Apelinergic system structure and function

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Abstract

Apelin and apela (ELABELA/ELA/Toddler) are two peptide ligands for a class A G-protein-coupled receptor named the apelin receptor (AR/APJ/APLNR). Ligand-AR interactions have been implicated in regulation of the adipoinular axis, cardiovascular system, and central nervous system alongside pathological processes. Each ligand may be processed into a variety of bioactive isoforms endogenously, with apelin ranging from 13 to 55 amino acids and apela from 11 to 32, typically being cleaved C-terminal to dibasic proprotein convertase cleavage sites. The C-terminal region of the respective precursor protein is retained and is responsible for receptor binding and subsequent activation. Interestingly, both apelin and apela exhibit isoform-dependent variability in potency and efficacy under various physiological and pathological conditions, but most studies focus on a single isoform. Biophysical behavior and structural properties of apelin and apela isoforms show strong correlations with functional studies, with key motifs now well determined for apelin. Unlike its ligands, the AR has been relatively difficult to characterize by biophysical techniques, with most characterization to date being focused on effects of mutagenesis. This situation may improve following a recently-reported AR crystal structure, but there are still barriers to overcome in terms of comprehensive biophysical study. In this review, we summarize the three components of the apelinergic system in terms of structure-function correlation, with a particular focus on isoform-dependent properties, underlining the potential for regulation of the system through multiple endogenous ligands and isoforms, isoform-dependent pharmacological properties, and biological membrane-mediated receptor interaction.

Cross-References

Cardiovascular system; G protein-coupled receptors and the G protein family; Obesity
Posttranslational processing of gut peptides; Structure and dynamic properties of membrane
proteins using NMR

Introduction

The apelinergic system is composed of two peptide ligands (apelin and apela) and a single class A (rhodopsin-like) G-protein-coupled receptor (GPCR) called the apelin receptor (abbreviated herein as AR; also known as APJ; gene name: *APLNR*). Each ligand has multiple endogenous bioactive isoforms (Table 1), with ligand binding to the AR exerting a variety of important functions, which include increasing heart contraction; vasodilation; modulation of glucose and insulin homeostasis; cardiovascular development, and more.

Furthermore, the apelinergic system has been linked with number of pathological conditions including chronic heart failure, diabetes, obesity, and HIV infection. For these reasons, the apelinergic system has been highlighted as having strong potential for therapeutic targeting. In this review, we highlight and summarize the important functions the apelinergic system plays in both physiological and pathological conditions, the complexities that arise from the presence of multiple bioactive isoforms, the signaling pathways involved, and focus on the correlation of the known structural and biophysical behavior of the ligands and the receptor to binding and subsequent function.

The apelin receptor

The AR is a class A rhodopsin-like GPCR with 380-residues, with the seven α -helical transmembrane (TM) segments characteristic of the GPCRs (Fig. 1). It was first identified by O'Dowd and co-workers through sequence identity, at 40% in the TM domain (Fig. 2 and Table 2), to the angiotensin AT₁ receptor (194). The AR TM domain is also highly homologous to the chemokine receptors CXCR4 and CCR5 (Fig. 2 and Table 2), both of which may also act as CD4 glycoprotein co-receptors during human immunodeficiency virus binding to cells (263). Based upon its approximately 40–50% sequence homology to AT₁R, the membrane receptor was initially speculated to bind to angiotensin II (AngII) (32, 194). However, AngII and the AR did not interact causing the receptor to remain orphaned until 1998.

Following the recent rapid increase in structural knowledge of GPCRs (e.g., (196)), including both CXCR4 (215, 277) and AT₁ (304, 305), a high-resolution AR structure was determined in 2017 by Ma *et al.* in complex with an apelin-mimetic peptide agonist (166). To allow this structural determination, several modifications to the AR were carried out in order to improve protein yield, homogeneity, and stability. These included N- and C-terminal truncations, three mutations at sites of post-translational modification, two mutations in the TM domain (in TM 3 and TM 6), and substitution of a 54-residue portion of rubredoxin (44) in place of most of intracellular loop 3.

As anticipated, AR exhibits the canonical seven TM α -helices with a short extramembrane eighth helix (Fig. 3) (166). Despite being co-crystallized with the agonist AMG3054, the AR was observed to be an inactive-like conformation. The mutations incorporated greatly reduced (if not ablated) the ability of the receptor to bind wildtype apelin-13, with a corresponding decrease in cAMP response upon activation. Hence, it is quite possible that the mutagenesis produced a constitutively inactive form of the AR. Notwithstanding this potential concern, significant insight is provided by the AR structure from Ma *et al.* This is further delineated as the known features of AR are dissected below.

It should be noted that while the overall TM domain structures of GPCRs appear very similar (Fig. 3), and are typically described as such (93), the architectural differences arising from helical kinks and disruptions are readily seen upon extrapolation from 3D coordinates to 2D topologies using, e.g., the MC-HELAN algorithm (144) (Fig. 4; also aligned relative to the AR in Fig. 2). Homology models of a given GPCR, therefore, will have features dependent upon the chosen template that may or may not be representative of the GPCR

itself. Furthermore, conformational variability of a GPCR as a function of activation state may also be significant (e.g., (293)). Taking a well-characterized GPCR as an example, the TM domain architecture of the β_2 -adrenergic receptor exhibits clear variation as a function of the class of ligand or effector bound (Fig. 5). Given that a crystal structure will only provide a representative snapshot of one particular conformation, even structural inferences from the AR structure from Ma *et al.* will require careful consideration of the constitutively inactive nature of this protein construct and its architecture (166).

In the past, to circumvent the potential issues of structural bias from choice of a particular homology model template, two strategies have been used for the AR. In the first, we employed NMR spectroscopy to study structure, dynamics, and topology sampled by the N-terminal tail and TM1 (AR55) (140, 143) recombinantly expressed in *E. coli*. Then, a hybrid of NMR spectroscopy derived structural data with a CXCR4-based homology model was developed and its behavior followed over time in molecular dynamics (MD) simulations to develop a structural model of the AR (143). A segment comprising the N-terminal and TM segments 1–3 has also been expressed recombinantly in *E. coli*, but has thus far only been partially characterized in various membrane mimetic conditions rather than being applied for structural modeling (196). In an alternative approach, Gerbier *et al.* carried out a comprehensive comparison of features in apelin-AR docking studies using a series of homology models of the AR based upon multiple templates (82). Although carried out prior to the AR structural characterization, both of these approaches have allowed for structurally-based functional inferences as detailed below. Notably, key features of the NMR-derived structural data on AR55, for example a kink in the TM1 helix at ~G42, correspond well to the crystal structure from Ma *et al.* (140, 143, 166) Future studies may ideally employ the now available AR structure as a starting point to understand the importance of conformational variation in the AR.

To date, several key residues have been identified within the AR through a combination of scanning mutagenesis studies and structurally-directed mutagenesis. These may be subdivided into mutations in extracellular regions, most likely involved in the apelin-AR binding; transmembrane regions, most likely involved in either receptor activation or folding/trafficking; and, intracellular regions, with effects upon signal transduction. For quick reference, these are summarized in the snake plot of the AR (Fig. 1) and highlighted, where included, in Figure 2.

Structure-function correlation in the extracellular domain

The extracellular domain (or ectodomain) of a GPCR typically has a ligand-binding pocket (e.g., (120, 129, 253)). In comparison to other GPCR classes, it should be noted that the class A GPCRs have a relatively small ectodomain (253). Assuming a GPCR ectodomain-ligand interaction as part of the activation process, the relatively short extracellular loops (ECLs) and, potentially, N-terminal tail must therefore be able to provide specificity in ligand selection without perturbation to the overall TM domain architecture.

Correspondingly, the ectodomains of class A GPCRs modulate ligand binding, selectivity, receptor activation, and signaling bias (120, 276). In the apelinergic system, therefore, the AR ectodomain must provide for specific recognition and binding of apelinergic system

ligands. In addition, multiple bioactive isoforms for each ligand with widely varying length (Table 1 apelin and apela) must be accommodated. Notably, this level of diversity in ligands for one GPCR is quite unusual (19).

In the first study dissecting AR-apelin binding, Zhou *et al.* focused on the AR N-terminal tail (316). Deletion of the first 10 residues did not affect apelin-13 binding, although cell surface targeting was reduced to ~80% of wildtype. Conversely, deletion of residues 1–20 completely ablated apelin-13 binding and biological activity although cell surface targeting was still ~65% of wildtype. This prompted an alanine-scan of 10 residues from Y10 to K25 (excluding: G12, N15, C19 and T22 as well as A13). Two mutations were shown to be deleterious to apelin-13 binding: E20A and D23A. Given the cationic nature of the apelin peptides, these residues were proposed to be involved in an apelin-AR electrostatic interaction.

Providing further context for the functional importance of E20 and D23 in apelin-AR binding, we employed NMR spectroscopy to structurally characterize the AR55 construct (residues 1-55 of the AR) (140, 143). Combining this with a homology model of the remainder of the AR, as introduced above, MD simulations in a hydrated phospholipid bilayer were carried out (143). In both our NMR structural ensemble of AR55 and over the course of the MD trajectories, the E20 and D23 side chains remained in close proximity to each other (in the MD simulations) and exhibited transient contacts with lipid molecules but not with other residues in the AR. This was upheld in a variety of membrane-mimetics, with the AR55 segment demonstrating similar behavior in all cases, particularly through the TM1 segment (140). These residues thus form an anionic patch proximal to the membrane, which would be appropriately situated for capturing of a membrane-associated apelin peptide consistent with our NMR studies demonstrating a propensity for apelin-17 binding to, and structuring upon binding to, membrane-mimetic micelles (141). This relates directly to the concept of membrane-catalyzed peptide-receptor binding, discussed below in greater detail.

Gerbier *et al.* carried out molecular modeling of apelin-AR binding, employing (as noted above) a series of homology model templates for the AR (82). This allowed identification of several ECL residues as candidates for involvement in apelin-AR binding. These candidates were tested through site-directed mutagenesis, with three acidic residues shown to be key for apelin binding: D94 (in ECL1), E174 (in ECL2), and D284 (in ECL3). The AR D94 was predicted to fall adjacent to a type II β -turn in ECL2, a positioning upheld in the recent crystal structure (166), providing relatively constrained positioning. In apelin, the “RPRL” sequence motif found in all bioactive isoforms (Table 1) has been shown to be functionally essential while exhibiting a unique biophysical behavior. These data are discussed in more detail later in the review, but the “RPRL motif” needs introduction to comprehensively discuss the AR ectodomain. Returning to Gerbier *et al.*, interactions between these anionic residues and apelin-13 were observed using a CXCR4 template. In particular, specific interactions between the Lys (at D94), the first Arg of the RPRL motif (E174) and second Arg of the RPRL motif (D284) of apelin-13 were proposed. None of the simulations exhibited situations where E20 and D23 interacted with apelin-13.

Kumar *et al.* recently used sets of multiple alanine substitutions in ECL1, 2, and 3 to test for perturbation to AR function (134). Interestingly, biased signaling was observed. The dialanine substitution employed in ECL1 (K103-L104) did not exhibit significant perturbation. A trialanine substitution at K268-T-L270 in ECL3 biased signaling to G_q-mediated mechanism. A tetraalanine substitution at M183-D-Y-S186 in ECL2, conversely, showed G_i-biased signaling. Consistent with previous observations of ECL regions being involved in activation (276), this implies that variations in apelin-AR ECL interactions lead to biased signaling. In this study, C281A in ECL3 was also shown to be inactive. This is indicative that the disulfide bond between C281 and C19, unambiguously present in the crystal structure (166) (Fig. 1), is essential. Notably, this disulfide would also have been absent when residues 1-20 were deleted (316).

Finally, in 2017, Ma *et al.* excitingly were able to unambiguously demonstrate the binding of a portion of the apelin analogue AMG3054 to the N-terminus and ECL2 of AR in the crystal structure of the complex (166). A short anti-parallel β -sheet formed between residues 8-10 of the ligand (consistent with the C-terminal two residues of the RPRL motif) and the Y21-D23 segment of the N-terminus. This was coupled with stabilizing main chain and side chain hydrogen bonding interactions. In ECL2, E174 and T176 interact with a His in the ligand analogue, which is at the same position in the sequence as endogenous His residue in apelin (seven residues from the apelin C-terminal shown in Table 1). Their interaction then anchors the ligand at the mouth of the TM domain pocket. The Gln immediately N-terminal to the RPRL motif also interacts with T177 in ECL2. Beyond highlighting the importance of the N-terminal tail and ECL2, with D23 and E174 being specifically implicated in AGM3054-AR binding, it is challenging to interpret the other mutagenesis results detailed from a static structural standpoint. Interestingly, Ma *et al.* also noted the presence of other potential ligand-interaction grooves on the surface of the AR (Fig. 3). Testing the involvement of these sites in binding to natural ligands of varying size and character will be extremely valuable.

Structure-function correlation in the TM domain

Subsequent to ectodomain binding, many polypeptide-based GPCR ligands (although likely not all (120, 129)) penetrate into the TM bundle as part of the receptor activation process (i.e., promote the transition to an activated conformation). The depth of ligand penetration can vary quite significantly (115), implying variability in the exact site of ligand interaction between GPCRs. Regardless, in many cases, ligand binding in this cavity toward the ectoplasmic face of the TM domain induces a conformational change at the so-called “transmission switch” (63). This is at the CWXP motif in TM6 (257), consistent with the extensive variation in topology observed for TM6 in β_2 AR between states (Fig. 5), leading to conformational transition to the GPCR active state through a rigid body motion coupled to the cytoplasmic side of the GPCR upon ligand interaction.

Iturrioz *et al.* showed that alanine or phenylalanine mutants to the tryptophan of the CWXP motif in the AR (W261; numbered as in human AR, vs. the rat AR numbering employed in by Iturrioz *et al.*) are capable of apelin binding and G α_i -protein coupling, but are unable to induce internalization in response to apelin-17 or lissamine-apelin-13 activation (113).

Mutation of the proximal F257 to alanine resulted in the same loss of internalization. Further evidence that these residues directly interact with apelin was shown through the fact that addition of steric bulk at position 257 with the F257W mutation ablated apelin binding, despite proper cell surface localization. Through modeling and MD simulations, these aromatic residues, alongside W154, were predicted to form a pocket that interacts with the C-terminal phenylalanine of apelin; mutagenesis to alanine or phenylalanine at W154 led to misfolded AR incapable of apelin binding. The loss of receptor internalization, while coupling to $G\alpha_i$ is retained, can also occur with either truncation or alanine-substitution of the C-terminal phenylalanine of apelin. Therefore, the “transmission switch” in TM6 of AR appears to be especially critical for β -arrestin coupling or regulating signaling bias rather than simply causing a conformational change towards an active receptor state.

Again, very excitingly, the AMG3054-AR crystal structure unambiguously shows a direct binding site for AMG3054 within the TM domain (166). As a whole, this is a nice demonstration of the potential applicability of a two-site binding mechanism for ligands to the AR, a mechanism which needs to be further fleshed out in future study. Despite similarities in penetration depths (within ~ 5 Å) observed between three other peptide-GPCR crystal structures (two in inactive-like states, one in an active-like state), the ligand penetration in the AMG3054-AR structure is not as deep as that predicted by Iturrioz *et al.* A variety of reasons may have led to this discrepancy. In one case, the homology model employed previously may have been incorrect, allowing for deeper ligand penetration than is possible in reality. Alternatively, the locking of the AR into an inactive state in the crystallized form may correspond to a ligand pose that is not consistent with the final, activated conformation. As noted above, penetration depth by peptidic ligands in GPCRs inferred by a variety of experimental methods is noted to be quite variable. Hence, a direct delineation of the pose of AR-apelin and AR-apela binding in the active vs. inactive states should be considered a priority for ongoing study, with the variety in bioactive ligand sizes for each of these peptides being particularly interesting for study.

The TM1 segment of GPCRs does not typically undergo conformational change in GPCR activation (253, 257). In our structural study of the AR55 segment (143), we found a kink in the region around the N46 residue that is highly characteristic of class A GPCRs (17, 257). Notably, this is maintained in the AMG3054-AR structure, where the kink is initiated at G42 (Fig. 3). The region of the AR TM1 where the kink occurs is quite hydrophilic (sequence GTTGNG) and it would be physically impossible for all hydrophilic side chain moieties to interact with the protein rather than facing the hydrophobic bilayer core if this were a canonical helix. Indeed, site-directed mutagenesis based on our AR55 structure identified G42, G45 and N46 in the kinked region of the TM1 to be highly sensitive to mutagenesis, with substitution of these residues leading to loss of cell-surface localization (143).

Intracellular domain mutagenesis

GPCR intracellular domains comprise three intracellular loops (ICLs) and a C-terminal tail and, as noted above, are critical for interactions with downstream effectors. Examining its sequence, AR exhibits the DRY motif immediately proximal to ICL2 in its TM3 (Figs. 1 and 2), corresponding to the “ionic lock” that may be essential for maintaining AR in an inactive

state (257). Following precedents in other class A GPCRs (109, 231), the AR C-terminal tail has been shown crystallographically to be initiated by an amphipathic, presumably membrane-associated helix (the so-called helix 8) immediately C-terminal to the NPXXY motif in TM7 (residues 305-309 in AR, Fig. 1 and 2) (166)). This is most likely followed by an intrinsically disordered segment covering the remainder of the C-terminal tail.

From an experimental perspective, only one study to date has examined the AR intracellular domain. Specifically, Chen *et al.* carried out mutagenesis in the intrinsically disordered C-terminal tail of the AR (39). In this study, three potential phosphorylation sites were mutated: S335A, S345A and S348A (all in intrinsically disordered C-tail). Notably, loss of the phosphorylation site at S348 specifically biased signaling towards G-protein dependent pathways, presumably through modulation of GRK/ β -arrestin interactions with the AR.

