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Structure–function relationship and physiological role of apelin and its G protein coupled receptor

Subhashree Murali[1] · **Gopala Krishna Aradhyam**[1]

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Abstract

Apelin receptor (APJR) is a class A peptide (apelin) binding G protein-coupled receptor (GPCR) that plays a significant role in regulating blood pressure, cardiac output, and maintenance of fluid homeostasis. It is activated by a wide range of endogenous peptide isoforms of apelin and elabela. The apelin peptide isoforms contain distinct structural features that aid in ligand recognition and activation of the receptor. Site-directed mutagenesis and structure-based studies have revealed the involvement of extracellular and transmembrane regions of the receptor in binding to the peptide isoforms. The structural features of APJR activation of the receptor as well as mediating G-protein and β-arrestin-mediated signaling are delineated by multiple mutagenesis studies. There is increasing evidence that the structural requirements of APJR to activate G-proteins and β-arrestins are different, leading to biased signaling. APJR also responds to mechanical stimuli in a ligand-independent manner. A multitude of studies has focused on developing both peptide and non-peptide agonists and antagonists specific to APJR. Apelin/elabela-activated APJR orchestrates major signaling pathways such as extracellular signal-regulated kinase (ERKs), protein kinase B (PKB/Akt), and p70S. This review focuses on the structural and functional characteristics of apelin, elabela, APJR, and their interactions involved in the binding and activation of the downstream signaling cascade. We also focus on the diverse signaling profile of APJR and its ligands and their involvement in various physiological systems.

Keywords Apelin receptor; Apelin · Elabela · GPCR · Signaling

Introduction

Class A G protein-coupled receptors (GPCRs) are the largest group of the seven-transmembrane proteins and a vast majority of the therapeutic agents developed are targeted toward these receptors (Hauser et al. 2017). GPCRs transduce signals by recruiting G-proteins which subsequently activate downstream pathways in response to ligand binding. Class A GPCRs undergo β-arrestin-mediated internalization (Rosenbaum et al. 2009). Apelin receptor (APJR), an important peptide-binding class A GPCR, was considered an orphan receptor until 1998, when it was found that the receptor binds to various isoforms of the endogenous peptide ligand apelin which shares 40% sequence homology with the angiotensin-1 receptor (Chapman et al. 2014). Apelin receptor transduces signals by coupling to $G\alpha_i$ and $G\alpha_q$ proteins and by activating the PLC-β, PI3/Akt and P70S6 kinase pathways in various cell types (Shin et al. 2017b).

Ma et al. reported the first crystal structure of APJR bound to AMG3054, a cyclic apelin mimetic peptide with 2.6-A resolution (Ma et al. 2017) (Fig. 1). Multiple studies involving site-directed mutagenesis, along with the latest APJR structure bound to a single-domain antibody with agonist properties, have given insights into the ligand-binding mode and its probable activation mechanism (Shin et al. 2017b). Since APJR is implicated in many physiological processes, novel peptides and cyclic analogues are being investigated for their potential therapeutic applications (Khan et al. 2010). Another recently discovered endogenous ligand, elabela, is known to mediate the development of the cardiovascular system at the embryonic level, though it remains to be characterized in humans (Wang et al. 2015). This review summarizes the structural determinants of ligand binding, activation and functional selectivity of APJR to its cognate downstream partners. Furthermore, recent progress in the characterization of its ligands apelin and elabela and the development of peptide-based

✉ Gopala Krishna Aradhyam
agk@iitm.ac.in

[1] Department of Biotechnology, Bhupat and Jyoti Mehta School of Biological Sciences, Indian Institute of Technology Madras, Chennai, India

agonists and antagonists are also discussed. Additionally, the review also elaborates on the role of the APJR/Apelin system in various pathophysiological conditions.

Ligands of apelin receptor

Overview

The endogenous ligands for APJR were initially isolated from bovine colostrum, where apelin isoforms of lengths 13, 17, and 36 amino acids were discovered (Tatemoto et al. 1998). Apelin is expressed and synthesized as a 77 amino acid pre-peptide that is then cleaved into its functional isoforms. Evidence suggests that the action of an endoprotease produces apelin-36, apelin-17, and apelin-13. Proprotein convertase subtilisin/Kexin 3 (PCSK3) or furin enzyme recognizes and cleaves at dibasic residues contained within the motif RR-XX-RR (Fig. 1) (Shin et al. 2013; Adam et al. 2016). The different isoforms are expressed widely with diverse distribution pattern among the cardiac, brain and endocrine tissues, possibly regulating distinctive signaling for each tissue (Shin et al. 2017b). Apelin-12 (Fig. 1) is the shortest active isoform and was found to be able to reduce blood pressure in rats (Tatemoto et al. 2001). The longest isoform, Apelin-55, which was previously considered to be a pre-peptide in the cleavage pathway of apelin, has been found to bind to APJR and cause activation of the ERK pathway (Shin et al. 2017a).

