhancement from the heteronuclear NOE at both 37 °C and 5 °C was determined using paired ¹H-¹⁵N HSQC experiments acquired in an interleaved manner with or without ¹H saturation during the recycle delay. All experiments were carried out using the standard pulse programs available in the TopSpin 2.1 library (Bruker Canada; Table S1 details experimental parameters).

Spectra were processed using TopSpin 3.1 and NMRPipe [20]. ¹H frequencies were referenced to DSS (0 ppm) and ¹⁵N and ¹³C frequencies indirectly referenced based on ¹H [21]. Sequential assignment, secondary chemical shifts (δ), chemical shift index (CSI) [22, 23], Dihedral Angles from Global Likelihood Estimates (DANGLE) [24], and heteronuclear NOE intensity ratios (intensity of saturated relative to unsaturated interleaved experiments) were analyzed using CcpNmr Analysis 2.3.1 [25]. The identified ¹H and ¹⁵N chemical shifts of apelin-55 at 37 °C were, in turn, used to assign ¹H-¹⁵N heteronuclear HSQC spectra for the shorter isoforms. Euclidian combined chemical shift displacement (CSD) [26] values between isoforms were determined using N and H_N chemical shifts.

3. Results and Discussion

3.1 Production and purification of apelin-36 and apelin-55

For apelin-55, our previously optimized *E. coli*-based expression method followed by TEV protease cleavage [3] allowed for cost-effective production of both isotope-enriched (~2 mg/L, ^{15}N and ^{13}C enriched) and natural abundance (~3–4 mg/L) apelin-55 with purity greater than 95% (Figures 1 and S1–S4), making apelin-55 highly amenable to biophysical characterization. Following from this success, we developed an *E. coli*-based system to produce apelin-36 both at natural abundance and isotopically-enriched. Specifically, we used an N-terminal His-SUMO fusion tag, as is frequently employed to enhance target protein expression and purification [27]. Overexpressed His-SUMO-apelin-36 remained highly soluble in *E. coli*, did not significantly perturb cell viability, and exhibited high affinity in Ni-NTA purification (Figure 1A). Interestingly, nearly complete His-SUMO tag removal could only be achieved when the total protein concentration was less than ~15 μM (Figure 1A). The final apelin-36 yield, following SUMO cleavage, was ~2 mg/L for apelin-36 at greater than 95% purity (Figures S2C and S5–S6). For NMR spectroscopy, ^{15}N incorporation of ~90% efficiency was achieved for apelin-36 with a final yield of ~1–1.5 mg/L (Figure S7). Finally, apelin-13 and -17 were synthesized (Figures S8–S9) and purified, following our previous methods [16], at greater than 95% purity (Figure S2A–B).

3.2 Apelin-55 can activate AR

To test the potential for apelin-55 to activate the AR, we employed our previously developed method, testing for ERK phosphorylation upon apelin treatment in HEK293 cells transiently transfected with AR [28]. Based upon our protocol and upon literature precedents [28, 29], a 5 min apelin treatment period with 1 μM dose was employed and the response to apelin-55 and -13 was compared. Notably, treatment with either apelin-55 or -13 led to a significant increase in ERK phosphorylation (pERK) in comparison to the non-stimulated control (Figure 2). To our knowledge, this is the first report identifying unprocessed apelin-55 as an isoform capable of activating the AR implying that apelin-55 is a longer bioactive isoform rather than strictly an inactive proprotein.

3.3 Apelin-55 has a similar potency to apelin-17 and -36

To better characterize the bioactivity of apelin-55, given that a 1 μM dose is not likely to correspond to the physiological condition [30], the potency (EC_{50}) of apelin-55 was directly compared to those of the other bioactive isoforms. The N-terminally pyroglutamate modified apelin-13 isoform (Pyr-apelin-13) was employed for these assays to reduce potential variability between experiments given that Pyr-apelin-13 spontaneously forms from apelin-13 [3, 31]. Pure Pyr-apelin-13 was thus used to avoid uncertainty about the relative quantities of apelin-13 and Pyr-apelin-13 employed. Testing of only Pyr-apelin-13 is expected to provide values of E_{max} and EC_{50} representative of both apelin-13 isoforms, since previous studies have shown apelin-13 and Pyr-apelin-13 to maintain highly similar backbone conformations [16] and to induce ERK phosphorylation with near identical potency [4] and efficacy [5].