Apelin

In 1998, Tatemoto and coworkers identified a family of peptides from bovine stomach tissue extracts which acted as ligands for the previously orphan GPCR AR (APJ). On the basis of this interaction, the family of peptides was named 'apelin', short for APJ Endogenous Ligand, with a specific nomenclature of apelin-n specifying that a given isoform has "n" amino acids (249). In this study, it was noted that the apelin (*APLN*) gene encodes 77-amino acid long protein, denoted as a preproprotein, with sequence conservation exhibited in humans, cattle, mice, and rats (Fig. 6) (249). Based upon our studies (*vide infra*), proapelin appears to not strictly be an inactive proprotein; hence, herein we refer to this 77-residue form of apelin as a pre(pro)protein to delineate this behavior from that of a classical preproprotein.

Closer examination of the pre(pro)apelin sequence demonstrates that its 23 C-terminal residues are completely conserved over human, cattle, mice and rats. Extending this analysis to non-mammalian species, the 12 C-terminal residues of the pre(pro)protein are identical over a wide range of fish as well as *Xenopus laevis* (265) (Fig. 6). Following from identification of the *APLN* gene on the X chromosome in humans (145), low levels of genetic variability have been observed in subsequent comparative genomics studies. These have typically been isolated to single nucleotide polymorphisms and, consistent with the high level of amino acid conservation over numerous species, have been found only outside of the *APLN* coding regions (123, 153, 154, 232, 308, 309).

Apelin Processing Mechanisms

The 77-residue pre(pro)apelin comprises a putative N-terminal 22-residue secretory signal peptide and a 55-residue C-terminal domain containing the receptor-binding site (89, 249). Dimerization of pre(pro)apelin occurs by cystine-mediated disulfide bridge formation and has been shown to be impaired through reduction by dithiothreitol (147). Both monomeric and dimeric forms of pre(pro)apelin were observed in both whole-cell lysate of transiently transfected COS-7 cells and in heart tissue of transgenic mice, while only the dimeric form was observed *in vivo* in mice (147). It should be noted that the only cysteines in pre(pro)apelin are located in the signal peptide domain (Fig. 6). Dimerization has previously been noted as a prerequisite step for proper processing in other prepropeptides, such as

somatostatin-II (180), suggesting that dimerization may also be a prerequisite step for proper pre(pro)protein processing in the apelinergic system. Following signal peptide cleavage, exclusively monomeric apelin would, thus, be expected as the bioactive form.

Upon the discovery and first report of apelin by Tatemoto *et al.* (249), it was suggested that removal of the signal peptide would result in a 55-residue isoform (apelin-55) that was presumed to be an inactive precursor denoted as proapelin. Analysis of the apelin-55 sequence demonstrated the presence of multiple di-basic amino acid motifs (249), indicative of the potential for proprotein convertase subtilisin kexin (PCSK)-mediated proprotein processing (230) at multiple sites. Based on these predicted sites of processing, Tatemoto *et al.* synthesized apelin-36, -17, and -13 alongside the N-terminally pyroglutamate-modified apelin-13 (Pyr-apelin-13) and compared them to the peptide isolated from bovine stomach tissue by gel filtration chromatography and polypeptide sequencing (249). From this analysis, they concluded that the apelin-36 isoform was the isoform isolated from bovine stomach tissue.

Despite identification of apelin-36 as the form of apelin isolated from stomach tissue, all four tested isoforms were shown to activate the receptor, as observed by a ligand-mediated acidification assay in CHO cells (249). Interestingly, the shortest 13-residue isoforms were the most active. Apelin-55, conversely, was not detected in bovine stomach extracts. This led to the conclusion that it was likely an inactive proprotein, with apelin-77 consequently labeled as a preproprotein (249). The three processed isoforms introduced by Tatemoto *et al.* (apelin-36, -17, and -13) have received by far the most attention, with by far the greatest emphasis being on apelin-13. This focus has likely arisen due both to the relatively high potency of (249) and the cost-effectiveness of obtaining this isoform. It was also hypothesized that only the N-terminally truncated versions of apelin-55 were bioactive, with length-dependent potency. This hypothesis led, in turn, to the suggestion that apelin processing occurred initially to produce apelin-36 from the 55-residue isoform, which is then processed further to shorter and more potent isoforms, apelin-13 or -17 (89, 131) (Fig. 7).

Corresponding to the identification of putative PCSK cleavage sites by Tatemoto *et al.*, we demonstrated that apelin-55 can be processed by PCSK3 (furin) *in vitro* (236). Notably, PCSK3-mediated processing of apelin-55 was direct and preferential, with only apelin-13 production being observed and without any evidence of other longer cleavage products by liquid chromatography-mass spectrometry. Despite the similarity in dibasic site cleavage motifs employed, PCSK1 and PCSK7, alternatively, did not process apelin-55. Apelin-36 was subsequently also shown to be processed by PCSK3 (1). In combination, these observations suggest that apelin processing mechanism is more diverse than initially envisioned and that specific isoforms can be directly produced depending on the processing enzymes present without the requirement for prior production of longer isoforms (Fig. 7).

Further complicating the situation, in 2011, Mesmin *et al.* detected intact/unprocessed forms of apelin-55 in both bovine colostrum and milk, with particularly high levels relative to other apelin isoforms in both pooled mature milk of 300 cows and in pasteurized commercial milk (178). This observation implies that apelin-55 may be present in other biological fluids and,

more importantly, demonstrates that apelin-55 can be secreted prior to processing. The direct secretion of apelin-55, in turn, indicates the potential for direct interaction with AR on the cell surface. This led us to hypothesize that apelin-55 may not function strictly as a proprotein. In testing this hypothesis, we found that apelin-55 has a similar potency to the shorter apelin-36 and -17 isoforms in terms of ERK phosphorylation for stably AR-transfected HEK 293T cells (235).

These results collectively indicate that apelin function and processing are dependent upon the active proteases available in a given environment. Logically, this implies that if available proteases vary between tissues or organs, the processing of apelin will correspondingly vary, leading to tissue- and organ-specific isoform production. This also suggests potential for extracellular processing, as processing enzymes can be released as soluble forms or membrane-anchored on the cell surface (229). In support of this, Tatemoto *et al.* detected apelin-36 in their mixture of bioactive peptides purified from bovine stomach tissues (249); conversely, Dray *et al.* detected only the Pyr-apelin-13 form in glucose-stimulated gastric secretion in mice (65). If apelin-36 were released from stomach cells, the differing isoforms detected intracellularly and extracellularly indicate that processing may occur extracellularly on apical membranes of gastric cells. To date, studies specifically demonstrating extracellular processing or the enzyme(s) involved in other apelin isoform (i.e. -36 and -17) productions have not yet been conducted. In summary, despite identification of both apelin-36 and -17 in physiological settings (both in stomach tissue (249) and elsewhere (*vide infra*)), the processing pathways by which these isoforms are produced have yet to be identified. This is a very important shortcoming in the current state of knowledge.

In addition to the various apelin isoforms that are obtained through proteolytic processing, apelin can be further post-translationally modified. One such modification, introduced above, is the spontaneous cyclization of the N-terminal glutamine of apelin-13 to pyroglutamate (236, 260), providing Pyr-apelin-13. This modified isoform has increased stability, as reflected in increased plasma half-life (312), likely through loss of the free primary N-terminal amine recognized by N-terminal exoproteases (261).

Another well characterized modification of apelin is the hydrolysis of C-terminal phenylalanine by angiotensin converting enzyme-2 (ACE2), a plasma metalloprotease, which removes the C-terminal phenylalanine of all apelin isoforms (264). Notably, removal of this phenylalanine has been linked to decreased cellular response in certain cases (264, 272), and has justly been attributed to be an apelin deactivation pathway and a key contributor to short half-lives observed for apelin peptides in circulation (272). However, discrepancies exist as to whether C-terminal truncation leads to significant decrease in physiological function and signaling biases (29, 284). As will be detailed in subsequent sections, the exact role of the C-terminal phenylalanine remains complex and somewhat controversial. Nevertheless, ACE2-mediated truncation can occur for all isoforms (apelin-13, -17, -36, and -55), as observed in mass spectrometric analysis of bovine colostrum and milk (178).

In light of potential activity upon C-terminal ACE-2 truncation, full deactivation processes likely involve more severe endo/exoprotease-mediated cleavage than the removal of a single

amino acid. In support of this, the zinc-dependent metalloprotease, neprilysin, was recently identified to cleave after RPR or RPRL in the “RPRL” motif (*vide infra*) of the conserved C-terminal 12 residues required for activity (175). This would lead to inactive N- and C-terminal fragments, as apelin peptides lacking up to three C-terminal residues (MPF) could not activate the receptor, as observed by Ca^{2+} mobilization assays, while peptides missing R, RP, or RPR residues of the RPRL motif could not decrease arterial blood pressure (250). Although only neprilysin has thus far been identified, other endo/exoproteases are likely to be involved in apelin processing (205). In support of this, shorter apelin fragments have been detected lacking various numbers of N-terminal residues were detected in bovine colostrum and milk (e.g. apelin-35, -18, and -15) (178), producing bioactive isoforms ranging beyond those first identified by Tatemoto *et al.* (250). In addition, isoforms lacking up to three C-terminal residues (i.e., the C-terminal MPF) have also been detected in colostrum and milk (178) as well as in plasma (185), suggesting that there are likely exoproteases other than ACE2 that are responsible for complete deactivation. Finally, additional degradation patterns are evident (185, 310), all of which provide clear evidence of a complex pathway modulated by numerous enzymes.

Apelinergic system expression in the body

Since the initial discovery of the apelin receptor (194) and then apelin (249) in the brain and stomach, respectively, the known tissue distribution of both the ligand and the receptor in the body have expanded to include the central nervous system, cardiovascular system, circulatory system, digestive system, reproductive system, and various peripheral tissues including adipose and skeletal muscles (69, 101, 128, 131, 145, 176) (Fig. 8A). The wide distribution observed of the apelinergic system in rats, mice, and humans has been reviewed in detail by O’Carroll *et al.* (192). In contrast to apelin, apela has thus far been found with a relatively limited distribution. Chng *et al.*, in the first report on apela, noted the presence of mRNA in adult human prostate and kidney (41). Since then, apela has been detected in adult hearts (203, 286), kidneys (61), and pluripotent stem cells (274). Although there are some differences between apelin and apela localization, as a whole, this is indicative of broad functionality for the apelinergic system.

In addition to expression in diverse tissues, the levels of apelinergic system ligands and the receptor can change with developmental state. During embryogenesis, apela and AR mRNA expression is enhanced, leading to apela-AR signaling which facilitates the migration of progenitor cells to the anterior lateral plate mesoderm through Nodal/ $\text{TNF}\beta$ -dependent signaling for proper cardiovascular development in zebrafish (41, 62, 199). However, levels of apela quickly drop subsequent to this and apelin mRNA levels are enhanced.

As mentioned earlier, the processing of apelin may vary between tissues or organs based on the active processing enzymes present in a given context, resulting in the potential for tissue-dependent preferential production of an apelin isoform (Fig. 8B). In support of this, dominant isoforms have been associated with various tissues using detection methods such as enzyme immunoassays (EIA) and radioimmunoassays (RIA) in combination with separation techniques such as gel exclusion chromatography (GEC) or high-performance liquid chromatography (HPLC). Through techniques of this nature, Pyr-apelin-13 was

identified as the predominant isoform in heart (169) and brain (60) while apelin-36 was predominant in lungs, testis, and uterus (128). Conversely, multiple dominant isoforms were shown to be present in or secreted from other tissues. For example, both Pyr-apelin-13 and apelin-36 are present at similar levels in mammary glands (128) and, as described earlier in depth, colostrum and milk contain apelin-55, -36, -17, -13 and many other modified isoforms (178). The physiological implications of ingesting milk containing multiple apelin isoforms, if any, are uncertain; however, mammary tissue clearly demonstrates production of a variety of isoforms. As a whole, tissues and organs demonstrate specificity in which, or even whether one, apelin isoform is predominantly produced and secreted over the others

At present, confidently stating the major form that is found in circulation is not straightforward due to discrepancies in the literature. Specifically, nearly all major isoforms have been identified in plasma through various combinations of techniques. The dominant plasma apelin isoform(s) have specifically been identified as: i) Pyr-apelin-13 using HPLC-MS (312); ii) Pyr-apelin-13 and -17 by GEC-RIA (60) and HPLC-RIA, with significantly less apelin-36 in comparison (10); iii) apelin-13 by GEC-HPLC-RIA (179); and, iv) isoforms longer than apelin-36 by GEC-RIA (76). It is interesting to note that identification of various isoforms in plasma is consistent with the results from colostrum and milk (178), which also demonstrated multiple isoforms. Thus, the discrepancies in the dominant form observed in plasma from study-to-study, and technique-to-technique, suggest that apelin can be secreted into biofluids other than milk without a requirement for intracellular processing.

The conflicting results observed in plasma apelin isoform levels likely stem from several possible causes, including as yet unknown physiological mechanisms such as the location of processing enzymes and regulatory molecules involved. However, these discrepancies may also arise from the technical limitations in the assay employed and/or physiological conditions in a given study. For example, the concentration of apelin can vary considerably over the range of picograms to nanograms per millilitre in the plasma of healthy subjects, as assessed through immunoassays (33). Alternatively, another study using HPLC-MS did not detect any apelin isoforms in plasma (89, 177). In combination, the development of novel techniques or improvements in current techniques in terms of both accuracy and precision are greatly warranted to reliably determine the predominant isoforms of apelin or, looking forward, apelin in plasma and other tissues/organs.

Role of apelin in physiological systems

Consistent with its diverse expression profile, apelin has been shown to play various physiological roles (Tables 3–12). As an example, multiple studies have examined the effects of intracerebroventricular (ICV) injection of apelin peptides, given the initial detection of AR in the brain. Apelin treatment caused changes in the level of fluid and food intake, implying that apelin-AR interaction centrally regulates physiological homeostasis. However, it is notable that role of apelin in fluid and food intake is controversial, since apelin treatments have demonstrated both positive and negative regulatory effects (Table 8). It should be noted that we have specifically tabulated the apelin isoform(s) examined in a given study, as this may specifically affect the resulting downstream function.

Since the discovery of apelin, the study of this bioactive peptide has most extensively focused around its roles in the cardiovascular system and associated diseases (recently reviewed by Yang *et al.* (285)), as summarized in Tables 3–5. Apelin is considered to be one of the most potent endogenous positive inotropes, as evidenced by its effects upon cardiac contractility and developed tension (245). In addition, apelin exhibits regulatory effects on blood pressure through endothelium- and nitric oxide synthase-dependent mechanisms (Table 3). In cardiovascular diseases, such as myocardial infarction, heart failure, and hypertension, apelin has a significant demonstrated protective effect. Specifically, apelin treatment resulted in reduced infarct sizes, improved cardiac parameters (e.g., cardiac output and contractility), decreased inflammation (e.g., coupled with tumor necrosis factor- α and interleukin-1 β), and increased cell viability (Table 4 and 5). Notably, apelin-independent AR activity has been linked to cardiovascular disease. For example, shear stress can activate β -arrestin signaling through the AR for endothelial cell polarization (137). Similar β -arrestin-mediated signaling has also been observed in cardiomyocytes in response to mechanical stretching, leading to myocardial hypertrophy (227). Although the role of apelin signaling in shear stress and endothelial cells is relatively unclear due to contrasting studies (27, 137), apelin peptide treatment was shown to prevent stretch-mediated cardiac hypertrophy through a G-protein-dependent mechanism (227). Considering all of these factors, the implication of apelin as a potential therapeutic target for multiple cardiovascular diseases has clear merit.

The role of apelin in regulating adipoinular axis function and ameliorating diabetes parameters has also been actively characterized, as detailed in recent reviews by Chaves-Almagro *et al.* (35) and Hu *et al.* (103). In one primary example, apelin has been shown to regulate glycaemia, likely through controlling glucose uptake, gluconeogenesis, and glycogenolysis (Tables 8–12). In addition, apelin can modulate both insulin and insulin receptor levels (79, 86, 219, 242, 313). The effects of apelin have also been extensively characterized in adipocytes, with apelin treatment significantly inhibiting white adipogenesis, decreasing free fatty acid release, and increasing brown adipogenesis (171, 254, 255, 296). All of these functional effects implicate apelin as a potential therapeutic target for diabetes. Correspondingly, apelin treatments in diabetic models decreased fat mass, hyperinsulinemia, and glycaemia while increasing pancreatic islet mass, mitochondrial biogenesis, fatty acid oxidation, and glucose uptake. Thus, apelin may also have a protective role against diabetes similarly to cardiovascular diseases.

It is important to note that the discrepancies in physiological functions mentioned above may be due to location of injection. For example, Reaux *et al.* and Cheng *et al.* employed an intravenous (IV) mode of injection, resulting in increased heart rate by apelin (40, 218). Dai *et al.*, conversely, observed a decrease in heart rate with ICV apelin treatment (56). In contrast, Kagiya *et al.* observed an opposite trend with ICV treatment in their experimental conditions, with the result being an increase in heart rate (125). Similar discrepancies have also been described in terms of hypotensive effects, where apelin treatments have been shown to both increase and decrease blood pressure (Table 3). Furthermore, apelin dosage-dependent changes have been demonstrated in cardiac contractility (190, 241), adding an additional layer of complication.

In functional modulation of the adipoinular axis, the role of apelin is also convoluted, with a variety of opposing and dosage-dependent effects having been noted for apelin. Specifically, Duparc *et al.* showed that low dose ICV (20 fmol) apelin treatment decreased glycaemia and improved glucose tolerance (68). Conversely, high dose ICV (40 fmol) treatment increased glycaemia (68). These findings illustrate the importance of dosage. Drougard *et al.* subsequently showed that a high dose treatment of apelin stimulated gluconeogenesis and glycogenolysis to cause hyperglycemia (66) and decreased energy expenditure and thermogenesis (67), which occurred in parallel to increased levels of reactive oxygen species (66) and inflammatory cytokines (67) in the hypothalamus. These studies, thus, indicated that central apelin administration at a high dose contributed to diabetes progression, while the opposite was true for low dosages. Furthermore, Ringstrom *et al.* observed that while high dose (1 μ M) apelin treatment resulted in a moderate increase in glucose-stimulated insulin secretion, low dose (10–100 nM) treatment robustly decreased insulin secretion (219).