The twelve amino acid residues at the C-terminus of all apelin isoforms are highly conserved across many species and are critical for receptor activation (Zhang et al. 2014). The N-terminal glutamine of apelin-13 is post-translationally modified to pyro-glutamate, making the peptide resistant to degradation by N-terminal exo-peptidases, thereby increasing its half-life in the plasma (Zhen et al. 2013).

There are characteristic differences in the potencies of apelin isoforms in activating the receptor. Function of these peptides seems to be size dependent, although in a context-dependent manner (Tatemoto et al. 1998). Shorter versions (apelin-13 and apelin-17) are the most potent isoforms as revealed by cell acidification assays in CHO cells. Apelin-13 and apelin-17 have been reported to be the more potent in inducing cell proliferation than apelin-36 (Wang et al. 2004; Kasai et al. 2004), while all isoforms showed comparable potencies when monitoring cell migration or activation of the ERK pathway assays (Medhurst et al. 2003; Gerbier et al. 2015).

Structural characteristics of apelin peptide

The structure–function relationship of apelin peptides has been studied in detail using specific mutations. Amino acid

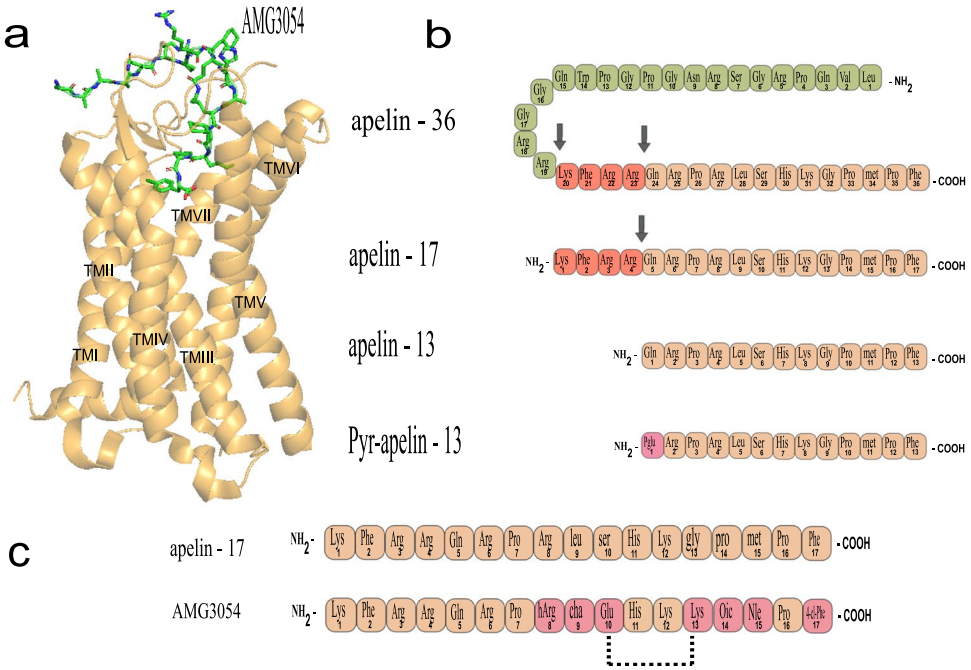

Fig. 1 **a** Representation of crystal structure of APJR-AMG3054 complex (PDB: 5VBL). The modified peptide ligand is depicted in green (sticks) and the receptor is shown in orange (cartoon). **b** Amino acid sequence of apelin peptide isoforms. Residues of Apelin-36, Apelin-17, Apelin-13, and Pyro-glutamate (Pyr) Apelin-13 are indicated. Grey arrows show the site of cleavage by PCSK3/furin enzyme in the larger isoforms, Apelin-36 and Apelin-17 **c** Comparison between Apelin-17 and AMG3054. The latter is a modified cyclic peptide (cyclised between Glu10 and Lys13, indicated by dotted lines). The modified residues of AMG3054 are coloured in pink. hARG, homoarginine; CHA, cyclohexylalanine; OIC, Octahydroindole-2-carboxylic Acid; NLE, Norleucine; 4-Cl-PHE, 4-choloro-Phenylalanine