Apelin isoform potencies were directly compared through development of a microplate-based In-Cell WesternTM assay using stably AR-transfected HEK 293A cells. Prior to determining EC₅₀ values for each isoform, activation of ERK by 1 μ M apelin treatment was monitored for 30 min to determine the optimal treatment period. Time-course analysis of ERK phosphorylation showed a maximum response at 5 min for all isoforms (Figure 3A–D), similar to previous reports by standard western blotting [28, 29], which progressively decreased to a near basal level by 30 min.

Under our assay conditions, all isoforms showed similar maximum pERK response (i.e., E_{max}) at 5 min (Figure 3E). Furthermore, apelin-17, -36, and -55 exhibited no significant difference in EC₅₀ (Figure 3E and Table 2). Thus, our results indicate that apelin-55 is as active as the shorter isoforms. In contrast, the EC₅₀ of apelin-13 was ~8 fold lower than the other isoforms, suggesting a significantly higher potency (apelin-13 vs. apelin-17; $P < 0.01$, vs. apelin-36; $P < 0.05$, vs. apelin-55; $P < 0.001$). A similarly increased potency for apelin-13 compared to longer isoforms has been reported previously [4, 8, 9, 32, 33]. In summary, our results clearly demonstrate that apelin-55 can bind to the AR and induce a transient pERK response similar to apelin-17 and -36.

One alternative to direct AR activation by apelin-55 would be extracellular processing prior to activation by a resulting shorter apelin isoform. This is unlikely under our assay conditions for three primary reasons. First, apelin-55 processing only occurs in the presence of specific processing enzymes, and does not spontaneously undergo proteolysis or degradation over hours [3], let alone within the 5 min assay period. Second, production of the shorter apelin-13 isoform to an appreciable level requires a significantly longer time period than 5 min, even under optimal *in vitro* proteolysis conditions, suggesting that apelin-55 is the predominant isoform during the treatment period [3]. Third, the level of cell surface AR was increased by transfection and, thus, likely would far outweigh the level of cell surface processing enzymes. In combination, it is more probable that apelin-55 will encounter and interact with an AR molecule than an endogenous processing enzyme during the 5 min incubation period. The observed ERK phosphorylation, thus, is most likely to result from a direct apelin-55—AR interaction rather than the processing of apelin-55 to a shorter isoform prior to activation.

3.4 Conformational comparison of apelin isoforms by far-UV CD spectropolarimetry

Given that all apelin isoforms activated the AR under our assay conditions and they share C-terminal residues required for the AR binding and activation [4], we hypothesized that they shared a similar conformation in the C-terminal region. Comparison of the secondary structure of apelin-55 and -36 by far-UV CD spectroscopy (Figure 4), the latter exhibiting spectra consistent with previous studies of synthetic apelin-36 [16, 34], did not imply any significant α -helical or β -sheet content. Rather, each isoform exhibits a strong negative band at ~200 nm consistent with random coil conformation [35].

In the shorter apelin-13 and -17 isoforms, we previously reported distinctive positive bands at ~194 nm and ~218 nm directly attributed to the C-terminal phenylalanine side chain, as they were eliminated by alanine substitution (i.e., in F13A-apelin-13) [16]. Interestingly, the F55A-apelin-55 mutant exhibited an increased magnitude of ellipticity relative to both

apelin-55 and -36 over the ~190–220 nm range (Figure 4), suggesting that residual band structure from the phenylalanine is still convoluting the backbone ellipticity in the longer isoforms. Apelin-36 also showed minor increases in ellipticity at ~194 nm and ~218 nm in comparison to apelin-55. Thus, our results imply that the negative band at ~200 nm in the longer isoforms (apelin-36 and -55) is accentuated by the increased number of backbone amide chromophores in comparison to the shorter isoforms (apelin-13 and -17), additively increasing in magnitude to outweigh the positive bands from the phenylalanine side chain. In combination, our CD results are consistent with the presence of bands from C-terminal phenylalanine regardless of the number of N-terminal residues.