The conflicting role of apelin in the adipoinular axis and in diabetes also translates to related diseases, such as diabetic nephropathy. As observed by Day *et al.* and Chen *et al.*, apelin can suppress of diabetes-induced glomerular hypertrophy, inflammation, and proteinuria (36, 58). This implies that apelin protects against development of diabetes nephropathy. However, Guo *et al.* and Zhang *et al.* observed an opposite trend, where apelin treatment aggravated the disease through abnormal glomeruli angiogenesis and increased proteinuria and glomerular permeability. Regardless, given the opposing effects of apelin observed in various diseases models, apelin has been suggested to play a “switch”-role in preventing or promoting disease progression.

Essential Components of Apelin

All isoforms of apelin introduced earlier and shown in Table 1 are capable of AR binding and activation (235, 249), consistent with a requirement for the C-terminal region of apelin for bioactivity. The importance of the entire C-terminal region is further implied by the absolute sequence identity over the 12 C-terminal residues of pre(pro)apelin for a wide variety of vertebrates (Fig. 6). Apelin-12, correspondingly, was shown to be an active isoform with greater potency than apelin-13 in reducing blood pressure in rats (250). A variety of strategies have subsequently been employed to further narrow the essential components of apelin for AR binding and activation.

As mentioned earlier, truncation at the N-terminus to isoforms shorter than apelin-13 demonstrated that such constructs are inactive. First, apelin-10 (a possible proprotein convertase product, based on basic residue positioning (230)) is inactive (59). Subsequently, apelin-11 alongside apelin-9 were also demonstrated to be inactive (250). Shorter isoforms were also investigated (176), with the ultimate finding that apelin cannot be N-terminally truncated to a form shorter than apelin-12 without loss of capability to bind AR and, correspondingly, signaling activity. Additional evidence for this requirement comes in consideration of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) use of the AR as a coreceptor for viral envelope protein binding to CD4. Although discrepancies are present, all isoforms of apelin from apelin-12 through to apelin-36 were

shown to inhibit HIV infection (319). This effect is highly isoform length-dependent with increased inhibition observed as a function of increasing isoform length. Notably, only apelin-36 consistently inhibited HIV infection in assays carried out by different research groups, while shorter isoforms varied in their inhibition (28, 315). Apelin-11 showed a complete lack of HIV inhibition, consistent with a lack of binding.

At the apelin-12 C-terminus, a number of potentially challenging to reconcile effects have been noted. This region of apelin has been proposed to penetrate deeply into the AR transmembrane helical bundle, thereby modulating the resulting downstream signaling (113). The recent crystal structure (166), as noted above, showed a lesser degree of penetration for the cyclic apelin-17 analogue employed for co-crystallization, with the reason for this discrepancy in penetration depth remaining unclear. Regardless of this uncertainty in TM domain ligand positioning in the active state (vs. inactive-like state of the crystallized AR), the fact that amidation of the apelin C-terminus leads to a 14-fold drop in *in vitro* affinity (124, 176) implies stringent steric requirements of apelin-AR interactions at the peptide C-terminus. Interestingly, this appears limited to the C-terminus itself, as comprehensive structure-activity relationship studies have demonstrated a great deal of latitude in sidechain size and functionality relative to the wildtype C-terminal phenylalanine without significant loss of binding affinity (186, 187). Despite the apparent relative plasticity to accommodate a wide variety of aromatic and aliphatic groups, the relative positioning of this sidechain is also essential as stereochemical inversion from L-phenylalanine to D-phenylalanine decreases affinity 20-fold (187). In rats, however, it should be noted that D-phenylalanine-apelin-13 still induced hypotensive effects associated with wildtype apelin-13 (145, 147).

Alanine substitution at the C-terminus of apelin has also led to some apparently contradictory results. In 2000, De Mota *et al.* reported that the F13A mutant of Pyr-apelin-13 abolished inhibition of forskolin (Fsk)-induced cAMP production in stably transfected CHO cells (59). Three years later, Fan *et al.* reported F13A-apelin-13 to have wildtype binding affinity and to be fully effective in intracellular Ca^{2+} mobilization and receptor internalization (72). In 2005, Lee *et al.* reported F13A-Pyr-apelin-13 to be antagonistic to hypotensive effects by *in vivo* (147). And, more recently, F17A-apelin-17 was shown by Iturrioz *et al.* to dramatically perturb internalization of the AR upon activation (113).

As a whole, these results appear challenging to reconcile. Adding to the picture, C-terminal truncation of apelin by up to two amino acids still allows for AR-binding and inhibition of cAMP production (70) as well as calcium mobilization (310). *In vivo*, the role of the C-terminal phenylalanine remains challenging to understand, as deletion of the C-terminal Phe removes the hypotensive ability of apelin-17 (70), just as with F13A-apelin-13 (147). But, Yang *et al.* found that Pyr-apelin-13 missing the C-terminal phenylalanine remained a potent inotrope and dose-dependently decreased blood pressure (284). At the signaling level, Ceraudo *et al.* showed that the presence or absence of the C-terminal phenylalanine of apelin-17 leads to biased signaling (29). Specifically, F17 of apelin-17 is not involved in $\text{G}\alpha_i$ activation or adenylate cyclase activation, but is critical for β -arrestin recruitment. This latter phenomenon is fully consistent with the perturbation of internalization noted for F17A-apelin-17 (113). Why this was not observed with F13A-apelin13 (72) is uncertain, but may

be reflective of differences either in the apelin-13 and -17 isoforms or in the specific assay conditions. The effects of substitutions and truncations at the apelin C-terminus therefore appear highly context-dependent and must be carefully and comprehensively characterized.

Further dissecting this minimally required core of 10 amino acids (e.g., apelin-12 with two residues truncated at the C-terminus), a number of substitution studies provide insight. Using alanine-scanning substitution (except at Pro and Gly), Medhurst *et al.* (based on numbering of apelin-13) identified R2, R4 and L5 as essential at all non-proline and –glycine residues (176). In the same year, Fan *et al.* showed decreased binding affinity for alanine-substitution at all positions except H7, P12 and F13; decreased Ca^{2+} mobilization with P3A, L5A, P10A and M11A; and, loss of internalization with R2A, P3A, L5A, K8A, P10A, and M11A alongside partial loss with R4A, S6A, and G9A (72). In D-amino acid scanning-substitution by Murza *et al.*, all sites were shown to elicit activity at 10 μM dose but significant losses in affinity were observed in the order $\text{R2} > \text{P3} > \text{P10} > \text{R4} > \text{S6} \sim \text{H7} \sim \text{K8} > \text{F13}$ with mild (2–11-fold) decreases for all other L- to D-amino acid substitutions (187). A number of structure-activity relationship studies (including those noted above regarding C-terminal substitutions) have built on these findings with additional non-natural amino acid substitutions and peptide functionalization, as recently reviewed by Narayanan *et al.* (191). In all of the studies to date, two primary regions of apelin appear critical: R2-P3-R4-L5 and P10-M11, with F13 being important both in binding affinity and in β -arrestin dependent signaling. For reference, these mutations have been summarized in Figure 9.

Structure-Function Correlation of Apelin

Using solution-state nuclear magnetic resonance (NMR) spectroscopy, we have compared both conformation and peptide backbone dynamics for all bioactive forms of apelin from apelin-55 to -12 (138, 235). Very notably, comparison of apelin isoforms demonstrated nearly identical chemical shifts for their shared C-terminal residues in solution at 37°C, suggesting that the chemical environment throughout the essential C-terminal region is independent of isoform length (235). This is consistent with a conformation that is independent of isoform length and with a lack of interaction between the N-terminal extensions of longer isoforms and the functionally critical C-terminal region of apelin. The independent behavior of segments within apelin means that features from biophysical studies on one apelin isoform should be directly applicable to the other isoforms up to apelin-55.

Apelin-17 has proven highly amenable to atomic-level conformational study (138). Shorter isoforms, conversely, tumble in a regime where the NMR phenomenon of the nuclear Overhauser enhancement used to obtain inter-atomic distance constraints for structural calculation is minimal in magnitude. Within apelin-17, at 35 °C, two relatively structurally converged four residue segments were observed within an otherwise disordered structure: K1-R4 and R6-L9 - the “RPRL motif” (Fig. 9). These NMR-derived data are also fully consistent with far-ultraviolet circular dichroism spectroscopy data, all of which imply an ensemble-averaged random coil conformation regardless of isoform length (72, 138, 235). Furthermore, the two structured regions in apelin-17 at 35 °C are also the regions of lowest conformational variability in longer apelin isoforms (apelin-36 and -55), further suggesting

that conformational properties of shared segments of apelin are applicable across the length range of apelin isoforms. Notably, ^1H - ^{15}N heteronuclear single quantum coherence spectroscopy-based assignment of four disparate sized apelin isoforms (apelin-55, -36, -17, and -13) at 37 °C demonstrated the presence of at least three exchanging conformations for the C-terminal GPMPF segment (235), further consistent with the lack of structural convergence in this region in the initial structure determination (138).

At low temperature (5 °C), apelin-17 became more structured (138). This is consistent with the stabilization of enthalpically favored nascent structure through decreased contribution of entropy to the Gibbs energy (reviewed in (139)). Under these conditions, the RPRL motif of apelin-17 becomes highly structured; the GPMPF region at the C-terminus also becomes convergently structured (Fig. 9). Notably, both of these regions of converged structuring correspond to regions shown to be essential for AR binding and/or signaling by the substitution and truncation studies detailed above.

In comparison to the structured motifs, the region spanning S10-K12 of apelin-17 showed significant dynamics, as observed by lack of superposable structure at either 35 or 5 °C (138). This was further evident in longer apelin isoforms, which indicate that this region acts as a flexible domain linking the structured regions (235). The N-terminal residues shared between apelin-36 and -55 also showed multiple potential conformations with practically indistinguishable chemical shifts, further emphasizing the applicability of biophysical data of shorter to longer isoforms of apelin. In combination, the similar conformations observed over all apelin isoforms regardless of length imply that all likely share a similar mode of receptor binding and/or activation and highlight the significance of the C-terminal residues and the potential consequences arising from mutation or truncation.

Within the RPRL motif, β -turn structure is prevalent and accentuated at low temperature (138). This is consistent with the hypothesis that β -turns are an important structural feature for recognition of peptidic ligands by GPCRs (259). Following from this finding, a number of apelin analogues were designed that induce β -turn structure in the RPRL motif. Macaluso and Glen showed that cyclized 6-7 residue truncated apelins with one additional residue N-terminal to and one to two residues C-terminal to the RPRL motif both induced β -turn formation and inhibited apelin-13 binding to AR (167). Extending upon this work, Macaluso et al. produced an apelin analogue with two cyclized β -turn RPRL motifs that acted as a competitive antagonist for the AR (168).

β -turn formation is also prevalent N-terminal to the RPRL motif, in the segment spanning the first four residues (KFRRQRPRL) of apelin-17. This would serve as the recognition site for PCSK3 processing to produce apelin-13. Interestingly, many PCSK3 substrates have β -turn conformation at or near their cleavage site (25). Notably, PCSK3-mediated processing did not produce any isoforms longer or shorter than apelin-13 (236) despite the numerous dibasic residue motifs and multiple β -turns present in the unprocessed apelin protein. This is especially hard to rationalize for apelin-17, as its cleavage site is also proximal to a dibasic residue motif a β -turn. Although it is not yet clear why apelin-13 is the only isoform produced by PCSK3, the observed β -turn conformations are likely an important factor for interaction with both the receptor and processing enzymes.

As noted above, the C-terminal phenylalanine of apelin is postulated to penetrate deeply into the AR transmembrane domain (113), albeit potentially shallower according to the AMG3054-AR crystal structure (166), with the capability to bias signaling upon its truncation (29). The relatively high degree of structuring of this region, particularly at low temperature (138), is consistent with a decreased energetic barrier for ligand-receptor interaction and immobilization upon binding (139). It is therefore notable that the C-terminal phenylalanine of apelin exhibits unusually intense far-ultraviolet CD spectral bands convoluting the ellipticity arising from backbone amides (138). Intriguingly, this phenomenon is observed in all isoforms from apelin-12 to -55 (235), with an appropriately decreasing intensity upon scaling to mean residue ellipticity due to the larger number of peptide backbone chromophores contributing to the ellipticity in the far-UV as the isoform length increases. The observed bands were unambiguously attributed to the C-terminal phenylalanine through alanine-substitution, both in apelin-13 (138) and apelin-55 (235). As a whole, this is consistent with elevated conformational restriction of the apelin C-terminal phenylalanine regardless of isoform length.

Each independently structured motif of the critical apelin C-terminal region therefore appears to have an important functional role and to behave highly similarly regardless of isoform length. The RPRL motif, especially when locked into a β -turn conformation, appears sufficient for binding (167, 168). In the GPMPF region at the C-terminus, retention of the methionine appears critical for $G\alpha_i$ activation (29, 70, 310), while retention of the C-terminal phenylalanine is essential for β -arrestin-mediated signaling and internalization (29, 113).

Corresponding to the importance of each motif in isolation, cyclization over a longer RPRL motif-containing segment of apelin-12, the R_1 PRLSHK₇ segment, while retaining the C-terminal GPMPF produced an AR agonist, albeit with an EC_{50} ~373 times that of Pyr-apelin-13 (91). Notably, however, cyclization at the C-terminus also produced an agonist with a somewhat lower EC_{50} (91). Effects on internalization were not examined as part of this study; therefore, based on the steric constraints at the apelin C-terminus (113, 124, 176), it seems likely that the analogue cyclized at the C-terminus would be biased towards G-protein activation and that its higher EC_{50} arises from a decreased affinity relative to Pyr-apelin-13.

Despite the importance of these motifs, binding and activation also depend upon the flexible linker region between them (of sequence SHK). This is implied both by the Ala-scanning study of Fan *et al.* (72) and by the D-scanning study of Murza *et al.* (187). Truncation to the octameric peptide Pyr-RPRLP-Nle-PF (where Nle is norleucine) and replacement of this linker segment by PEG or a tetra-Ala motif also greatly increased EC_{50} and obviated both $G\alpha_i$ and β -arrestin mediated signaling (187). Despite the sensitivity of this region to substitution, Murza *et al.* recently successfully developed an alternative strategy, where macrocyclic functionality is used to link the RPRL motif and PF of the GPMPF motif (189). Through a variety of structure-activity relationship studies in this macrocycle-linked context, a number of highly promising analogues with high affinity capable of eliciting biased signaling were derived.

In all, two primary and semi-independent functionally critical motifs in apelin have emerged through correlated consideration of structural features, substitution studies, structure-activity relationship studies, and functional assays. The RPRL motif, particularly in a β -turn conformation, appears sufficient for AR binding but not activation. This is consistent with one of the two observed AMG3054 binding sites in the co-crystal structure, where the AMG3054 segment analogous to the RPRL motif interacted with residues in the N-terminus and ECL2 of the AR. Notably, conformational restriction of the RPRL motif does not prevent AR activation, implying that significant conformational change within this region is not required following binding. Conversely, for activation, a C-terminal extension beyond RPRL up to at least the methionine of the GPMPF motif appears essential for $G\alpha_i$ signaling. This is consistent with both the AMG3054-AR crystal structure, where the cyclized C-terminal segment of AMG3054 penetrates into and binds within the TM domain of the AR. Full extension to the C-terminal phenylalanine, or a variety of sterically bulky and stereochemically appropriate substitutions, appears key for β -arrestin-mediated signaling and internalization.

The behavior of this pair of motifs brings to mind a fly-casting type mechanism (237). Specifically, the RPRL motif would serve as an “anchor” for a given apelin isoform, with the C-terminal tail then sampling various conformations and interactions with AR in a restricted manner until finding the ultimate binding site. This may be similar in posture to the AMG3054-AR interactions within the inactive-like AR TM domain or, perhaps, an additional conformational rearrangement within the TM domain may be required allowing a deeper ligand penetration. The fly-casting mechanism, in turn, provides a context for both the bioactivity of and variation in functionality observed with longer apelin isoforms. Provided that an additional N-terminal extension to apelin does not occlude the binding site of the apelin C-terminus, nor does it lead to intramolecular interactions that modulate its conformation, a fly-casting scenario would still be fully valid for apelin-AR activation following binding through the RPRL motif. An N-terminal extension in longer apelin isoforms would then provide for the increased affinity noted in some studies (*vide infra*), given the potential for an increased apelin-AR complex stabilization through favorable intermolecular interactions between the apelin N-terminal extension and AR extracellular domain (e.g., unoccupied surface grooves noted in the ECD by Ma *et al.* (166), Fig. 3)

Pharmacological differences between apelin isoforms

Although all apelin isoforms likely activate the AR in a similar manner given their common C-terminal conformations (138, 235), the initial report by Tatamoto *et al.* demonstrated length-dependent potency, with apelin-13 and Pyr-apelin-13 being the most potent isoforms in cellular acidification assay (249). Since then, numerous studies have identified shorter isoforms, typically apelin-13, as being more potent in activating various signaling pathways. As an example, apelin-13 was shown to be more potent than apelin-36 in stimulating gastric cell proliferation in STC-1 cells (270). Furthermore, only apelin-13 enhanced cell proliferation while apelin-36 did not in RF/6A cells (127), demonstrating that isoforms can differ in both potency and efficacy.