substitutions of the N-terminal ^{2}RPRL^{5} motif in apelin-13 (Fig. 1) lead to loss of receptor binding (Medhurst et al. 2003). The same study showed that changes in Q^{1}, S^{6}, and H^{7} residues produce minimal disruption in receptor binding. These results emphasize the indispensable role of the ^{2}RPRL^{5} motif in APJR activation. The structural and conformational importance of this domain was further emphasized by cyclic analogues of apelin-13 peptide. Cyclization of the ^{2}RPRL^{5} motif led to higher affinity to the receptor when compared to other peptide analogues that were lacking cyclization of the motif (Macaluso and Glen 2010). NMR analysis of the apelin isoforms shows that the RPRL motif forms a characteristic β-turn (Langelaan et al. 2009) that could be vital in specific recognition by the receptor, as observed in many class A GPCRs with peptide ligands (Tyndall et al. 2005).

The C-terminal F^{13} residue of apelin isoforms seems to play a crucial role in activating the receptor even though reports are somewhat contradictory (Murza et al. 2015). In the AMG3054 bound APJR crystal structure, the C-terminal modified residue 4-Cl-F is seen to make multiple highly conserved contacts with the transmembrane region of the receptor (Ma et al. 2017) (Fig. 1, Table 1). Alanine substitution of the conserved F^{13} in apelin peptide did not affect its binding to the receptor and activation of the cAMP pathway (Ceraudo et al. 2014), although it did lead to loss of receptor internalization, indicating its primary role in mediating structural changes that activate the β-arrestin pathway. Interestingly, replacing F^{13} with hydrophobic, unnatural amino acids led to an increase in its binding affinity and potency in lowering blood pressure in mouse models (Murza et al. 2015). Additionally, angiotensin-converting enzyme- 2 (ACE2) was found to inactivate apelin peptides by selective cleavage of two C-terminal residues—P^{12}, F^{13} (number based on apelin-13, Fig. 1). Wang et al. produced peptide analogues that were resistant to ACE2 cleavage by substituting the C-terminal M^{11} and F^{13} residues, and these modified 12-residue peptides displayed cardioprotective effects (Wang et al. 2016). The details of the structural determinants of apelin are summarized in Table 1.

Structure–function relationship of elabela

Apelin receptor early endogenous ligand (APELA or Elabela) was recently discovered as another endogenous ligand for the APJR (Murza et al. 2016). It is expressed in the early developmental stages in humans and has been demonstrated to be essential for the proper formation of the cardiovascular system in zebrafish (Ho et al. 2015). The 54 amino acid pre-peptide is cleaved into isoforms with -32, -22, and -11 amino acid long peptides (Fig. 2a), of which elabela-11 is the only isoform detected in the plasma (Shin et al. 2017b). Elabela isoforms also elicit the same downstream signaling pathways as activated by the apelin peptides, such as $G\alpha_i$-mediated cAMP inhibition and ERK activation (Wang et al. 2015). There are significant differences in potencies among the isoforms, with elabela-11 demonstrating the lowest potency in binding, $G\alpha_i$ activation as well as β-arrestin recruitment (Trân et al. 2021). Like apelin, elabela isoforms contain seven C-terminal amino acids that are completely conserved across different classes of animals (Fig. 2b). Additionally, it also contains two conserved cysteine residues, which contribute to the dimerization of elabela-11 (Huang et al. 2017). The two peptide ligands apelin and elabela have the same clusters of amino acids in the N-terminal and the C-terminal regions at similar positions, indicating their important role in mediating the binding and activation to the receptor. (Fig. 2C).

Alanine scanning studies have demonstrated the pivotal role of the C-terminal residues H^{15}, R^{17}, V^{18}, P^{19}, F^{20}, and P^{21} (Fig. 2d). These residues are very important for receptor binding and G-protein activation (Fig. 2d). (Couvineau et al. 2020). However, mutations in N–terminal basic residues in elabela peptide do not significantly affect APJR signaling (Murza et al. 2016). These studies establish that ^{8}QRR^{10} and ^{18}VPFP^{21} in the peptide are important motifs in eliciting downstream signaling. The apelin and elabela peptides display a distinct structure–function relationship despite their sequence similarity.

APJR agonists and antagonists

Apelin peptides are potent initiators of the inotropic effect, and their role in regulating the cardiovascular system and fluid homeostasis has led to the development of peptide analogues that have shown promise as potential pharmacological agents. One such cyclic analogue, MM07, has been successfully characterized and shown to effectively activate the Gαi pathway over β-arrestin mediated signaling (Yang et al. 2019), with an increase in cardiac output in animal models (Brame et al. 2015). Gerbier et al. synthesized the chemically modified apelin-17 analogs P92 and LIT01–196, which were extra stable in the plasma and caused vasorelaxation with increased cardiac contractility in mice (Gerbier et al. 2017). A cyclic analogue, containing two ^{2}RPRL^{5} motifs, demonstrated competitive antagonism towards APJR (Macaluso et al. 2011).