3.5 Structure and dynamics of apelin-55 at 5 °C and 37 °C

To allow characterization of apelin-55 at the atomic-level, sequential assignment was carried out. Prior to conducting the heteronuclear NMR experiments required for the main chain-directed assignment approach, we tested the stability of apelin-55 in various NMR buffer conditions (NaCH₃COO (pH 5), MES (pH 6), NaH₂PO₄/Na₂HPO₄ (pH 6 & 7), Bis Tris (pH 6 & 7), and HEPES (pH 7)). Our analysis showed that apelin-55 was stable in all potential NMR buffer conditions for at least one week at 37 °C, room temperature, and 5 °C, as analyzed through SDS-PAGE (Figure S10). The stability of apelin-55 in NaH₂PO₄/Na₂HPO₄ buffer at pH 6 is further demonstrated by a lack of perturbation to the ¹H-¹⁵N HSQC peak pattern following 9 days of triple-resonance experiments (Figure S11).

Triple-resonance NMR experiments allowed sequential assignment in NaH₂PO₄/Na₂HPO₄ buffer (pH 6) using uniformly ¹⁵N- and ¹³C-enriched apelin-55 at both 5 and 37 °C. H_N, N, C_α, C_β, and C' alongside asparagine N_δ-H_δ and glutamine H_ε-N_ε chemical shifts were assigned for all residues except 0S (remaining residue from TEV cleavage), 1G, 49H and 50K at 37 °C (Table S2). In contrast, H_N, N, C_α, C_β, C', asparagine N_δ-H_δ, and glutamine H_ε-N_ε chemical shifts were assigned for all residues except 0S at 5 °C (Table S3). Interestingly, cross-peaks with chemical shifts suggestive of asparagine H_δ-N_δ and/or glycine H_N-N could be detected quite downfield relative to the typical values expected for ¹⁵N (glycine) or ¹H (asparagine) chemical shifts at both 5 °C and 37 °C (Figures 5A and S12; green annotations) [36]. Using a ¹⁵N-edited TOCSY-HSQC experiment, H_α and the majority of side chain H_β, H_γ, and H_δ ¹H chemical shifts were also assigned. For reference, H_N-N resonance assignments are annotated on ¹H-¹⁵N HSQC spectra (Figure 5A at 37 °C and Figure S12 at 5 °C).

The lack of assignable resonances for residues 0S and 1G was expected due to fast exchange with solvent molecules at 37 °C [37], given the reliance upon H_N in the triple-resonance experiments and the TOCSY-HSQC experiment. In the case of 49H and 50K, our structural characterization of apelin-17 demonstrated that these residues were located in a highly dynamic region [16]. Specifically, the region encompassing 10S, 11H, and 12K in apelin-17 (equivalent to 48S, 49H, and 50K in apelin-55) could not be superposed over the ensembles of NMR structures calculated at either 5 °C or 35 °C in buffer, indicating that it is effectively acting as a flexible linker between two relatively more structured regions. The lack of assignable spin-systems for residues 49H and 50K in apelin-55 is, thus, consistent with poor structural convergence similar to apelin-17 with the implication that this region is

undergoing dynamics on the intermediate time scale from an NMR perspective [19]. In support of this, we could clearly identify spin systems for 1G, 49H, and 50K upon decreasing the temperature from 37 °C to 5 °C (Figure S12). This is consistent with a temperature-dependent modulation of the exchange rate upon cooling, likely from the intermediate (ms) towards the slow (s) exchange regime.

Although apelin-55 only contains one tryptophan (33W), three cross-peaks attributable to the side chain H_{e1} - N_{e1} spin-pair were visible in the HSQC spectrum (Figure 5B; one major, two minor cross-peaks). These additional cross-peaks imply the sampling of conformational states in the slow exchange regime [19]. In support of this, additional distinct sets of backbone 1H and ^{15}N chemical shifts were assignable for 33W and for a variety of other amino acids (Figure 6A). These additional assignments were predominantly localized to the region of apelin-55 N-terminal to apelin-17 and to the C-terminal GPMPF region. Despite evidence of slow exchange in these regions of the backbone, it was not possible to sequentially assign any additional chains of multiple amino acids that would be consistent with a second major conformation of the apelin-55 backbone.