Although length-dependent potencies and/or efficacies were observable through various assays, many also showed discrepancies, bringing controversy into the concept of ligand-dependent properties. For example, in terms of inhibition of forskolin-stimulated cAMP production, Pyr-apelin-13 was identified as a more potent isoform than apelin-36 (89, 128), while no difference in potency was observed between Pyr-apelin-13 and -17 (59). Conversely, Medhurst *et al.* identified no significant differences in potencies between apelin-13, Pyr-13, -17, and -36 (176). In chemotaxis, both Pyr-apelin-13 and apelin-36 could cause cell migration in CHO cells expressing AR, with increased potency of Pyr-apelin-13 in comparison to its longer counterpart. For RF/6A cells and lymphatic endothelial cells, however, apelin-13 and Pyr-apelin-13 showed comparable potency to apelin-36 in terms of enhancing cell migration (127, 226). In terms of ERK signaling, we found Pyr-apelin-13 to be significantly more potent in its capacity to phosphorylate the signaling molecule in comparison to its longer counterparts (-17, -36, and -55) (235). However, Gerbier *et al.* observed no significant differences between Pyr-apelin-13 and apelin-17 under their ERK assay conditions (81). Furthermore, despite the potential for potency differences, all isoforms have been shown to exhibit similar efficacy in terms of ERK signaling (235, 238). As a whole, this implies that both assay conditions and the reported parameters must be chosen with care.

Length-dependent properties observed at the cellular level also translate to physiological function. For example, in the cardiovascular system, apelin-13 was shown to be more potent than apelin-36 in decreasing blood pressure (250). However, the discrepancies observed in cellular assays also translate to organism-level studies. In terms of hypotensive action, apelin-17 was shown to be more potent than Pyr-apelin-13 (70), demonstrating that shorter isoforms are not necessarily more potent. Further complicating things, apelin-13 and -36 were shown to have a comparable hypotensive effect in rats alongside a similar inotropic effect (169).

Regardless of the discrepancies observed between assays, the demonstration of isoform-dependent pharmacological and physiological properties indicates that apelin processing may serve as a potential mechanism for regulation of apelin function. On this note, Adam *et al.* showed that both apelin-13 and -36 could inhibit thrombosis, but that an apelin-36 mutant that was incapable of being processed by PCSK3 could not (1). This finding suggests that apelin-36 must be processed in order to inhibit thrombosis. However, it is important to note that the effect of apelin on thrombosis is actually somewhat controversial as apelin-13 treatments result in increased tissue factor expression, consistent with a prothrombotic role for apelin (47). Despite this complication, it seems plausible that differential apelin processing is a potential underlying cause for discrepancies between isoforms mentioned above.

Post-translational modification of apelin isoforms (e.g., processing and deactivation processes) may be responsible, at least in part, for the discrepancies discussed above. Since modification may contribute to variations in the half-lives of apelin isoforms (272), the concentrations of all available apelin isoforms will be regulated by available endo- and exoproteases in extracellular environments such as blood/plasma. Thus, depending on the physiological environment, concentrations of various apelin isoforms may be expected to

change rapidly, in turn altering the observed potency. However, it is unlikely that processing will occur rapidly under *in vitro* assay conditions, as verification of peptide stability should be provided as a routine control under the given experimental conditions. Differences in half-lives of the various apelin isoforms between *in vivo* and *in vitro* assay conditions may, thus, also be responsible for the discrepancies observed. This fact serves to reiterate the importance of carefully choosing assay conditions and parameters.

Another cause for discrepancies may involve AR-independent mechanisms. Picault *et al.* showed that apelin-13, Pyr-13, and -36 could all inhibit apoptosis in LoVo cells through deactivation of caspase-dependent pathways (206). However, in retinal ganglion cells, N-methyl-D-aspartic acid (NMDA)-induced cell death could be inhibited by apelin-36, but not by apelin-13 (220). Interestingly, in this study, Sakamoto *et al.* noted that inhibition of cell death was likely through a mechanism independent of the AR, as inhibitors of AR and various protective signaling pathways of the AR did not impair apelin-36 activity. Based on these results, Sakamoto *et al.* suggested that apelin-36 may protect against neuronal cell death by directly influencing a non-AR receptor (i.e., the NMDA receptor in their case). Although the potential for apelin interaction with the NMDA receptor remains uncharacterized, involvement of AR-independent mechanisms may also be a plausible source of literature discrepancies in apelin function.

Adding support to the potential of another receptor for apelin, Galon-Tilleman *et al.* also showed that apelin-36, introduced through use of adenovirus to achieve lasting systemic expression, reduced cholesterol levels, glycaemia, and body weight (80). Conversely, systemic expression of apelin-13 or -17 resulted in low or negligible effect using the same model. Thus, this study indicated the potential for another membrane receptor that recognizes and is activated by the N-terminal residues of apelin-36. However, the presence of another receptor is rather controversial since treatment with apelin-13 or -17 peptides has been shown to similarly reduce glycaemia (64) through increased uptake of glucose (3, 9, 64, 77) and also to decrease body weight (30). Galon-Tilleman *et al.*, rightly, highlight that differences in AR oligomerization state or other intermolecular interactions, in turn being modulated differently by apelin-36 vs. by shorter isoforms, may be the source of the differences observed.

In short, although there are potential discrepancies in function between apelin isoforms, the number of publications that have relied upon the use of one isoform (usually either apelin-13 or Pyr-apelin-13) is staggeringly greater than the number of publications that have compared the properties or effects of multiple isoforms. Given that there may be pharmacological differences – in some cases quite dramatically so – between these isoforms, the exclusive use of one isoform is worrisome. Serious critical thought should be given to the importance of comparison of multiple isoforms, particularly if the relative levels of physiologically-relevant isoforms in a given setting are unclear.

Differences in receptor regulation by apelin isoforms

The differences between the pharmacological properties of apelin isoforms are difficult to rationalize in terms of their affinities for the AR (Table 13). Theoretically, if the N-terminal residues impeded binding, then the affinity of longer isoforms should decrease with a

corresponding decrease in potency. Conversely, the observed increased affinity upon N-terminal extension of apelin to longer isoforms should increase potency. However, experimental data are suggestive of a perplexing combination of these two hypotheses. Some studies have noted a more stable interaction (101, 128) and higher affinity (112, 128) between the longer apelin isoforms and the AR compared to shorter isoforms, while potency has been noted to be greater for the shorter isoforms, as observed through the various assays as introduced above. As a whole, these findings indicate that potency is likely not limited to interaction between ligand and receptor, and indicates the existence of another mechanism (or more than one alternative mechanism) to regulate the activity of the apelinergic system.

Although it is not clear exactly what mechanisms underlie the variation observed between isoforms, one of these may rely upon variation in ligand-dependent receptor internalization and regulation. This was clearly demonstrated by Hosoya *et al.*, who showed that apelin-36 remained strongly associated with the AR through the demonstration that radiolabeled apelin-13 did not displace AR-bound apelin-36 in CHO cells while AR pre-treated with apelin-13 readily bound radiolabeled apelin-13 (101). This study indicated that the interaction between the longer apelin-36 and the AR is stronger than that between apelin-13 and the AR. This, logically, led to the hypothesis that a stable complex formed between apelin-36 and the AR would be likely to lead to receptor internalization with subsequent regulation of signaling. Conversely, weak binding between apelin-13 and the AR would allow for rapid disassociation of the ligand from the receptor, allowing for receptor recycling to the cell surface.

In support of this hypothesis, using HEK cells stably expressing fluorescently tagged AR, Lee *et al.* clearly showed that receptor regulation (i.e., the balance of cell surface recycling vs. degradation) is ligand-mediated (146). Specifically, apelin-13 treatment resulted in agonist-mediated internalization of the AR to intracellular vesicles. However, upon agonist washout, the activated AR reappeared at the cell surface within 1 hour. Apelin-36 treatment resulted in similar internalization of AR. In contrast to apelin-13, however, apelin-36 treatment did not result in AR recycling back to the cell surface even after agonist washout. Analysis of the receptor regulation pathway demonstrated that apelin-13 activation resulted in recruitment of β -arrestin to the cell surface, but that the arrestin molecules did not internalize as a complex with the AR. Apelin-36 also recruited β -arrestin, but β -arrestin remained associated with the receptor during internalization. Similarly, Evans *et al.* demonstrated that the AR recruited β -arrestin to the cell surface upon activation by apelin-13 (71). Notably, they detected internalization of apelin peptides, but did not observe significant alterations in the distribution of β -arrestins. This supports the concept that AR activated by apelin-13 undergoes endocytosis but dissociates promptly from β -arrestin close to cell surface facilitating rapid recycling of the receptor back to the cell surface.

In addition to relying upon β -arrestin, AR internalization has been shown to be clathrin-dependent (70, 212), as pretreatment with hypertonic sucrose to block clathrin-mediated endocytosis inhibited receptor internalization. Furthermore, clathrin-mediated internalization likely also involves the transferrin receptor, since co-localization of the AR and transferrin receptors were observed intracellularly (314). Like other GPCRs, the AR depends on Rab proteins for receptor regulation, where the receptor is internalized with Rab5 for endosomal

trafficking as observed by co-localization of fluorescent AR and Rab5 by confocal microscopy. Interestingly, Rab4 was required for recycling of agonist-activated AR from intracellular vesicles (146). This was evident as cells expressing an inactive Rab4 mutant could not recycle the AR back to the cell surface even for apelin-13 treated cells. In contrast, Rab7 was recruited for receptor trafficking to the lysosome for degradation, as constitutive Rab7 expression directed the AR to lysosomes while inactive Rab7 expression resulted in no co-localization with the AR.

Notably, the differential internalization observed between ligands also translates to receptor desensitization (42, 174). By probing for cAMP inhibition and ERK phosphorylation, Masri *et al.* showed that apelin-36 treatment resulted in significantly diminished level of inhibition in response to repeated exposure (174). Conversely, a similar loss in signal with repeated exposure was not observable when treated with apelin-13 (174). Furthermore, a similar desensitization was observed through a Ca^{2+} response assay in NT2.N neurons (42).

It is important to note that the four N-terminal residues of apelin-17 (KFRR) may also play a major role in controlling the downstream effects of apelin. Using internalization assays, El Messari *et al.* observed significant differences between the potencies of apelin-13 and -17 toward induction of AR internalization (70), suggesting that the KFRR motif regulates receptor internalization. Given the similar conformation shared for the 12 core C-terminal residues (138, 235) and the two-site binding mode detailed above, these N-terminal residues are likely responsible for stabilizing the ligand-receptor interaction. Furthermore, the stabilization likely arises from electrostatic interactions between positively charged N-terminal residues of apelin isoforms and key negatively charged surface residues of the AR (82, 113, 143, 316), as mutations of these key acidic residues on the receptor results in loss of binding. This is consistent with some of the observed AMG3054 interactions with the AR N-terminus and ECL2 segments, with the presence of anionic grooves on the receptor surface likely to provide additional sites for electrostatic stabilization of apelin-AR binding (166). Furthermore, given the isoform-dependent affinity and variable regulatory binding partners, it seems likely that both ligand-induced receptor conformation through allosteric effects and the resulting receptor dynamics would vary as a function of isoform. Such variation offers biophysical justification for isoform-dependent preferential interaction with regulatory molecules.

In summary, the longer apelin isoforms lead to β -arrestin binding to the AR, causing internalization and degradation of the AR; apelin-13, conversely, allows for disassociation of the receptor from β -arrestin and subsequent recycling of the receptor following internalization. Based on these findings, Lee *et al.* hypothesized that the levels of different apelin isoforms available may control the level of AR on target cell surface, i.e., the level of receptor desensitization (146). Correspondingly, isoform-dependent potencies may in part be affected by this differential regulation, since receptor desensitization will result in a higher dose requirement of apelin for function. Conversely, potency may increase for β -arrestin signaling for longer peptides. Given that a more stable interaction is promoted with arrestin molecules, potency will be improved to reflect increased binding. In support of this, El Messari *et al.* demonstrated that apelin-17 was more potent in causing β -arrestin-mediated decrease in blood pressure in comparison to Pyr-apelin-13 (70). In combination, isoform-

mediated differences are likely due to intricate differences in receptor interaction, leading to downstream effects upon binding partner interactions and signaling cascades. It is also interesting to note that if the ratio of long to short apelin isoforms can determine the level of AR desensitization, then this ratio will directly determine whether apelin treatment plays a physiological or pathological role.

Apelin conformation in membrane-mimetic conditions

Another mechanism that may functionally differentiate apelin isoforms is differences in interaction with biological membranes. The membrane catalysis hypothesis, developed by Sargent and Schwyzer (224), states that ligand-receptor binding will be preceded by an initial step of ligand-membrane interaction (Fig. 10). This initial peptide-membrane interaction restricts diffusion of the ligand from a three-dimensional to two-dimensional process, substantially improving the probability of a diffusional ligand-receptor encounter. A favorable peptide-membrane interaction also serves to increase the local concentration of the peptide near the cell surface receptor, further increasing the probability of ligand-receptor interaction. Finally, membrane interaction may also induce structural changes within the ligand, with this membrane-induced conformation being favored for ligand recognition by the receptor (for a detailed review, see (142)). All of these factors will result in an enhanced rate of ligand-receptor binding and lead to the designation of the cell membrane as a “catalyst” for the binding event. Consistent with this theory, numerous peptide hormones, including both apelin and apela, have been shown to interact with membrane-mimetics such as micelles, leading to changes in both structure and dynamics of the peptide (49, 105, 141, 142, 160, 170, 184) (Fig. 10).

Both structural and dynamic changes have been characterized at the atomic-level for apelin in the presence of membrane-mimetic micelles (141). Of the apelin isoforms characterized under membrane-mimetic conditions thus far, the most extensive characterization has been for apelin-17, following from its amenability (as noted above) to NMR characterization in solution (138). In the presence of anionic micelles, composed of either sodium dodecyl sulfate (SDS) or of 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (LPPG), apelin-12, -13, -17, and -36 all showed spectral perturbation by far-UV CD spectroscopy, indicative of membrane-induced conformational change. However, in the presence of zwitterionic dodecylphosphocholine (DPC) micelles, apelin isoforms showed relatively limited changes by CD spectroscopy. These global conformation data indicated a preferential interaction between apelin and anionic headgroup micelles, which is consistent with the high number of cationic residues found in apelin (Table 1).

The preferential interaction was further validated using pulsed field gradient NMR spectroscopy-based diffusion ordered spectroscopy (DOSY) experiments (183) for direct measurement of the translational diffusion rate of a given species. Comparison of the translational diffusion rates of the ligand and the micelle in isolation vs. when mixed, in turn, allows quantitation of the fraction of ligand bound to the micelle. Simply put, a decreased diffusion rate for apelin in the presence of a micelle relative to the free state would be representative of an interaction between apelin and micelles leading to a decrease in its translational motion. By DOSY, the diffusion of apelin-17 was decreased in the presence of

anionic SDS and LPPG micelles, but was less impeded in the presence of zwitterionic micelles. Thus, apelin-17 quantitatively demonstrated a preference for binding to anionic micelles over zwitterionic micelles by DOSY in agreement with the structural perturbation implied by CD spectropolarimetry (141).

Using solution-state NMR spectroscopy, the high-resolution structure of apelin-17 was determined in the SDS micelle-bound state. Highly converged structuring was observed over the segments R6-K12 and M15-F17 (Fig. 9). Notably, R6-K12 spans the RPRL motif (R6-L9) previously noted to be conformationally converged in buffer. Strikingly, the number of nuclear Overhauser enhancement distance constraints providing evidence of short-range inter-atomic distance constraints was increased ~2-fold in the presence of micelles vs. in buffer. This is indicative of a significant enhancement in structuring over this region with β -turn formation propensity in apelin-17 in the presence of membrane-mimetic micelles. Conversely to the RPRL motif, despite becoming highly structured upon micelle binding, the M15-F17 region was not structurally converged in buffer at 35 °C. This segment of apelin-17 is therefore consistent with the membrane catalysis hypothesis, where induced structuring by the apelin-micelle interaction is consistent with a unique structuring postulated to facilitate recognition by the receptor.

Paramagnetic relaxation enhancement (PRE) NMR experiments were also employed to differentiate between regions of apelin-17 that were solvent exposed residues vs. those localized on the micelle surface and/or penetrating into its hydrophobic core. PRE clearly demonstrated that, of the two structured motifs, the RPRL motif interacted with and was bound to the micelle surface while the M15-F17 region was solvent exposed. Akin to the fly-casting mechanism introduced above, albeit in a different context, this suggests that apelin may indeed use the RPRL motif as an “anchor”, which undergoes a conformational change to secure the peptide on its target membrane and prevent dissociation. Structuring of the C-terminal MPF domain may then serve to prime the ligand for enhanced receptor interaction.

It is important to note that membrane-induced structuring may have a broader context than simply enhancement of receptor interaction. Membrane catalysis may also be critical for promoting and modulating interactions of apelin with binding partners other than the AR, including the PCSKs. In support of this contention, PCSK3 and a number of other PCSKs are membrane-anchored (229); thus, apelin-membrane interactions may serve to enhance the probability of encounter between a given PCSK and an apelin isoform containing PCSK recognition sites. Furthermore, the increased β -turn structuring in the RPRL motif in the presence of membrane may serve to further enhance PCSK-mediated processing. In the case of apelin-17, the putative PCSK recognition site giving rise to apelin-13 cleavage (KFRRQRPRL; recognition site underlined) is not in direct contact with the micellar headgroup, meaning that its processing should not be impeded by an apelin-membrane interaction.

Although the membrane-interactive properties of apelin isoforms longer than apelin-17 have not yet been characterized, the conformational restriction observed in the 17-residue isoform is within the core 12 C-terminal residue region required for receptor binding and activation;

thus, longer isoforms would be hypothesized to share a similar membrane binding capability to apelin-17. However, if the N-terminal residues are in contact with the membrane surface and undergo a conformational change in response to this interaction, this may impede both peptide processing and receptor binding through restricted availability of a given binding site.

As a further regulatory mechanism, discrepancies may also exist between isoforms in their membrane headgroup preference (as we saw with apela, detailed below), with such differences affecting the pharmacological properties of the ligand in various ways. In terms of membrane catalysis, variation in the local concentration of a ligand near its membrane-spanning or -anchored binding partners would affect its potency, albeit not in a trivially predictable manner. In addition, given that the physiological function of apelin can vary depending on dose, modulation of local concentration would also affect the final physiological function. Furthermore, membrane interactions may also affect the localization of a ligand inside the body, where the membrane composition of a target cells, tissues, or organs may control the local concentration of the ligand and downstream effect. All of these possibilities provide a strong incentive to characterize the membrane interactions longer apelin isoforms and of bioactive peptides more generally.

Apela

For 15 years, apelin was considered to be the only ligand for the AR. In this period, APJ was even renamed after the ligand, which had, in turn, been initially named for APJ. However, one clear line of evidence had always suggested that there was more to the story. Specifically, AR knockdown caused impairments in cardiovascular development during the embryonic stages; however, these impairments were not observed upon apelin knockout (34, 198, 228, 300). Based on these discrepancies, AR signaling was speculated to occur through either an apelin-independent mechanism or to be mediated by an alternative ligand during embryonic development. Using zebrafish as a model, two research groups independently identified a new ligand for the AR nearly simultaneously in 2013. This ligand was named differently by the two groups, as ELABELA (41) and Toddler (199). The recommended name for the gene is now “*APELA*”, coming from *Ap*elin *R*eceptor *E*arly *E*ndogenous *L*igand (6). In keeping with this recommendation, we refer to the peptides resulting from processing of this gene product as apela herein. It should be explicitly noted, however, that the majority of literature still refer to this peptide as ELABELA, often shortened to ELA.

The *APELA* gene, located on chromosome 4, was initially identified as producing a non-coding RNA (41, 199). However, analysis by Chng *et al.* and Pauli *et al.* independently identified the gene as encoding a 54-residue long preprotein in humans (58-aa in zebrafish), with an N-terminal α -helical hydrophobic 22-residue signal peptide (41, 105, 199) (Table 1 and Fig. 11). Upon removal of the signal peptide, the longest bioactive isoform, apela-32, is produced. As in apelin-55, analysis of the amino acid sequence of apela-32 demonstrates the presence of multiple dibasic sites, indicating potential proprotein convertase-mediated processing to yield 22, 21 or 11-residue isoforms (apela-22, -21 and -11) (Table 1). All isoforms contain a highly conserved set of 7 C-terminal residues (Fig. 11).

Of these isoforms, only apela-11 has been endogenously observed via mass spectrometry in embryos expressing apela mRNA (199). However, other isoforms likely do exist *in vivo*, as incubation of apela-32 in rat plasma produced an N-terminal domain containing the first 9 residues and apela-22 (188). This is suggestive of cleavages between R9 and R10 and between R10 and K11. Although this indicates the potential production of apela-23, it has not yet been determined whether apela-23 is produced or whether the N-terminal domain released from apela-22 is further processed by exoproteases. Regardless, this result indicates the presence of processing enzymes in plasma. Corresponding to the relatively recent identification of apela, no enzymes have yet been implicated in apela processing and/or deactivation. Furthermore, it is entirely possible that apela isoforms other than those identified as following dibasic sites corresponding to PCSK-mediated cleavages may exist physiologically. Apela-32, for example, has an N-terminal glutamine as with apelin-13. This residue, similarly to apelin-13, spontaneously converts to pyroglutamate (105, 188), which likely increases the stability of this isoform.

One feature that clearly differentiates apelin and apela is the absence vs. presence, respectively, of cysteine residue(s) following signal peptide cleavage. In apelin, as detailed above, cysteines are located only in the signal peptide, resulting in dimer formation potentially important for signal peptide processing (147). In apela, conversely, the putative 22-residue signal peptide does not have any cysteines. Rather, apela-54 contains two highly conserved cysteines at positions 39 and 44 (41, 199) (Fig. 11). This allows for potential intra- (for apela-32) and inter-molecular disulfide bridge formation. In support of this, apela-11 spontaneously dimerizes (105). However, it is presently unclear what role dimerization plays in apela peptide function, as substitutions that prevent disulfide bridge formation resulted only in minor conformational differences in apela-11 (105) and did not significantly affect the potency of apela-14 analogues via AR-mediated G-protein-dependent or independent pathways (188). It is also unclear whether disulfide linkages occur physiologically. *In vitro*, neither HPLC nor mass spectrometry showed evidence of intramolecular or intermolecular disulfide bridge formation in apela-32 in recombinant production and purification (105), but minor differences by far-UV CD spectropolarimetry were observable between reducing and oxidizing conditions suggestive of disulfide bridge formation. Thus, further studies are required to characterize the dimerization state of endogenous apela isoforms and the physiological purpose that these cysteine residues may play.

Physiological effects of apela

As introduced above, apela was initially discovered as a second ligand for the AR that is expressed during embryogenesis and is important for cardiovascular development. Specifically, finely regulated control of apela levels was shown to be essential as its absence or overproduction led to inappropriate migration of mesendodermal cells during zebrafish gastrulation (199). Its expression was also noted to be tied directly to developmental stage in zebrafish, as apela expression was upregulated during embryogenesis and development, vs. in adults where apelin expression was upregulated (41, 199). Furthermore, supplementation of apelin in apela knockout zebrafish could rescue the mutant phenotype (199), suggesting that apelin and apela have overlapping functions mediated by the AR during embryogenesis

and that their difference in bioavailability is the reason for discrepancies in phenotypes between apelin and AR knockout models.

Exogenous treatment using apela peptides results in similar downstream physiological effect to apelin, which is perhaps expected as both ligands activate the AR. For example, apela can regulate blood pressure (188); fluid homeostasis (61); food intake (223); cardiac contractility (203); and, angiogenesis and vasodilation (274) (Table 14). In addition to these functions, apela activates the canonical downstream signaling molecules that have been associated with apelin-mediated signaling, including G-proteins (61, 188), ERK (61), and β -arrestin (188). Thus, it is likely that apela activates signaling pathways in a similar, or like, manner to apelin to exert the corresponding physiological effect. Consistent with this, apela-11, the isoform with the lowest potency for β -arrestin recruitment, showed the lowest capacity to decrease mean arterial pressure (MAP) (188). This corresponds to previous work showing that β -arrestin activation and the resulting AR internalization regulate MAP (70, 147).

Despite these apparent similarities in apelin and apela function, some clear differences are also apparent. For example, apela triggers vascular relaxation in a nitric oxide-independent manner (274). This was clearly evident as relaxation still occurred even when blood vessels were treated with the nitric oxide inhibitor L-NAME (274). Apela and apelin also differ in their potencies with respect to particular physiological effects. Apela-32, for example, bound to the AR with increased affinity, increased ERK phosphorylation response, and had a greater impact on fluid homeostasis than apelin-13 (61). Similarly, Yang *et al.* demonstrated that apela-32 was more potent than apelin-13 in inducing cardiac contractility and output (286).

It is also highly noteworthy that apela appears likely to have another receptor that it may signal through. In human pluripotent embryonic stem cells (ESCs), apela inhibited cell apoptosis and promoted self-renewal (98). However, these undifferentiated stem cells did not express the AR. Only after differentiation, approximately three days later, was AR expression detected. This is highly suggestive of the presence of an additional, as yet unknown, receptor for apela. Indeed, pre-treating these cells with trypsin resulted in decreased apela binding, supporting the hypothesis that apela binds to another protein on the cell surface. Moreover, exogenous apela treatment resulted in clear internalization of the peptide hormone, as observed by intracellularly-localized biotin-labeled apela. In addition, treatment with methyl- β -cyclodextrin, an inhibitor of clathrin-independent endocytosis, prevented internalization. All of these data suggest the presence of a non-AR receptor for apela. The presence of an additional receptor may also correlate to the differences in amino acid composition between apelin and apela (Table 14 apela function), which may serve to facilitate the prevention of apelin binding to this unknown receptor. At the present level of knowledge, this mechanism remains hypothetical.

Rather compellingly, apela can have varying effects on ESCs derived from different species. As an example, human and murine pluripotent ESCs respond differently to exogenous apela treatment. In human ESCs, apela-32 treatment inhibited apoptosis through the unknown receptor noted above (98). In mice, apela may cause apoptosis in a non-peptidic manner. Specifically, Li *et al.* identified *Apela* RNA as a regulatory RNA to control DNA

damage-induced apoptosis (152). By binding to heterogeneous nuclear ribonucleoprotein L (hnRNPL), *Apela* RNA freed p53 from the hnRNPL/p53 complex and allowed apoptosis to occur.

In combination, although apela was initially identified as playing a major role during development, it is not necessarily limited to this role. Furthermore, apela expression in adults may be limited to specific tissues/organs unlike the more ubiquitous apelin, but it still appears to share some functions with apelin, with the potential to act in conjunction with apelin. Thus, the discovery of the apela peptides provides an alternative peptide family with potential therapeutic application highly similar to apelin, expanding the potential target range for medicinal chemistry efforts.

Correlations between activity and essential components of apela isoforms

Given the relatively recent discovery of apela, this ligand has not yet received a similar level of scrutiny as apelin in terms of determination of key residues and motifs. Despite this, the currently published studies clearly demonstrate that apela exhibits isoform-dependent differences in potency and efficacy. Key residues and domains have also been delineated that control its potency and efficacy. Pharmacologically, apela-32 presented significantly greater potency compared to apela-11. Specifically, in terms of AR binding affinity, G-protein interaction, and β -arrestin recruitment, apela-32 exhibited ~40-, ~8-, and ~15-fold increases, respectively, relative to the apela-11 isoform (188). In contrast, apela-32 and -21 showed similar binding affinity for the AR (61). Characterization of isoforms shorter than apela-11 has not yet been reported in terms of determination of the shortest apela isoform capable of AR interaction and activation.

The amino acid compositions of apelin-36 and apela-32 are relatively similar, with both peptides being basic and relatively hydrophilic (Table 1 and Fig. 12). Comparative analysis of the apela and apelin primary structures demonstrates the presence of several conserved residues that are similar in position (Fig. 12A). Based upon characterization already carried out for apelin, several residues were speculated to be key modulators of receptor interaction (61, 188). For example, residues Q41, R42, and R43 directly N-terminal to the apela-11 cleavage site (based on apela-54 numbering, and falling at the N-terminus of “apela-14”) bring to mind the N-terminal QRPRL region of apelin-13, containing the critical RPRL motif. Interestingly, Murza *et al.* demonstrated that apela-11 was nearly 15-, 3-, 23-fold less potent than apela-14 in terms of AR binding affinity, G-protein binding, and β -arrestin recruitment, respectively (188). Similarly, Yang *et al.* showed that apela-32, -21, and -14 were significantly more potent than apela-11 in terms of AR binding and β -arrestin recruitment (286). In contrast, apela-14 only exhibited relatively minor losses of binding affinity, namely ~5-fold (188) and ~2-fold (286), relative to apela-32. These findings suggest that apela-AR binding is likely enhanced by the QRR segment at the N-terminus of apela-14. In support of this, alanine substitutions resulted in statistically significant loss in receptor binding affinity for these arginine residues, but not for the N-terminal glutamine (188). As with apelin, the residues involved in apela-AR interaction thus seem likely to be localized to the C-terminal region, with optimal apela-AR binding being dependent upon basic residues.

R2 and R3 in apela-14 (i.e. R42 and 43 in apela-54) also resemble the diarginine motif directly N-terminal to the apelin-13 cleavage site (KFRRQRPRL). Using an In-Cell Western-based ERK phosphorylation assay, we noted that these residues could regulate potency, as observed in comparison of apelin-13 and -17 (235). Specifically, extension from apelin-13 to -17 resulted in decreased potency in terms of ERK signaling (235), while previous studies demonstrated enhanced binding affinity and β -arrestin recruitment (70). Thus, whether this diarginine motif in apela isoforms play a role more closely tied to the arginine residues in the KFRR or QRPRL motifs of apelin remains to be determined. Regardless, basic residues in close proximity in the N-terminal extensions of both apelin and apela beyond the shortest bioactive isoforms appear to play important regulatory roles between isoforms in terms of both AR binding affinity and subsequent β -arrestin recruitment. This may, in turn, directly relate to the anionic binding grooves recently noted on the AR ECD (166).

Alanine-scanning dissection of apela-14 also identified several other residues of importance in AR binding and signaling, the most critical of which were located in the C-terminal half of the peptide (61, 188). Specifically, L47, H48, R50, V51, P52, F53 and P54 (based on apela-54 nomenclature) were identified as the residues most severely sensitive to alanine substitution in terms of receptor binding affinity, $G\alpha_i$ interaction, and β -arrestin-2 recruitment (188). It should be noted that P52, F53, and P54 are highly similar to the PMPF domain of apelin, further highlighting the importance of the C-terminal residues in receptor binding. Deng *et al.* also identified similar perturbations to receptor binding when F53 and P54 were substituted with alanine (61). The implications of these mutations are summarized in Figure 13.

For F53, bringing to mind the C-terminal phenylalanine in apelin, AR docking studies employing apela-11, in direct comparison to previous studies with apelin-13 (26), implied that the binding cavity in the AR TM domain was large enough accept an additional amino acid (i.e., P54) (286). This is hard to fully reconcile with the results noted above of functional perturbation resulting from incorporation of steric hindrance in the vicinity of apelin's C-terminus. Taking these docking studies at face value, the inference may be made that the penultimate phenylalanine of apela makes a similar contact in the binding cavity of AR as the C-terminal phenylalanine of apelin. This is consistent with the severe perturbation in apela function upon substitution of this phenylalanine to alanine (61, 188). Alternatively, the C-terminal proline of apela may provide similar steric contacts as apelin's C-terminal phenylalanine, if docking studies that indicate the lack of significant space to accommodate functionality C-terminal to this site (286) are more accurate. This would be consistent with the loss of affinity observed for the P14A-apela-14 analogue (188). Apela analogues with replacements to aromatic and/or bulkier substituents for the C-terminal residue have not, to our knowledge, yet been examined; short of high-resolution structural data involving more physiological ligands than AMG3054, these would allow for better distinguishing of these alternatives.

Notably, Deng *et al.* determined that P32A-apela-32 could inhibit wildtype apela-32-mediated ERK signaling and fluid homeostasis (61). Whether this is due to direct inhibition or due to biased signaling, as observed in apelin (*vide supra*), is unclear. Further

complicating this, Murza *et al.* showed lower binding affinity but similar potency for both G-protein and β -arrestin signaling for P14A-apela-14, analogous to the P32A-apela-32 (188). This finding demonstrates C-terminal proline-to-alanine apela mutants as being incapable of acting as an antagonist or inhibitor. In addition, the C-terminal amide derivative of apela-14 was much less efficient at β -arrestin recruitment than P14A-apela-14 (188), implicating the negatively charged C-terminus as the critical regulator of binding and signaling, rather than the explicit identity of the C-terminal residue of apela. Overall, the role of the C-terminal residue in apela seems context-dependent, similar to apelin, and will require additional analysis to decipher its exact role.

As described earlier, macrocyclic analogues of apelin have yielded new analogues with varying signaling biases. Thus far, synthetic cyclization has only been carried out for apela-11, which showed negligible differences to the linear version (286). Notably, however, apela contains two cysteine residues (C39 and C44 based on apela-54 numbering), making some apela isoforms capable of forming an intramolecular disulfide bridge and forming a macrocycle without the requirement of chemical modification. However, unlike apelin, where cyclization modulated affinity and activity, linear and cyclized forms of apela-16 exhibited similar potencies in receptor binding affinity, $G\alpha_i$ interaction, and β -arrestin-2 recruitment (188). Furthermore, alanine-scanning experiments demonstrated that the C4A-apela-14 did not have significantly different receptor binding or signaling relative to the wildtype (188). This study by Murza *et al.* implied that neither the disulfide-linked cyclization nor the presence of cysteine residues at either position in apela-32 (or shorter isoforms) are key for AR activation.

It is possible that the cysteine residues in apela play a bigger role in binding to the as yet-unidentified receptor for apela implicated as having a role in human ESCs (98). Intramolecularly disulfide-bonded apela-32 was shown to activate this unknown receptor. However, it is not fully clear as to the final state of the disulfide bond upon receptor binding. Uncertainty in this regard comes from the fact that C22A-apela-32 (equivalent to C44 in apela-54) resulted in a significant loss in receptor binding and internalization, while C17A-apela-32 (i.e. C39 in apela-54) did not (98). The disproportionate effect observed for these two mutations suggests that the two cysteine residues independently affect receptor binding, with the effect of an intact disulfide still unknown.

The N-terminal cysteine of apela-11 (i.e., C44 of apela-54) undergoes spontaneous dimerization during recombinant production (105), suggesting the potential for apela-11 to dimerize *in vivo*. Given the importance of this cysteine in apela-32 binding to the unknown receptor (98), apela-11 dimerization may also have consequences for receptor binding. However, exactly how dimerization either hampers or promotes interaction with the unknown receptor remains unknown.

Binding to the unknown receptor was also dependent on R31 and R32 (based on apela-54 numbering), as double R9G- and R10G-apela-32 mutant could not bind to the receptor or cause internalization (98). Furthermore, this diarginine mutation in apela-32 resulted in complete loss of the ability of apela to promote cell viability and growth. Other diarginine mutations at R42 and R43 (based on apela-54 numbering) also led to a loss of receptor

binding and internalization as observed in double R29- and R30-apela-32 mutant, but was not to the same extent. It is noteworthy that R31 and R32 in apela-54 are the proposed cleavage sites for a PCSK to produce apela-23 and -22, respectively. This is consistent with, as mentioned earlier, the observation that incubation of apela-32 in plasma resulted in production of apela-22 and the first 9 N-terminal residues of apela-32 (188). Given that these residues also play an important role in binding to the unknown receptor (98), it is logical to hypothesize that processing of apela-32 into the 22-residue isoform will prevent binding to and activation of this receptor. Thus, processing by a plasma endoprotease may serve to deactivate apela-32 from signaling through this unknown receptor and/or bias the apela ligands for the AR.

Although the effects of apela-22 in comparison to apela-32 have not yet been characterized in the human ESC model, the discussion above implies that apela processing may be an important regulator of its function in different developmental and physiological settings. Furthermore, as discussed earlier, the apelin-AR interaction appears likely to involve electrostatic interactions between key basic residues on the ligand and acidic residues on the receptor. It seems probable that similar interactions may also be involved with apela binding to its unknown receptor, but it is unlikely that the binding site architectures of this unknown receptor and the AR are highly similar since apelin-13 has been shown to have no functional effect on human ESCs.