In further support of chemical exchange giving rise to the additional chemical shifts observed, apelin-55 at 5 °C exhibited a different pattern of potential assignments on a residue-by-residue basis in comparison to those observed at 37 °C (Figure 6). This modulation in exchange may arise from a temperature-dependent change in the equilibrium populations of different states. This would, in turn, change the propensity of observing a lower populated state. Alternatively, or in concert, lowering of temperature may slow the rate of an exchange process from the intermediate toward the slow exchange regime. Interestingly, increased evidence of slow exchange was especially clear at 5 °C for residues near 33W. Correspondingly, 33W H_{e1} - N_{e1} presented an altered HSQC peak pattern, both with respect to chemical shifts and through the presence of one additional major cross-peak relative to 37 °C (Figure 5C). Unlike at 37 °C, where only one set of chemical shifts could be assigned sequentially corresponding to one major conformation, additional sets of chemical shifts could be sequentially assigned at 5 °C (Figure 6B, yellow highlights; Table S3). In combination, these results indicate that apelin-55 undergoes exchange at the intermediate through slow time scales.

All proline residues except 23P and 45P exhibit multiple sets of chemical shifts at both 5 °C and 37 °C, indicative of slow exchange. Correspondingly, multiple sets of chemical shifts were frequently observed for residues N-terminal to or flanked by prolines. One likely source of conformational exchange for proline arises from its altered *cis-trans* isomerization equilibrium relative to other amino acids [38]. This may result in a significant population of *cis* peptide bonds in slow exchange with the *trans* isomer. Correspondingly, we previously observed two major conformers in the C-terminal GPMPF region of apelin-17, one with both proline residues having *trans* peptide bonds and the other with both *cis* alongside less populated conformers with one residue *trans* and one *cis* [16]. The C_{β} chemical shift of proline is diagnostic of the presence of either a *cis* (av. 34.8 ppm) or *trans* (av. 32.2 ppm) peptide bond, even in disparate environments [39, 40]. The presence of both *cis* and *trans* isomers is clear for 52P and 54P at both 5 °C and 37 °C, while *cis* isomers are also clear for 5P at 37 °C and 32P at 5 °C (Table S2 and S3). Correspondingly, the proline-induced

conformers identified as being in slow exchange in apelin-17 may also exist in the C-terminal region of apelin-55.

The reason that residues in the N-terminal half of apelin-55 exhibit multiple sets of chemical shifts independent of proline is not as clear-cut. Whether this is the result of proline-mediated isomerization being propagated along the backbone or simply less stringent degree of backbone conformational restriction remains to be determined. However, N-terminal flexibility may hypothetically be required to facilitate interaction with diverse binding partners (e.g., the AR, proprotein processing enzymes, or as-yet unidentified partners) or other cellular factors that regulate the rate of receptor interaction. In future, we intend to further characterize the N-terminal residues to investigate the physiological purpose of such conformational variability.

Interestingly, the lowest degree of conformational variation was observed over 37R-48S at both 5 °C and 37 °C. This region encompasses the 39K-42R and 43R-46L segments, which were converged in apelin-17 (apelin-17 numbering 1K-4R and 6R-9L) [16]. Decreased conformational variation in this region, similar to apelin-17, further bolsters the hypothesis that apelin-55 may have a similar converged conformation in the shared C-terminal region facilitating receptor interaction.

Chemical shift analysis of apelin-55 implied no extended segments of secondary structure according to either the CSI (Figure 7) or the Bayesian algorithm DANGLE (data not shown) at both 37 and 5 °C. Furthermore, a comparison of chemical shifts to expected random coil chemical shifts only showed minor differences throughout the majority of the peptide backbone (Figure 7). These results correlate well with the random coil CD spectral characteristics observed (Figure 4). It should be noted that β -turn character may still be present, as previously observed in apelin-17 [16]. Neither DANGLE [24] nor CSI [22, 23] unambiguously identifies β -turns, despite their utility for more extended secondary structures.

At higher temperature, analysis of the ^1H - ^{15}N heteronuclear NOE enhancement factor on a residue-by-residue basis also implies that apelin-55 is highly dynamic along the entire backbone at the ps-ns time scale, further supporting the random coil conformation suggested by CD and the lack of extended secondary structuring predicted by DANGLE and CSI (A). In contrast, analysis of the ^1H - ^{15}N heteronuclear NOE enhancement factor at 5 °C showed decreased dynamics throughout apelin-55 (Figure 7B), which may simply be due to the expected increase in viscosity of water at this temperature [41]. Interestingly, NOE analysis identified the greatest dynamics in the N-terminal end of the protein at both temperatures, corresponding to the lack of assignable resonances for N-terminal residue(s). Similar to apelin-55, the shorter isoforms were also highly dynamic in buffer at higher temperature [16]. Their dynamics at 35 °C were only decreased upon interaction with membrane mimetic detergents, which also resulted in striking conformational changes [16, 42]. It is possible that apelin-55 may undergo similar changes in the presence of detergents, providing an incentive for future studies.