Comparing and contrasting apela and apelin structural features and membrane interactions

To date, only apela-54, -32, and -11 have been characterized through biophysical techniques (105). The full-length gene product and longest isoform, apela-54, was predicted to be a preprotein containing a 22-residue hydrophobic N-terminal signal peptide. Characterization of recombinantly expressed apela-54 supported this prediction, with helical content observable by far-UV CD spectropolarimetry in a fluorinated alcohol environment. Apela-54 was also insoluble in aqueous solution, consistent with significant hydrophobicity for the preprotein. Apela-32, in contrast, exhibited a significant loss in helicity relative to apela-54 and was readily soluble in aqueous solution, consistent with a hydrophobic helical domain located in the N-terminal signal peptide region of apela-54 (Fig. 13).

In terms of general secondary structure, both apela-32 and -11 exhibit random coil conformations in solution, as observed by both far-UV CD and solution-state NMR spectroscopy (105). Interestingly, apela exhibited conformational variation as a function of isoform, in striking contrast to apelin (*vide supra*). Specifically, NMR chemical shift assignment of apela isoforms as a function of backbone position demonstrated that while C-terminal residues (29V and 31F) of apela-32 and -11 sampled similar conformations, the remainder of the shared peptide region showed larger differences. This implies that the C-terminal 11-residues of apela isoforms do not behave entirely independently of the rest of the peptide, in striking contrast to apelin where the conformation of shared residues was independent of peptide length. This difference may, in turn, modulate the favorability of

interaction with the AR, providing a structural and dynamic justification for the significant differences observed in pharmacological properties between apela isoforms.

Interestingly, residues V51 and F53 (based on apela-54 numbering) near the C-terminus of apela-32 and -11 exhibited four exchanging conformations in solution. These two residues are adjacent to prolines, similar to the glycine, methionine, and phenylalanine in the GPMPF motif of apelin. As detailed earlier, apelin presented at least three potential exchanging conformations for its C-terminal region (138). Although it is not yet clear what the exact functional ramifications are of these additional conformations in both apelin and apela, the presence of two prolines in the C-terminal region of both apelin and apela suggests that these proline residues are likely important for receptor interaction. Correspondingly, alanine substitutions at P10 (72) for apelin-17 and at P52 (188) and P54 (61) for apela (based on apela-54 numbering) resulted in loss of receptor interaction and/or signaling. Given the relative constriction in conformations available to the imino acid proline vs. the 19 standard amino acids, its presence is likely important for restricting the number of potential conformations sampled by the C-terminal region to facilitate favorable receptor interactions for recognition, binding and/or activation following binding.

Atomic-level structural determination of apela in solution has not yet been conducted; thus, it is unknown whether apela isoforms also obtain β -turn structure similar to apelin. However, in the presence of membrane mimetic conditions, far-UV CD spectra of apela-32 and -11 were consistent with the presence of β -turn structures. Thus, membrane catalysis may also enhance the interaction of apela with the AR, similar to apelin, through the induction of β -turn structural content.

Notably, apela-32 and -11 further distinguish themselves from each other, and from apelin, in terms of micelle interaction. As evidenced by CD spectroscopy, solution-state NMR-based backbone assignment, and hydrodynamics characterization by DOSY, the shorter 11-residue isoform presented a striking bias towards interaction with micelles composed of detergents with negatively charged headgroups (SDS and LPPG) over those with zwitterionic headgroups (DPC) (105). This behavior is highly reminiscent of apelin (141). In strong contrast, the longer apela-32 isoform exhibited strong interactions with both classes of detergent headgroup. In addition, as observed through NMR spectroscopy, apela-32 adopted α -helical structuring in the presence of both zwitterionic and anionic detergents in the N-terminal region (T6-L12) and in the middle of the peptide (H15-20R), respectively (Fig. 13). Apela-11, conversely, did not exhibit evidence of either α -helical or β -sheet structuring in response to micelle interaction, instead presenting evidence of β -turn formation. Notably, the segments of apela-32 which became helical upon micelle binding do not encompass apela-11; thus, apela-11 is not expected to maintain the micelle-mediated helicity.

PRE NMR experiments have not been conducted for apela, as were used with apelin-17 (141). This would potentially allow the identification of specific region(s) of apela in direct contact with the micelle surface. However, in zwitterionic micelles, the α -helical domain spanning T6-L12 was the only region with differences in comparison to apela in solution (105). Furthermore, apela-11, which lacks this segment, showed negligible chemical shift

differences in the presence of zwitterionic micelles. Thus, it seems most likely that the helical domain is in direct contact to the micelle surface. In the presence of anionic micelles, the number of conformations observed for the C-terminal V29 and 31F residues were reduced for apela-32. Furthermore, apela-11 showed large differences in response to anionic micelles by both CD and NMR spectroscopy. This implies that the direct interaction between anionic micelles and apela is localized to the C-terminal residues, but the exact residues involved remain to be determined.

It is important to note that all NMR-based characterization of apela was conducted under reducing conditions (105). The α -helical domain observed in apela-32 in the presence of anionic detergent spans one of the cysteine residues capable of forming the potential intramolecular disulfide bridge. Thus, intramolecular disulfide formation may affect micelle interactions due to the constrained conformation arising from cyclization. Conversely, micellar or, by inference, bilayer interactions may impair intramolecular cyclization. Furthermore, if lipid interactions reduce the propensity of disulfide bridge formation, C44 would be free to interact with the unknown receptor, explaining why mutation of C44 impairs binding to this receptor.

Potential ramifications of membrane interaction in the apelinergic system

The observed differences in membrane binding from apela-32 relative to both apela-11 and the apelin isoforms may translate to the observed variations in potency. As mentioned above, apela-32 was significantly more potent than (i) apela-11 in both $G\alpha_i$ and the β -arrestin-mediated signaling pathway activation (188), and (ii) Pyr-apelin-13 in ERK phosphorylation, fluid homeostasis (61), and cardiac contractility and output (286). Since the outer membrane of most cells is largely composed of zwitterionic headgroup lipids (262), a greater quantity of apela-32 would be localized onto the cell membrane in comparison to its shorter apela-11 counterpart and the apelin isoforms, according to the membrane catalysis hypothesis (*vide supra*). Furthermore, this would increase the probability of apela-32-receptor complex formation and provide a correspondingly increased potency for the longer apela isoform.

More specifically, one may imagine that apela-32, while mostly disordered in the extracellular fluid, undergoes a reduction in its conformational space upon reaching the cell membrane. The peptide could first be drawn to zwitterionic regions of the membrane via localized interactions at the N-terminal α -helical region of apela-32, resulting in a decrease in dynamics and allowing for lateral diffusion. Once encountering a region of negative charge on the membrane, conformational change would be promoted at the central and C-terminal regions, priming the peptide for receptor binding. For apela-11 (and apelin-13 and -17), interaction with zwitterionic regions of membrane would be limited. Only in the presence of an anionic patch of the cell surface would local peptide concentration increase, with the induction of a conformation enhancing receptor binding. Any intrinsic or membrane-induced variation in structure among the two families of AR ligands and their isoforms could then result in the activation of specific signaling pathways (e.g., $G\alpha_i$ vs. β -arrestin pathways) with distinct intracellular and physiological consequences.

Another hypothesis that logically follows from the isoform-dependent differences in both structure and function is that different mechanisms of signaling (e.g. autocrine vs.

endocrine) may occur due to variations in membrane interactions. Again, with the mammalian cell membrane outer leaflet being composed of mostly zwitterionic lipids, peptide hormones that favorably interact with zwitterionic lipids (e.g., apela-32) would likely bind to the host cell or nearby cells upon secretion and, hence, act in an autocrine or paracrine fashion (Fig. 14A). On the other hand, hormones without such a preference (e.g., apela-11 and apelin-17), would have the potential to much more readily diffuse away from the cell, favoring endocrine signaling (Fig. 14B).

Implications of multiple bioactive peptide isoforms in the apelinergic system

The studies detailed above provide a clear demonstration that apelin and apela isoforms differ in their pharmacological and biophysical properties. Hypothetically, these differences may have wildly varying consequences. Specifically, if one isoform of one ligand is predominantly produced, the level of AR recycling or degradation will be correspondingly controlled by this isoform, resulting in either continuous or transient signaling. Logically, isoform-dependent differences in pharmacology will contribute to either physiological or pathological effects in the apelinergic system. This also translates to the location of signaling (i.e., at the cell membrane or intracellularly) and the signaling pathway (e.g., G-protein vs. β -arrestin), since longer isoforms cause prolonged receptor internalization and more potent β -arrestin signaling relative to shorter isoforms.

If a given tissue requires one class of signaling over another for an appropriate downstream physiological effect, tissue-dependent processing of apelin/apela peptides may, arguably, be the simplest way to achieve the desired effect. Specifically, by regulating which isoform is predominantly produced from the corresponding preprotein, it should be possible to directly regulate the function of apelin and apela at the tissue level. However, if mixtures of isoforms of apelin, apela, or both peptides are released, the isoform closest to the AR will be likely to bind the receptor first, determining the final physiological effect. If so, factors other than simply preferential isoform processing may be required in order to elevate the local concentration of the required isoform near the receptor. Following from the membrane catalysis hypothesis and the noted potential for differences in isoform-specific membrane interaction, changing the composition of lipid headgroups at the cell surface may provide a means of assisting in altering the level of a required isoform on the cell surface. In other words, differences in the membrane binding preferences of apelin and apela isoforms may be exploited to regulate the downstream signaling. Furthermore, since apelin and apela are secreted prior to interaction with their cell-surface receptor, both preferential processing and membrane interaction propensities may simultaneously act to fine-tune regulation in the apelinergic system.

Unfortunately, the exact purpose of the production of multiple apelin and apela isoforms remains speculative, at best, and the precise mechanisms underlying the regulation of relative levels of isoforms remain elusive. Notwithstanding this lack of mechanistic understanding, the currently available data clearly indicate that isoforms have distinct signaling bias (i.e., $G\alpha_i$ vs. β -arrestin signaling pathways), with distinct intracellular and physiological consequences. As a whole, therefore, further elucidation of apelin and apela processing (e.g., other proteases involved; mechanisms regulating production of various

isoforms; more comprehensive characterization of isoform levels as a function of location and pathophysiological state; etc.) is critical to fully define the regulation and function of this important signaling system.

Conclusion

In summary, the components of the apelinergic system are expressed throughout development and throughout the body, with interactions between a given apelin or apela isoform and the apelin receptor being responsible for regulation of numerous physiological functions. These include key roles in central nervous system, cardiovascular system, and adipoinular axis regulation, with clear relation to a variety of pathological situations. Both apelin and apela can be processed into multiple N-terminally truncated isoforms, in each case retaining the C-terminal residues of the respective preprotein to interact with their cell surface receptor. All isoforms of a given ligand likely interact with and activate the AR through a similar mechanism, given their common amino acid composition and relatively similar conformations, explaining their shared physiological effect in the body. Although the overall physiological effects are similar between isoforms of apelin and apela, potencies and efficacies are observed to vary between isoforms. A large number of studies have attempted to explain these discrepancies through identification of: (i) key residues on both the ligands and receptor involved in interaction; (ii) sequence motifs, in some cases with clear structural properties, that regulate receptor binding and signaling downstream; and (iii) pharmacological differences between isoforms.

Specifically, key basic residues on the ligands and acidic residues on the receptor have been identified as being functionally critical, indicating the potential of ionic interactions. The recent crystal structure of apelin-17 analogue AMG3054 bound to human AR is consistent with at least one electrostatic interaction involving the N-terminal tail of the AR, with the inference made that longer isoforms may interact with anionic grooves on the extracellular surface of the receptor. Structural characterization of apelin demonstrated highly similar backbone conformations for all isoforms, implying the potential of similar modes of receptor interaction for all isoforms; however, the apela isoforms did not share this trait, with similar but non-identical conformations. The discrepancies in biophysical behavior between apelin and apela become even more apparent in the presence of membrane-mimetic micelles, where apela-32 did not exhibit a preferential interaction with anionic micelles, unlike either apela-11 or a variety of apelin isoforms. Finally, apelin-13 has been shown to differ significantly from apelin-17 and -36 in terms of receptor regulation, and similar differences in regulation have been observed between apela-11 and -32. In combination, isoform-dependent modulation of signaling is clear, indicating that peptide processing would provide a mechanism to finely regulate the apelinergic system. Furthermore, functional differences may arise from isoform-dependent variation in membrane interaction, changes in receptor conformation, and differences in receptor desensitization or recycling.

Unfortunately, however, despite these discrepancies observed between isoforms, many of the physiological roles of apelin have only been studied for the isoform identified as being the most potent: apelin-13 and the highly similar, but more stable, Pyr-apelin-13. The potential

for similarly limited studies of apela isoforms to take place is concerning, particularly given a lack of knowledge of which isoform(s) are found in a given physiological context.

In short, although structure-function correlations are starting to become clear in the apelinergic system, many important aspects of this system remain to be fully characterized. In particular, the exciting report of an apelin analogue-bound AR structure provides a new avenue for development of structurally-informed hypotheses with regard to interactions between AR and each ligand. This will be critical not only in terms of biomedical research, where both apelin and apela have been suggested and applied as scaffolds for therapeutic design, but also in terms of understanding the fundamental basis of ligand-receptor binding, receptor regulation, and signaling pathway activation. The diverse pool of endogenous ligands for the AR provides, to our knowledge, an unprecedented range of bioactive ligands to probe receptor conformation and dynamics. This offers a rare opportunity to characterize a membrane-bound receptor without the requirement for non-physiological ligands, such as small molecule modifiers. This, in turn, will expedite development of novel therapeutics and expand our understanding of molecular mechanisms behind GPCR activation and regulation by peptide ligands.

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Didactic Synopsis

Major teaching points:

- the apelinergic system comprises two peptide ligands, apelin and apela, and their cognate G-protein-coupled receptor (GPCR), the apelin receptor
- the apelinergic system is widely distributed, with (patho)physiological effects ascribed, e.g., in regulation of metabolism, the cardiovascular system, and the central nervous system
- apelin and apela have multiple bioactive isoforms widely ranging in size
- key motifs for each ligand have distinctive structural features, membrane-interactive properties, and functional effects
- apelin and apela processing to different isoforms modulates biophysics, pharmacological properties, signaling, and receptor regulation
- membrane-ligand interactions appear likely to facilitate recognition by and binding to the apelin receptor through the membrane catalysis mechanism
- regulation of the apelinergic system may, thus, rely on: whether apelin or apela is produced in a given setting; balancing of isoform processing; cell surface composition; and, modulation of receptor level on the cell surface

Figure 1.

Human apelin receptor (AR) sequence illustrated in “snake plot” format, with seven transmembrane (TM) helices delineated. Additional structural features observed in the AR crystal structure (166) are illustrated: a short β -sheet in the second extracellular loop; the 8th helix, immediately C-terminal to TM7; and, the two extracellular domain disulfide linkages (denoted by distinct dagger symbols). Residues shown by mutagenesis to have functional importance are shown by filled circles. Important motifs common to class A GPCRs (106, 257) are shown by red circles: the Trp toggle (CWXP) in helix 6; the ionic lock (DRY) in helix 3; and, the NPXXY motif in TM7. Membrane interface positioning is as estimated by TMDET (258).

Teaching points: This figure demonstrates the topology of the apelin receptor. It has the canonical G-protein-coupled receptor (GPCR) topology with seven transmembrane helices, an extracellular N-terminal tail, intracellular C-terminal tail, and three loops connecting TM helices on each side of the membrane. Additional structural features determined crystallographically are also shown, including a β -sheet in the second extracellular loop and a short “8th helix” immediately following transmembrane helical segment 7. A number of studies have now also demonstrated functionally important residues through mutagenesis and functional study, as are highlighted.

Figure 2.

Sequence comparison of the seven transmembrane (TM) regions of human apelin receptor (AR; colored red) with 7 other GPCRs (human unless specified): bovine rhodopsin (Rh); fellow HIV-1 co-receptors CCR5 and CXCR4; angiotensin-II receptor isoform 1 (AT₁); and, β_2 -adrenergic receptor (β_2 AdR), adenosine A_{2A} receptor and turkey β_1 -adrenergic receptor (β_1 AdR). Alignments were performed using consensus residues defined by Baldwin *et al.* (17). Boxes surround conserved/homologous residues in >4 (or 4 including AR) of the GPCRs. Numbering above the alignments refer to the standard GPCR numbering used in the GPCRDB project (100) with green highlights showing putative TM region, while numbering at each end of the sequence refers to the sequential numbering for the full-length protein. Yellow highlights indicate residues in AR where mutagenesis has shown perturbation to either function or localization (113, 134, 143).

Teaching points: The sequence of each of the seven apelin receptor transmembrane segments is compared to those known for several related class A GPCRs. Although sequence conservation is reasonably high, particularly for the angiotensin-II receptor isoform 1 (AT₁), clear distinctions are apparent in all of these receptors. Correspondingly, although a canonical GPCR architecture is expected in each case, differences in structure, intramolecular dynamics, ligand binding, and activation mechanisms are not unexpected due to the variations in primary structuring.

Figure 3.

Representations of the crystal structure of the apelin receptor in an inactive-like state in complex with the agonistic apelin-17 analog AMG3054 (PDB entry 5VBL; (166)). Cartoon and cylinder diagrams are colored from blue (N-terminus) to red (C-terminus), with the ligand shown as grey sticks. Surface representations colored (as indicated on lower right) from a charge of -2 as red to +2 as blue were generated using the PyMol (Schrödinger, Cambridge, MA) adaptive Poisson-Boltzmann Solver plugin. Membrane positioning is as estimated by TMDET (258).

Teaching points: The first apelin receptor crystal structure is illustrated. The apelin-17 analog, AMG3054, was bound to the receptor, as shown. The GPCR architecture is clear in the structure and analysis of its features allowed determination of the fact that the state

observed is an “inactive-like” conformation despite the fact that AMG3054 is an agonist. This was likely due to the mutagenesis required in order to obtain a stable construct for crystallographic studies. An important feature is the high degree of anionic character on the extracellular face of the receptor, with a number of unoccupied grooves being apparent. These grooves have been postulated to provide natural binding sites for longer apelin isoforms.