3.6 Atomic-level comparison of apelin isoforms by NMR spectroscopy

The potential conformational similarities between apelin-55 and the shorter apelin isoforms were probed using ^1H - ^{15}N HSQC experiments at 37 °C for ^{15}N -enriched apelin-55 and -36 and for apelin-17 and -13 at natural abundance (Figures 8A and S13). Comparison of the HSQC spectra of all apelin isoforms showed near perfect spectral overlay for any cross-peaks shared between longer and shorter isoforms, with a greater number of cross-peaks corresponding directly to increased isoform length. Furthermore, inference of the amino acids giving rise to the correlations observed in the ^1H - ^{15}N HSQC for each of the shorter apelin isoforms corresponded directly to the shared amino acids assigned in apelin-55. The observed chemical shifts were practically indistinguishable from apelin-55, with only very minor CSD perturbation (CSD ~ 0.02 ppm; Figure S14).

Additional sets of chemical shifts assigned to 51G, 53M and 55F (likely arising from multiple conformations in the C-terminal region of apelin) were observable in all of the shorter apelin peptides, inferring that all isoforms maintain a similar pool of *cis/trans* isomers in the C-terminal GPMPF motif (Figure 8C–E). The observation of similar chemical shifts between apelin isoforms extends from our previous work on shorter isoforms, where we demonstrated highly similar chemical shifts between apelin-12, -13, Pyr-13, and -17 [16]. Inferring chemical shift assignments from apelin-55 did not allow for unambiguous identification for 1K-4R (equivalent to 39K-42R) in apelin-17. This may be due to increased dynamics near the N-terminus and the low natural abundance of ^{15}N (0.37%). Spectral comparison of tryptophan $\text{H}_{\text{e}1}$ - $\text{N}_{\text{e}1}$ spin-pairs for apelin-55 vs. -36 also showed minor differences in conformational biases. In particular, one additional cross-peak was observed for apelin-36 (Figure 8B), which was visible near the noise level for apelin-55.

In summary, our results clearly indicate that the common C-terminal residues (Table 1) share a highly similar conformation, and provide a structural rationale to explain the observation of receptor activation by all apelin isoforms, including the putative proprotein. Furthermore, our NMR spectroscopic results imply that the critical C-terminal residues remain conformationally unhindered by the N-terminal residues in all isoforms, which is likely fundamental to the processes of receptor binding and activation. Conversely, N-terminal residues remain unobstructed by C-terminal residues to interact with either the AR (*vide infra*) other binding partners such as PCSK3.

3.7 Structure-function correlation of apelin isoforms

Practically identical ^1H - ^{15}N chemical shift patterns across all apelin isoforms from apelin-13 to -55 imply nearly identical backbone conformations and conformational sampling, leading to our hypothesis that all isoforms are recognized by, bind to, and activate the AR through their shared C-terminal conformational behaviour. This is further exemplified by the fact that all isoforms were capable of activating downstream signalling *via* the AR. One significant distinction was, however, clear in that apelin-13 was observed to be $\sim 8\times$ more potent than the longer isoforms (-17, -36, and -55), all of which displayed comparable potency.

It is interesting to note that the near identical conformations between apelin isoforms are also in contrast to the other ligand of the AR, apela [43]. Although apelin and apela have

relatively similar amino acid composition and bind to the same receptor, apela isoforms (-32 and -11) presented differences in their conformations [43] and ability to activate various signalling pathways [14], suggesting the potential of ligand conformation-dependent potencies for apela isoforms.

For apelin, one possible explanation for isoform-dependent potency is interference in receptor binding arising from the additional N-terminal residues of the longer isoforms, specifically by the KFRR that falls immediately N-terminal to apelin-13 in the longer isoforms. In contrast to this, however, several studies have shown longer isoforms to have greater affinity for the receptor than apelin-13 [9, 10, 44]. This has been proposed to be the result of electrostatic interactions between positively charged N-terminal residues of apelin isoforms and key negatively charged surface residues of the AR [28, 45–47]. This may relate to the conformational flexibility we have observed in the N-terminal region of apelin-55, allowing partner-dependent folding upon binding [48], including to the AR. Furthermore, the observed lack of interaction between N- and C-terminal regions of apelin-55 would provide additional freedom for favourable ligand-receptor interactions to increase affinity.

Perplexingly, the observed increased affinity for longer isoforms seems counterintuitive with respect to their lower potency relative to apelin-13. Although the isoforms may vary in their biophysical and pharmacological properties *in vivo*, we did not detect significant isoform-dependent variation between $E_{\max}$ or conformation under our experimental conditions. Thus, it seems likely that the difference in potency stems from as yet unknown intricacies in ligand-receptor interactions, which would ultimately result in isoform-dependent modulation of receptor conformation and/or dynamics [49–51].

3.8 Isoform-mediated impact on physiological responses

Isoform-dependent differences in AR signalling can distinctly affect downstream physiological responses. For example, Lee *et al.* showed striking differences between apelin-13 and -36 in their effects on receptor regulation [10]. While apelin-13 allowed for receptor recycling, apelin-36 caused intracellular sequestering of the receptor. Furthermore, Adam *et al.* showed that apelin-13 injection inhibited platelet function [52]. In the case of apelin-36, PCSK3-mediated processing to apelin-13 was required for activity, as mutants incapable of being processed did not inhibit platelet activation. Thus, apelin isoforms can have significantly different functions and the ratio of apelin isoforms has been proposed to be an important regulatory method *in vivo* [10].

The introduction of apelin-55 as an additional, significantly longer bioactive isoform considerably expands the apelinergic system. Since apelin-55 can exist extracellularly [7] and is capable of activating the AR, the *in vivo* phenomena discussed above are likely not limited solely to apelin-36 and -13. Furthermore, this implies that the level of various isoforms or their half-lives will need appropriate regulation to modulate the final physiological response(s). Whether this regulation is conducted intracellularly or extracellularly remains to be characterized, and further elucidation of apelin processing mechanisms and proteases involved are required to understand the physiological purpose of apelin-55. Intriguingly, an alternative as-yet unidentified receptor has also been implicated for apelin-36 [53]. The effect of N-terminal extension to apelin-55 upon this alternative

signalling pathway remains uncharacterized. Regardless, our identification of apelin-55 as an additional bioactive isoform indicates greater complexity in the apelinergic system than previously recognized.

4. Conclusion

The two distinct endogenous ligands of the AR, apelin and apela, each can be processed into multiple isoforms with varying binding affinities and potencies for activating the receptor. Thus, the AR presents an exciting opportunity to characterize a GPCR using series of native ligands rather than synthetic alternatives. We have demonstrated herein that instead of being an inactive proprotein, apelin-55 is an additional, longer bioactive apelin isoform. Through NMR spectroscopy, similar shared C-terminal conformations are apparent for apelin-55 and the shorter isoforms, suggesting that similar modes of AR recognition, binding, and activation are likely.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AR	apelin receptor/APJ/APLNR
BSA	bovine serum albumin
CD	circular dichroism
CSD	chemical shift displacement
CSI	chemical shift index
DANGLE	dihedral angles from global likelihood estimates
ERK	extracellular signal-regulated kinase
ESI-MS	electrospray ionization mass spectrometry

FBS	fetal bovine serum
GPCR	G-protein coupled receptor
HSQC	heteronuclear single quantum coherence
IPTG	isopropyl β -D-1-thiogalactopyranoside
HPLC	high performance liquid chromatography
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
PCSK3	proprotein convertase subtilisin kexin subtype 3
Pyr	pyroglutamate
RP	reverse phase
TFA	trifluoroacetic acid
TOCSY	total correlation spectroscopy

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Figure 1.

Apelin-36 and -55 expression, fusion protein cleavage, and purification. SDS-PAGE gels demonstrating Ni-NTA column purification of (A) His-SUMO-apelin-36 (HS-AP36) and (C) His-TEV-apelin-55 (HT-AP55) and subsequent cleavage reaction by (A) SUMO protease to produce His-SUMO (HS) tag and apelin-36 (AP36) and (C) TEV protease-mediated His tag removal to produce apelin-55 (AP55). Preparative RP-HPLC chromatograms of (B) apelin-36 and (D) apelin-55 (confirmed product elution denoted by asterisks (*)). Acronyms: L-ladder, FT-flow through, W-wash, E-elution, UC-uncleaved, C-cleaved.