Figure 4.

Structural and topological comparison of the apelin receptor to four other class A GPCRs crystallized in inactive-like conformations. *Left column:* Comparison of GPCR architecture (see, e.g., Hanson et al. (93) for a detailed discussion of these comparator GPCRs) colored from blue (N-terminus) to red (C-terminus), with the corresponding PDB entry codes provided with co-crystallized, bound agonist/antagonist molecules given in brackets; retinal is present in the rhodopsin structure shown. Each GPCR is shown in a cartoon representation in the same orientation and with the same color scheme. *Right column:* Comparison of topologies with TM helix kinks identified as bends (*black circles*) or disruptions (*black lines*) and TM helices shown as colored rectangles with position and angle correct relative to

membrane boundaries (blue lines). Topologies were determined by the MC-HELAN algorithm (144) with TM orientation and membrane boundaries defined by the TMDET algorithm (258). Residues at start and end of each TM region and at kinks are indicated (see also Fig. 2). Loops connecting TM helices are shown as black lines (independent of length), while the N- and C-termini are not represented. Kink angles cannot be preserved in translation from 3D structure to 2D topology diagram, but are calculated correctly by MC-HELAN.

Teaching points: The apelin receptor structure is compared to those of four other class A GPCRs, all crystallized in inactive-like conformations. Although the overall architecture is the same in all cases (cartoon illustrations in left-hand column), the topology of each GPCR in the transmembrane domain is distinct with some helices being more variable than others.

Figure 5.

Comparison of β_2 -adrenergic receptor topology as a function of activation state. PDB entry is given, alongside its resolution and the nature of the bound ligand or effector protein. Topologies were predicted by MC-HELAN (144) with TM helix kinks identified as bends (*black circles*) or disruptions (*black lines*) and TM helices shown as colored rectangles with position and angle correct relative to membrane boundaries (blue lines) defined by the TMDET algorithm (258).

Teaching points: The topologies of four distinct crystallographically-characterized states of the β_2 -adrenergic receptor, a relatively extensively characterized class A GPCR, are illustrated. Notably, even for a single GPCR, the topology in the transmembrane domain for the structural snapshots varies as a function of activation state.

Figure 6.

Sequence conservation of apelin. Residues that are fully conserved over the six illustrated species are indicated with a black background; partially conserved with varying shades of grey; and, variable positions with white.

Teaching points: The sequence alignment for pre(pro)apelin over a variety of species is shown. The N-terminal 22 residues are the predicted signal peptide, cleaved to produce a bioactive 55-residue form of apelin (apelin-55). Initially, apelin-55 was not believed to be bioactive and was referred to as proapelin. Alignment demonstrates very clearly that the C-terminal 12 residues are identical, directly corresponding to the requirement of these residues for receptor binding and activation. More variability is seen N-terminal to this region. Despite this variability, dibasic motifs associated with proprotein convertase processing are still frequently found, implying the potential of multiple isoforms being produced in all of the species compared.

Figure 7.

Apelin processing pathways. A) Previously theorized “apelin-36 precursor” processing pathway. B) Current “myriad” processing pathway theory based on new publications, which also identifies proapelin as apelin-55 as an additional bioactive member of the apelinergic system. Note that this processing pathway does not show other post-translational modifications such as ACE-2-mediated C-terminal phenylalanine removal.

Teaching points: Two apelin processing pathways are compared. In each case, the 77-residue pre(pro)protein is initially processed to produce a 55-residue form (apelin-55). Initially, this was believed to be an inactive proprotein, but subsequently apelin-55 secretion was demonstrated and it has now been shown to be bioactive. Despite this, apelin-55 may be processed intracellularly to shorter isoform(s) prior to secretion. Mechanisms by which shorter isoforms are produced remain ill characterized, with only two studies to date demonstrating that the proprotein convertase PCSK3 processes either apelin-55 or apelin-36. Further study is needed to flesh out these pathways.

Figure 8.

Apelinergic system expression and isoform localization profile. A) Apelin, apela, and AR localization as a function of tissue/organ system. B) Predominant apelin isoform(s) detected, to date, in specific organs or body fluids.

Teaching points: Localization of each of the components of the apelinergic system is quite widespread (panel A). This is better characterized for apelin and the AR, given the recent discovery of apela. Tissue- and fluid-specific isolation of apelin isoforms has also been demonstrated (panel B). This has implications in terms of physiological function, given that distinct apelin isoforms lead to differences in downstream signaling and receptor regulation.

Figure 9.

Summary of structure-function correlations for apelin. Apelin-17 is illustrated as it has been the most widely-studied isoform in terms of biophysics and structure. Functional effects of mutagenesis or truncation are indicated directly on the peptide sequence (symbols denoted in the legend). For direct comparison, regions of apelin-17 exhibiting structural convergence (138) and membrane-interactive properties (141) are delineated alongside the segments of the apelin-17 analog AMG3054 that interact with the AR in the co-crystal structure (PDB entry 5VBL (166)).

Teaching points: Motifs within the apelin-17 isoform are illustrated. The 12 residues in the C-terminal region of apelin that are widely conserved over many species demonstrate the most importance in terms of functional effects upon substitution or truncation, structural convergence, and membrane-interaction. The “RPRL” motif at the N-terminus of apelin-12 demonstrates structuring under a variety of conditions and is important in membrane interaction. In the AMG3054 analog, this region also interacts with an extracellular anionic groove. The C-terminal “GPMPF” motif exhibits distinct structural differences as a function of condition. In AMG3054, this region penetrates into the transmembrane domain of the AR, with a kink at the His. Also of note, the C-terminal Phe of apelin has a number of features ascribed in the literature. Signaling bias based upon its presence or absence appears to be the most likely source for these discrepancies.

Figure 10.

Membrane catalysis hypothesis as applied to binding of apelin-17 to the apelin receptor. Sequentially, (1) apelin is proposed to bind to the membrane, increasing the likelihood of (2) its interaction with and recognition by an unliganded apelin receptor (AR) on a cell surface followed by (3) receptor binding and activation. Structures of apelin-17 in buffer (BMRB entry 20029 (138)) and bound to SDS micelles (BMRB entry 20082 (141)); and, of AR in absence of ligand with anionic patch residues E20 and D23 illustrated (143) and bound to apelin-17 analog AMG3054 (PDB entry: 5VBL (166)) were employed.

Teaching points: The membrane catalysis hypothesis is illustrated using structural data from the apelinergic system. In the first step, the “RPRL” motif is modestly structured in solution and binds to membrane; this pre-structuring would lead to a decreased entropic penalty upon membrane binding. The receptor encounter likelihood of the membrane-bound apelin is then increased due to the reduction of the diffusional search from a 3D to a 2D process, the increased local concentration of ligand on the membrane, and the induction of structuring, particularly at the apelin C-terminus (the MPF within the “GPMPF” motif). Upon receptor interaction and recognition, an additional conformational change will take place during binding. An anionic patch on the apelin receptor (AR) N-terminal tail may also facilitate this step. Binding is likely to be a two-step process. An initial binding step (not illustrated) is likely whereby the apelin N-terminal region interacts with the AR extracellular domain. This would facilitate a “fly casting” mechanism by which the C-terminal tail of apelin is facilitated in finding and penetrate into the transmembrane domain of the AR. A subsequent, additional conformational change is then likely to go from an inactive bound state (as observed crystallographically) to an active bound state.

Figure 11.

Sequence conservation of apela. Residues that are fully conserved over the five illustrated species are indicated with a black background; partially conserved with varying shades of grey; and, variable positions with white. Hyphens indicate residues absent from a given species.

Teaching points: The sequence alignment for preapela over a variety of species is shown. The N-terminal 22 residues are the predicted signal peptide, cleaved to produce a bioactive 32-residue form of apela. Alignment demonstrates very clearly that the C-terminal 9 residues are identical, with a very high degree of conservation through the 13 C-terminal residues. As with apelin, this is indicative of the requirement of these residues for receptor binding and activation. Some additional variability is seen N-terminal to this region. Following the precedent of apelin, dibasic motifs associated with proprotein convertase processing are identical, implying the potential of the same sets of isoforms being produced in all of the species compared.

Figure 12.

Comparison of apelin-36 and apela-32. A) Sequence comparison of apelin-36 (top) and apela-32 (bottom). Dashed lines represent residues falling in similar positions implying the potential for similar structural and/or functional roles. B) Comparison of amino acid composition.

Teaching points: Apelin-36 and apela-32 exhibit similar, but non-identical, amino acid compositions. A number of key motifs observed for one peptide may be translated to the other, as indicated by dashed lines. These, however, are frequently translated in position.

Apela-32Zwitterionic Micelle
Induced StructuringAnionic Micelle
Induced Structuring **Figure 13.**

Summary of structural and functional studies for apela. Apela-32 is illustrated, although it should be noted that a number of studies have focused on shorter isoforms. Functional effects of mutagenesis or truncation are indicated directly on the peptide sequence (symbols denoted in the legend). Regions exhibiting structuring in the presence of the indicted type of micelle (105) are delineated.

Teaching points: Motifs within the apela-32 isoform are illustrated. Although substitution and truncation studies have been more limited, to date, relative to apelin, a number of key sites for both binding and signaling have been identified (as illustrated.) Distinct regions of apela-32 also become structured, depending upon the surface charge properties of membrane-mimetic micelles employed.

Figure 14.

Implications of membrane catalysis for the regulation of signaling. A) Autocrine and B) paracrine or endocrine signaling of apelin or apela (denoted as “AP”) isoforms may be regulated by variation in preferential membrane headgroup association. Ligand-mediated apelin receptor (AR) activation is represented by G-protein binding and subsequent ERK phosphorylation (pERK), although many other signaling pathways are possible (Tables 13–14).

Teaching points: This figure illustrates different possible fates of an apelin or apela (“AP”) ligand upon secretion. If there is a strong potential for interaction with the membrane of the cell from which it is secreted, this would bias toward autocrine signaling (A) through apelin receptor (AR) molecules on the same cell. Alternatively, the ligand may encounter and bind to a nearby cell (i.e., paracrine signaling) or more distant cell (i.e., endocrine signaling) as in panel B, exerting functional effects through AR on the cell in question. In any instance, further processing of the secreted peptide may take place prior to receptor interaction, either with cell surface localized proprotein convertases or other processing enzymes (autocrine, paracrine, or endocrine signaling) or with circulating enzymes (likely most applicable to endocrine signaling). The potential for post-secretion processing is not explicitly illustrated, but should be kept in mind as an important additional regulatory mechanism.

Table 1

Amino acid sequence of human apelin and apela isoforms

Amino acid sequence	Identity
<u>MNLR</u> LCVQALLLLWLSLTAVCGGSLMPLPDGNGLEDGNVRHLVQPRGSRNGPGPWQGGRRKFRRQRPRLSHKGPMMPF	Apelin-77
GSLMPLPDGNGLEDGNVRHLVQPRGSRNGPGPWQGGRRKFRRQRPRLSHKGPMMPF	Apelin-55
LVQPRGSRNGPGPWQGGRRKFRRQRPRLSHKGPMMPF	Apelin-36
KFRRQRPRLSHKGPMMPF	Apelin-17
*<ERPRLSHKGPMMPF	Pyr-apelin-13
QRPRLSHKGPMMPF	Apelin-13
<u>MRFQQFLFAFFIFIMSL</u> LLISGQRPVNLTMRRLKLRKHNCQRRRCMPLHSRVFPF	Apela-54
QRPVNLTMRRLKLRKHNCQRRRCMPLHSRVFPF	Apela-32
*<ERPVNLTMRRLKLRKHNCQRRRCMPLHSRVFPF	Pyr-apela-32
KLRKHNCQRRRCMPLHSRVFPF	Apela-22
LRKHNCQRRRCMPLHSRVFPF	Apela-21
CMPLHSRVFPF	Apela-11

Underlined residues represent signal peptides.

<E represents pyroglutamate (Pyr).

Table 2

Pairwise identity to human apelin receptor (AR) for the transmembrane (TM) regions of each indicated GPCR: bovine rhodopsin (Rh); human chemokine receptors CCR5 and CXCR4; human angiotensin-II receptor isoform 1 (AT₁); human adenosine A_{2A} receptor; and, turkey β_1 - and human β_2 -adrennergic receptors (β_1 AdR and β_2 AdR). Pairwise homology values are even higher, as illustrated in Fig. 2. The regions considered are based upon homology for class A rhodopsin-like GPCRs determined by Baldwin *et al.* (17), as shown in Fig. 2.

	TM1	TM2	TM3	TM4	TM5	TM6	TM7	All TM
Rh	22%	31%	21%	27%	18%	21%	16%	22%
CCR5	34%	38%	32%	23%	24%	30%	26%	30%
CXCR4	34%	44%	44%	23%	24%	27%	29%	33%
AT ₁	28%	50%	56%	27%	36%	24%	39%	38%
A _{2A}	22%	25%	28%	19%	18%	21%	36%	24%
β_1 AdR	31%	25%	28%	19%	18%	24%	23%	24%
β_2 AdR	19%	25%	29%	12%	27%	27%	25%	24%

Table 3

Roles of apelin in the cardiovascular system

Function	-13	Pyr-13	-17	-36	Other isoforms	References
Decrease blood pressure (adenovirus-mediated for Galon-Tilleman)	✓ (++)	✓ (+)	✓ (++++)	✓ (+)	-12(+), -11(-), 10(-), -9(-)	(40, 70, 145, 218, 250) (56, 74, 80, 110, 122, 147, 186, 188-190, 241, 272, 284)
Increase blood pressure	✓	✓				(52, 54, 83, 125, 233, 292, 303, 307)
Increase cardiac force contractility	✓ (+)	✓ (+)		✓ (+)	-16	(169, 203, 204, 245, 284)
Increase cardiac index/output	✓	✓ (+)		✓ (+)		(7, 18, 26, 117, 213, 286)
Increase cardiac contractility (LV dp/dt)		✓	✓	✓		(7, 81, 117, 197, 286)
Promote cardiomyogenesis	✓					(55)
Increase heart rate	✓	✓ (+)			-16, -12, -10 (-)	(20, 40, 52, 125, 218, 245, 307)
Decrease heart rate	✓					(56)
Increase nitric oxide (NO) concentration/nitric-oxide synthase (NOS) activation	✓	✓	✓	✓		(12, 45, 64, 68, 77, 108, 110, 121, 132, 151, 216, 250, 288, 299)
Increase sarcomere shortening					-16	(73, 267)
Increase sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) activity/ expression	✓ (+)	✓ (+)	✓	✓ (+)		(108, 169, 211, 284)
Cause vasodilation	✓ (+)	✓ (+)		✓ (+)		(18, 26, 116, 169)
Inhibit vehicle-mediated vasoconstriction	✓	✓	✓			(5, 81, 88, 108, 112, 221)

✓ represents isoform(s) used.

(++++/+++/++) represents potency and/or efficacy differences between isoforms compared in a single publication where number of + signs represents increase in given functional property.

Table 4

Roles of apelin in cardiovascular disease models at the physiological level (myocardial infarction/heart failure/salt-loading hypertension/ischemia/aortic banding/atherosclerosis/hypertrophy)

Function	-13	Pyr-13	-36	Other isoforms	References
Increase cardiac index/output	✓	✓			(7, 18)
Ameliorate lowered cardiac contractility ($\pm$ LV dp/dt)	✓	✓	✓		(7, 12, 46, 122, 190, 197, 216, 241, 271, 299, 301)
Aggravate changed cardiac contractility ($\pm$ LV dp/dt; high dose)	✓				(190, 241)
Increase cardiac contractile force				-12	(57)
Inhibit cardiac hypertrophy	✓	✓			(13, 30, 78, 151, 162, 200, 227)
Increase cell viability	✓ (+)	✓		-12(+)	(13, 23, 24, 46, 151, 207, 299)
Decrease infarct size	✓ (+)	✓	✓ (+)	-12	(11-13, 23, 24, 132, 200, 208, 209, 216, 238, 248, 252, 287, 301, 306)
Ameliorate lowered left ventricular end systolic pressure (LVESP)	✓		✓		(122, 299, 301)
Ameliorate elevated left ventricular end diastolic pressure (LVEDP)	✓	✓	✓		(12, 122, 190, 216, 268, 299, 301)
Increase lowered mitochondria content	✓				(23, 24)
Regulate neointimal formation and vessel area		✓			(45, 133)
Increase neovascularization	✓				(151, 252)
Recruit stem cells	✓				(46, 151, 252, 306)
Increase stroke volume	✓			-12	(7, 208, 271)

✓ represents isoform(s) used.

(+/+/+/++) represents potency and/or efficacy differences between isoforms compared in a single publication where number of + signs represents increase in given functional property.