Figure 2.

Apelin-55 and -13-mediated ERK phosphorylation in HEK293A cells transiently expressing the AR. HEK293 cells pre-transfected with an AR encoding plasmid were treated (+) with 1 μ M of given apelin isoform for 5 min. (A) Levels of pERK and ERK were determined by western blot analysis, as illustrated for representative experiments carried out on the same membrane. (B) pERK/ERK levels were quantified by densitometry and compared to controls without stimulation (–). Statistical significance was determined by one-tailed unpaired Student's t-test (N=4, *** P < 0.001 compared to no stimulation control).

Figure 3. Apelin isoform-mediated ERK phosphorylation. (A) Pyr-apelin-13, (B) apelin-17, (C) apelin-36, and (D) apelin-55 (1 μ M for isoforms) show time-dependent ERK phosphorylation by In-Cell WesternTM assay. (E) Non-linear regression analysis of apelin isoform dose-response.

Figure 4. Comparison of apelin isoforms by far-UV CD spectropolarimetry. Apelin-13 and -17 data are re-plotted from Langelaan *et al.* [16].

Figure 5.

Comparison of apelin-55 at 37 °C and 5 °C. (A) ^1H - ^{15}N HSQC spectrum at 37 °C, with cross-peaks annotated with by number in the sequence and single-letter amino acid code. Additional resonance assignments from alternative conformations are differentiated by a question mark. Orange lines connect asparagine and glutamine side chain amides and green lines connect potential asparagine side chain amides. Inset shows overlay of apelin-55 at 5 °C (blue) on 37 °C (red). 33W $\text{H}_{\text{e1}}\text{-N}_{\text{e1}}$ side chain amides are also compared between (B) 37 °C and (C) 5 °C.

Figure 6. Number of potential spin system assignments per residue of apelin-55 based upon sequential assignment at (A) 37°C and (B) 5°C. The yellow boxes identify chains of more than three residues exhibiting more than one sequentially assigned series of chemical shifts at 5 °C (detailed in Table S3).

Figure 7.

$^1\text{H}-^{15}\text{N}$ heteronuclear NOE enhancement factor, secondary chemical shift (δ), and CSI for apelin-55 at (A) 37 °C and (B) 5 °C. * Residue not observable in the heteronuclear NOE-modulated $^1\text{H}-^{15}\text{N}$ HSQC experiment.

Figure 8.

Conformational similarities between apelin isoforms. (A) Overlay of ^1H - ^{15}N HSQC spectra for all isoforms. (B) Tryptophan $\text{H}_{\text{e}1}$ - $\text{N}_{\text{e}1}$ spin-pair of apelin-55 and -36. Overlays of HSQC regions corresponding to (C) 51G, (D) 53M, and (E) 55F in the C-terminal GPMPF motif shared between all apelin isoforms.

Table 1

Amino acid sequences of apelin isoforms

Amino acid sequence	Apelin
* <u>MNLR</u> LCVQALLLLWLSLTAVCGGSLMPLPDGNGLEDGNVRHLVQPRGSRNGPGPWQGGRRKFR ₇₇ RRPRLSHKGMPMF	–77
GSLMPLPDGNGLEDGNVRHLVQPRGSRNGPGPWQGGRRKFR ₅₅ RRPRLSHKGMPMF	–55
LVQPRGSRNGPGPWQGGRRKFR ₃₆ RRPRLSHKGMPMF	–36
KFR ₁₇ RRPRLSHKGMPMF	–17
QR ₁₃ PRLSHKGMPMF	–13

* Underlined residues represent the predicted signal peptide sequence.

Table 2Pharmacological properties of apelin isoforms (mean $\pm$ SEM)

	Pyr-apelin-13	Apelin-17	Apelin-36	Apelin-55
EC ₅₀ (nM, 5 min) ^a	2.05 $\pm$ 0.65	16.35 $\pm$ 3.73**	16.54 $\pm$ 6.51*	16.91 $\pm$ 2.86***

^aThe EC₅₀ values (based on Figure 3E) for each isoform were extrapolated and averaged from the curves of individual experiments.

Statistical significance compared to Pyr-apelin-13 was determined using one-tailed unpaired Student's t-test (* P < 0.05, ** P < 0.01, and *** P < 0.001).