Table 5

Roles of apelin in cardiovascular disease models at the molecular level

Function	-13	Pyr-13	-17	-36	Other isoforms	References
Decrease angiotensin-1 expression	✓	✓				(12, 252)
Increase angiotensin-2 expression	✓					(252)
Increase angiotensin receptor (Tie2) expression		✓				(12)
Increase angiotensin II levels		✓				(2)
Decrease angiotensin converting enzyme (ACE) levels		✓				(132)
Decrease angiotensin II receptor type 1 (AT1R) levels		✓				(132)
Increase lowered B-cell lymphoma 2 protein (Bcl-2) levels	✓	✓				(13, 23, 24)
Decrease elevated Bcl-2-associated X protein (Bax) levels	✓	✓				(13, 23, 24)
Increase Ca ²⁺ mobilization					-12	(57)
Decrease elevated Ca ²⁺ uptake	✓					(268)
Decrease elevated caspase-3 levels	✓	✓				(13, 23, 24)
Increase lowered catalase expression					-12	(208)
Increase CD39 (ATPase) activity	✓					(96)
Increase CXC chemokine receptor type 4 (CXCR4) expression	✓					(151)
Increase endothelin 1 levels		✓				(2)
Increase lowered glutathione peroxidase (GSHP) levels					-12	(208)
Decrease interleukin-1 β and -6 (IL-1 β , IL-6) levels	✓	✓				(23, 132)
Increase Jagged-1 expression	✓					(150, 151)
Decrease lactate dehydrogenase (LDH) levels	✓ (+)	✓		✓	-12(+)	(11, 122, 207-210, 216, 299)
Decrease malondialdehyde (MDA) levels	✓	✓		✓	-12	(11, 122, 208, 268, 299)
Decrease MB-creatine kinase (MB-CK) levels		✓			-12	(11, 208-210)
Delay mitochondrial permeability transition pore opening	✓ (+)			✓ (+)		(238, 287)
Decrease monocyte chemoattractant protein (MCP-1) levels		✓				(132)
Decrease elevated myeloperoxidase (MPO) levels	✓					(23)
Decrease expression of β -myosin heavy chain	✓					(151, 162)

Function	-13	Pyr-13	-17	-36	Other isoforms	References
Decrease natriuretic peptide expression	✓					(23, 151, 162)
Increase Notch-3 expression	✓					(151)
Decrease nuclear factor-κB (NF-κB) activation		✓				(132)
Decreased elevated reactive oxygen species (ROS) levels	✓ (+)				-12(+)	(23, 207, 268)
Increase sarcoplasmic reticulum Ca ²⁺ -ATPase (SERCA) activity	✓					(268)
Increase stromal cell-derived factor 1 (SDF-1α) expression	✓					(151)
Increase lowered superoxide dismutase (SOD) levels	✓				-12	(208, 299)
Decrease elevated troponin levels	✓					(23)
Decrease tumor necrosis factor-α (TNF-α) levels	✓	✓				(132, 240)
Increase vascular endothelial growth factor (VEGF) expression	✓	✓				(12, 151, 252)
Increase VEGF receptor (VEGFR) expression		✓				(12)

✓ represents isoform(s) used.

(+/-/+/+/++) represents potency and/or efficacy differences between isoforms compared in a single publication where number of + signs represents increase in given functional property.

Table 6**Roles of apelin in angiogenesis/lymphogenesis**

Function	-13	Pyr-13	-17	-36	Other isoforms	References
Increase angiotensin receptor (Tie2) levels	✓	✓				(12, 301, 302)
Increase capillary-like tube formation (Cells)	✓ (+)	✓ (+)		✓ (+)		(12, 21, 127, 135, 226, 288, 301)
Increase permeability (glomerular and cardiac)	✓					(85, 301, 302)
Increase VEGF receptor (VEGFR) levels	✓	✓				(12, 301, 302)
Increase vessel formation/branching	✓	✓				(51, 102)

✓ represents isoform(s) used.

(+/+/+/++) represents potency and/or efficacy differences between isoforms compared in a single publication where number of + signs represents increase in given functional property.

Table 7

Roles of apelin in thrombosis

Function	-13	Pyr-13	-17	-36	Other isoforms	References
Increase bleeding time	✓ (+)			✓ (+)		(1)
Decrease platelet aggregation	✓ (+)	✓		✓ (+)		(1, 136)
Increase tissue factor (TF) levels and activity	✓ (+)				-12 (-)	(47)

✓ represents which isoform was used.

(+/+/+/++) represents potency and/or efficacy differences between isoforms compared in a single publication where additional + represent increased pharmacological properties.

Roles of apelin in fluid and energy homeostasis

Function	-13	Pyr-13	-17	-36	Other isoforms	References
Decrease core body temperature	✓					(67)
Increase core body temperature	✓					(118)
Increase diuresis	✓	✓	✓			(60, 61, 107, 108, 188)
Decrease food intake	✓				-12	(48, 165, 195, 244)
Increase food intake	✓					(75, 155, 202, 251, 260, 266)
Decreased water/fluid intake	✓	✓				(48, 218)
Increase water/fluid intake	✓	✓				(61, 145, 246)

(+ / + / + / +) represents potency and/or efficacy differences between isoforms compared in a single publication where number of + signs represents increase in given functional property.

Table 9

Roles of apelin in regulating adipoinsular axis

Function	-13	Pyr-13	-17	-36	Other isoforms	References
Decrease abdominal adiposity		✓				(296)
Increase brown adipose tissue (BAT) temperature	✓					(171)
Increase citrate synthase activity	✓					(79)
Increase cytochrome c oxidase (COX1) activity	✓					(79)
Decrease energy expenditure	✓					(67)
Decrease free fatty acid (FFA) release		✓				(254, 296)
Decrease free glycerol release		✓				(296)
Increase gluconeogenesis	✓					(66)
Increase glucose absorption from intestine		✓				(65)
Increase glucose uptake	✓	✓				(8, 64, 77, 281, 295)
Increase glucose-stimulated insulin secretion (high dose)				✓		(219)
Decrease glucose-stimulated insulin secretion (low dose)		✓		✓		(86, 219, 242)
Increase glucose transporter (GLUT2 and 4) levels		✓				(65, 77)
Increase glycogenolysis	✓					(66)
Decrease glycaemia (low dose Duparc)	✓					(64, 68, 77)
Regulate hormone-sensitive lipase (HSL) activation		✓				(296)
Cause hyperglycemia (high dose Duparc)	✓	✓				(66, 68)
Increase β -hydroxyacyl CoA dehydrogenase (β HAD) activity	✓					(79)
Increase interleukin 1 β (IL-1 β) expression	✓					(67)
Decrease insulin levels in serum	✓					(79)
Decrease leptin levels		✓				(296)
Increase mitochondrial import and assembly components	✓					(79)
Decrease PR-domain containing protein 16 (PRDM16) levels	✓					(67)
Decrease proliferator activated receptor- coactivator-1 α (PGC1 α)	✓					(67)
Increases proliferator activated receptor- coactivator-1 β (PGC-1 β) levels	✓					(79)

Function	-13	Pyr-13	-17	-36	Other isoforms	References
Decrease sodium glucose transporter-1 (SGLT1) levels		✓				(65)
Decrease uncoupling protein (UCP1) levels	✓					(67)
Increased uncoupling protein (UCP1) levels	✓					(171)

✓ represents isoform(s) used.

(+/+/+/++) represents potency and/or efficacy differences between isoforms compared in a single publication where number of + signs represents increase in given functional property.

Table 10

Roles of apelin in regulating adipocytes

Function	-13	Pyr-13	-17	-36	Other isoforms	References
Inhibit adipogenesis		✓				(254)
Decrease acetyl-CoA carboxylase (ACC) expression		✓				(254)
Decrease adipocyte protein 2 (aP2) expression		✓				(254)
Increase adiponectin release		✓				(256)
Increase catalase levels		✓				(256)
Decrease CCAAT/enhancer binding protein- α (C/EBP- α) expression		✓				(254)
Increase CCAAT/enhancer binding protein- β (C/EBP- β) expression		✓				(255)
Increase cell death-inducing DNA fragmentation factor A-like effector A (CIDE-A) expression		✓				(255)
Increase cytochrome c oxidase subunit 1 (COX1) levels		✓				(255, 256)
Decrease fatty acid synthase (FAS) expression		✓				(254)
Increase glutathione peroxidase (GPx) levels		✓				(256)
Increase insulin receptor recycling		✓				(313)
Decrease interleukin-6 (IL-6) release		✓				(256)
Decrease leptin expression		✓				(254)
Increase lipid droplet size		✓				(254)
Decrease NADPH oxidase subunit (p47 phox) levels		✓				(256)
Increase O ₂ consumption		✓				(255)
Decrease perilipin activation (phosphorylation)		✓				(254)
Increase peroxisome proliferator-activated receptor- coactivator-1 α (PGC-1 α) levels		✓				(255, 256)
Decrease peroxisome proliferator-activated receptor- (PPAR-) expression		✓				(254)
Increase peroxisome proliferator-activated receptor- (PPAR-) expression		✓				(255)
Increase PRD1-BF1-RIZ1 homologous domain containing 16 (PRDM16) expression		✓				(255)
Decrease reactive oxygen species (ROS) production		✓				(256)
Increase succinate dehydrogenase complex subunit A (SDHA) levels (normal/vehicle induced)		✓				(256)
Increase superoxide dismutase (SOD1) levels		✓				(256)

Function	-13	Pyr-13	-17	-36	Other isoforms	References
Decrease tumor necrosis factor- α (TNF- α) release		✓				(256)
Decrease triglyceride level	✓	✓				(87, 254)
Increase uncoupling protein 1 (UCP1) expression		✓				(255)
Increase Wnt10b expression		✓				(254)
Inhibit vascular hyperpermeability	✓					(225)

✓ represents isoform(s) used.

(+/+/+/+/++) represents potency and/or efficacy differences between isoforms compared in a single publication where number of + signs represents increase in given functional property.

Table 11

Roles of apelin in diabetes models at the physiological level

Function	-13	Pyr-13	-17	-36	Other isoforms	References
Increase body temperature	✓					(97)
Decrease body weight (adenovirus-mediated for Galon-Tillemann)	✓ (+/-)			✓ (+)		(30, 80)
Decrease cholesterol levels (adenovirus-mediated for Galon-Tillemann)	✓ (+)		✓ (-)	✓ (++)	-16 (-)	(80)
Increase CO ₂ release	✓					(9)
Decrease fat mass	✓					(9, 97)
Increase fatty acid oxidation	✓					(9)
Increases glucose turnover	✓					(64)
Increase glucose uptake		✓				(77, 318)
Decrease glycaemia (adenovirus-mediated for Galon-Tillemann)	✓ (+/-)	✓	✓ (+/-)	✓ (++)	-16 (+)	(3, 9, 64, 77, 80)
Increase lowered glycogen synthesis in hepatocytes	✓					(43)
Decrease hyperinsulinemia	✓	✓				(3, 9, 30, 97)
Increase lowered insulin content	✓					(37)
Decrease insulin resistance (glucose tolerance test)		✓				(77)
Increase mitochondrial biogenesis	✓					(9)
Increase lowered pancreatic islet mass	✓					(37)
Increase O ₂ consumption	✓					(9, 97)
Decrease triglyceride levels	✓					(9, 97)

✓ represents isoform(s) used.

(+/-/+/++) represents potency and/or efficacy differences between isoforms compared in a single publication where number of + signs represents increase in given functional property.

Table 12

Roles of apelin in diabetes models at the molecular level

Function	-13	Pyr-13	-17	-36	Other isoforms	References
Decrease acylcarnitine levels	✓					(9)
Increased adiponectin levels	✓	✓				(97, 318)
Increase lowered autophagy related protein-7 (Atg7)	✓					(30)
Decrease elevated calnexin level	✓					(37)
Decrease elevated C/EBP homologous protein (CHOP) activation/levels	✓					(30, 37, 248)
Decrease elevated chaperone binding protein (BiP)	✓					(30)
Decrease elevated 78 kDa glucose-regulated protein (GRP78) levels	✓					(248)
Increase glucose transporter 4 (GLUT4) translocation under insulin-resistant setting		✓				(77, 318)
Increase glycogen synthase kinase (GSK) activation	✓					(43, 287)
Decrease elevated heat shock protein 70 (HSP70) level	✓					(37)
Decrease elevated inositol requiring enzyme 1 α (IRE1 α) activation	✓					(37)
Decrease elevated JNK activation (ER stress marker)	✓					(248)
Increase lowered light chain 3B I and II (LC3BI and LC3BII)	✓					(30)
Decreased leptin levels	✓					(30, 97)
Increase lowered miRNA levels (-133a, -208, -1)	✓					(30)
Increase mitochondrial transcription factor A (TFAM) expression	✓					(9)
Increase nuclear respiratory factor 1 (NRF1) expression	✓					(9)
Increase peroxisome proliferator-activated receptor- coactivator-1 α (PGC-1 α) expression	✓					(9)
Decrease elevated PKR-like eukaryotic initiation factor 2 α kinase (PERK) activation	✓					(37)
Increase uncoupling protein (UCP-1 and 3) levels	✓					(97)

✓ represents isoform(s) used.

(+/+/+/++) represents potency and/or efficacy differences between isoforms compared in a single publication where number of + signs represents increase in given functional property.

Table 13

Comparison of apelin isoforms

	-13	Pyr-13	-17	-36	Other isoforms	References
Competitive	✓ (++++)	✓ (++++)	✓ (+)	✓ (+)		(61, 72, 81, 101, 112, 113, 186-189)
noninhibition of	✓ (+)	✓ (+)	✓ (+)	✓ (++++)	-12 (+), -11 (-)	(70, 101, 128, 176)
	✓	✓				(15, 39, 186, 188)
inhibitor-mediated)	✓	✓ (+)		✓ (+)		(291)
	(+)	✓ (+)	✓ (++)	✓ (+)		(81)
	(+)	✓ (+)	✓ (++++)	✓ (++++)	-16, -12(+), -11(-), -10(-)	(81)
	(+)			✓ (-)		(146)
ling	(+)			✓ (-)		(146)
e trafficking	(-)			✓ (+)		(146)
	(+)		✓ (+)	✓ (++++)		(42, 174, 212)
P production	(+)	✓ (++++)	✓ (++++)	✓ (+)	-12(+), -11(-), -10(-)	(16, 39, 59, 70, 80, 81, 86, 89, 112, 113, 128, 176, 182, 186, 187, 206, 217, 218, 284)
	(+)	✓ (+)	✓ (+)	✓ (+)	-12 (+)	(21, 27, 37, 53, 64, 84, 87, 111, 132, 148, 151, 156, 157, 163, 173, 174, 181, 193, 197, 206, 234, 238, 239, 243, 247, 248, 255, 256, 268, 269, 278-280, 287-289, 295, 297, 299, 311, 318, 320)
se (AMPK)	✓	✓				(8, 9, 37, 64, 65, 200, 248, 255, 256, 281, 288, 290, 295, 296)
CC) activation	✓	✓				(9, 64, 281, 295, 296)
	(+)	✓ (++++)	✓ (+)	✓ (+)	-55 (+), -16	(14, 16, 21, 37, 39, 50, 53, 61, 81, 104, 111, 148-150, 156, 157, 172-174, 193, 197, 201, 204, 234, 235, 238, 239, 243, 248, 254, 256, 278, 283, 287, 289, 297, 299, 320)
ivation	✓	✓		✓	-16	(31, 50, 95, 157, 159, 172, 173, 204, 267, 268)
on	✓ (++++)	✓ (++++)	✓ (++)	✓ (+)		(101, 249, 319)
	✓ (+/-)	✓ (+)		✓ (+)		(4, 21, 38, 50, 51, 53, 90, 102, 161, 206, 220, 247, 248, 273, 275, 280, 282, 297, 298, 311, 317, 320)
-12 activation	✓ (+)	✓ (+)		✓ (+)		(38, 119, 130, 206, 247, 248, 280, 282, 297, 298, 311, 320)
	✓ (++++)	✓ (+/-)		✓ (+/-)	-12 (-)	(22, 51, 92, 94, 127, 135, 149, 150, 156, 270, 279, 294)
						(14, 27, 38, 99, 102, 201, 214, 222, 275, 283, 288, 301, 302)
	✓ (+)	✓ (++++)		✓ (+)		(21, 38, 51, 101, 114, 126, 127, 133, 135, 158, 161, 164, 214, 222, 226, 269, 273, 282, 288, 301, 302)

represents potency and/or efficacy differences between isoforms compared in a single publication where number of + signs represents increase in given functional property.

Table 14

Apela physiological functions

Function	-32	Pyr-32	-21	-11	Other isoforms	References
Receptor binding (both AR and unknown receptor)	✓ (++)	✓ (++)	✓ (++)	✓ (+)	-16 (++)-14 (++)	(61, 188, 203, 286)
Receptor internalization (both AR and unknown receptor)	✓ (++)		✓ (++)	✓ (+)		(98, 199, 274, 286)
Inhibit cAMP production	✓ (+)		✓ (+)	✓ (+)	-14(+)	(61, 274, 286)
G-protein interaction		✓ (++)		✓ (+)	-16 (++)-14 (++)	(188)
β-arrestin interaction	✓ (++)	✓ (++)	✓ (++)	✓ (+)	-16 (++)-14 (++)	(188, 286)
G-protein activation	✓					(61)
ERK activation	✓					(61, 203, 274, 286)
eNOS activation	✓					(286)
PRAS40 activation (unknown receptor)	✓					(98)
SMAD3 activation (Nodal/TGFβ pathway; unknown receptor)	✓					(98)
p70-S6K activation (mTORC1 pathway; unknown receptor)	✓					(98)
Akt activation (unknown receptor)	✓					(98)
Primes ESCs for differentiation (observation of multiple gene expressions; unknown receptor)	✓					(98)
Increase cell proliferation (unknown receptor)	✓					(98)
Increase translation (protein production for mitosis; unknown receptor)	✓					(98)
Inhibit apoptosis (vehicle-induced; unknown receptor)	✓					(98)
Regulate cell migration (mesodermal movement)			✓			(199)
Increase diuresis	✓	✓ (+)			-14(++)	(61, 188)
Increase water intake	✓					(61)
Decrease food intake			✓			(223)
Increase c-Fos expression in the brain			✓			(223)
Increase Ca ²⁺ mobilization	✓		✓			(223, 274)
Increase angiogenesis (tubule formation)	✓					(274)
Increase cardiac force contractility	✓					(203)
Relaxes blood vessel	✓					(274)

Function	-32	Pyr-32	-21	-11	Other isoforms	References
NO-mediated effect (inhibitor-mediated, L-NAME)	✓					(274)
Increase left ventricular ejection fraction (LVEF)	✓					(286)
Increase right ventricular ejection fraction (RVEF)	✓					(286)
Increase cardiac output	✓					(286)
Increase cardiac contractility (dp/db)	✓					(286)
Increase left ventricular developed pressure (LVDP)		✓			-14 (+)	(188)
Decrease mean arterial blood pressure (MABP)		✓ (+++)		✓ (+)	-14 (++)	(188)
Reduce hypertrophy (vehicle-induced)	✓					(286)

✓ represents isoform(s) used.

(+ / ++ / +++) represents potency and/or efficacy differences between isoforms compared in a single publication where number of + signs represents increase in given functional property.