In addition to modified peptide analogues, many studies have been performed to identify non-peptide agonists and antagonists. For instance, a compound known as E339-3D6, was discovered to trigger internalization, reduce forskolin-induced cAMP formation, and cause vasodilation in rats (Iturrioz et al. 2010a). Subsequent studies showed that E339-3D6 derived compounds, named 19 and 21, exhibited better signaling and increased plasma stability (Margathe et al. 2014). Another small molecule, CMF-019, was shown to be biased towards $G\alpha_i$ signaling and was less effective in activating β-arrestin pathways. It also increased heart contractility and vasodilation in mice in addition to protecting endothelial cells from apoptosis (Read et al. 2016). Similar reports of small molecule agonists for APJR include compounds 15a and ML233 (Khan et al. 2010; Su et al. 2019). Detailed scanning of various macrocyclic compounds of apelin-13 led to compounds numbered 39 and 40, which were produced with truncation of important residues (Q^{1}, R^{2}, P^{12}, F^{13}) and substitution of

Table 1 Molecular specifics of apelin-13 peptide in binding and activation of the APJ receptor. Amino acids are represented in their single-letter codes. ↑ represents an increased effect and ↓ depicts an attenuation effect

Apelin molecule (no. based on apelin-13)	Interacting residues in APJR	Effect	Mutation studies	Reference
Q^{1}	$T177^{ECL2}$	Hydrogen bonding between sides chain of Q and T177	Mutation to alanine increases binding affinity and cAMP inhibition ($G\alpha_i$ mediated)	(Ma et al. 2017)
R^{2}			Substitution by D-isoform ↓ binding; mutation to alanine significantly ↓ binding	
P^{3}			Substitution by D-isoform ↓ binding affinity	
R^{4}	$Y21^{N-TERM}$ $D23^{N-TERM}$	β-sheet formed between the R8-E10 of the modified peptide and N-terminal of the receptor	Substitution by D-isoform ↓ binding affinity	
L^{5}			Substitution by D-isoform did not affect affinity; mutation to alanine significantly ↓ affinity and intracellular Ca^{2+} increase. ($G\alpha_i$ mediated)	
S^{6} (OiC14 in AMG3054)			Substitution by D-isoform ↓ affinity; mutation to alanine ↑ binding affinity and intracellular Ca^{2+} increase. ($G\alpha_i$ mediated)	
H^{7}	$E174^{ECL2}$	Electrostatic interaction between H and E174 of ECL2	Substitution by D-isoform decreased affinity; mutation to alanine ↑ binding affinity and intracellular Ca^{2+} increase. ($G\alpha_i$ mediated)	
K^{8}			Substitution by D-isoform ↓ affinity	
P^{10} (OiC^{14} in AMG3054)	$L173^{ECL2}$, $Y182^{ECL2}$, $Y185^{ECL2}$	Hydrophobic contacts	Substitution by D-isoform ↓ affinity	
M^{11} (Nle^{15} in AMG3054)	$Y271^{6.58}$, $F291^{7.35}$	Interacts with the aromatic side chains of Y271 and F291	Mutation to alanine ↑ intracellular Ca^{2+} ($G\alpha_i$ mediated); Tolerates F and L substitution, V totally abolishes signaling	(Medhurst et al. 2003; Murza et al. 2012; Zhang et al. 2014; Ma et al. 2017)
P^{12}	$W24^{N-TERM}$, $Y93^{ECL1}$, $R168^{4.64}$	Pyrrole ring interacts with N-term and ECL1 residues; causes kink in peptide; hydrogen bond with R168	Substitution by D-isoform did not alter affinity significantly;	
F^{13} (4-Cl-F in AMG3054)	$Y35^{1.39}$, $W85^{2.60}$, $Y88^{2.63}$, $Y93^{ECL1}$, $V117^{3.40}$, $R168^{4.64}$, $W261^{6.48}$, $Y299^{7.43}$, $K268^{6.55}$, $Y264^{6.51}$	Vanderwaals contact with hyrdrophobic binding cavity lined by residues in TM; polar interactions with K268 and Y294; electrostatic interaction with R168	Substitution to alanine does not impact binding affinity or $G\alpha_i$-mediated signaling; Impairs internalization and β-arrestin Substitution by D-isoform did not alter binding affinity significantly	(Iturrioz et al. 2010b; Murza et al. 2015; Ma et al. 2017)

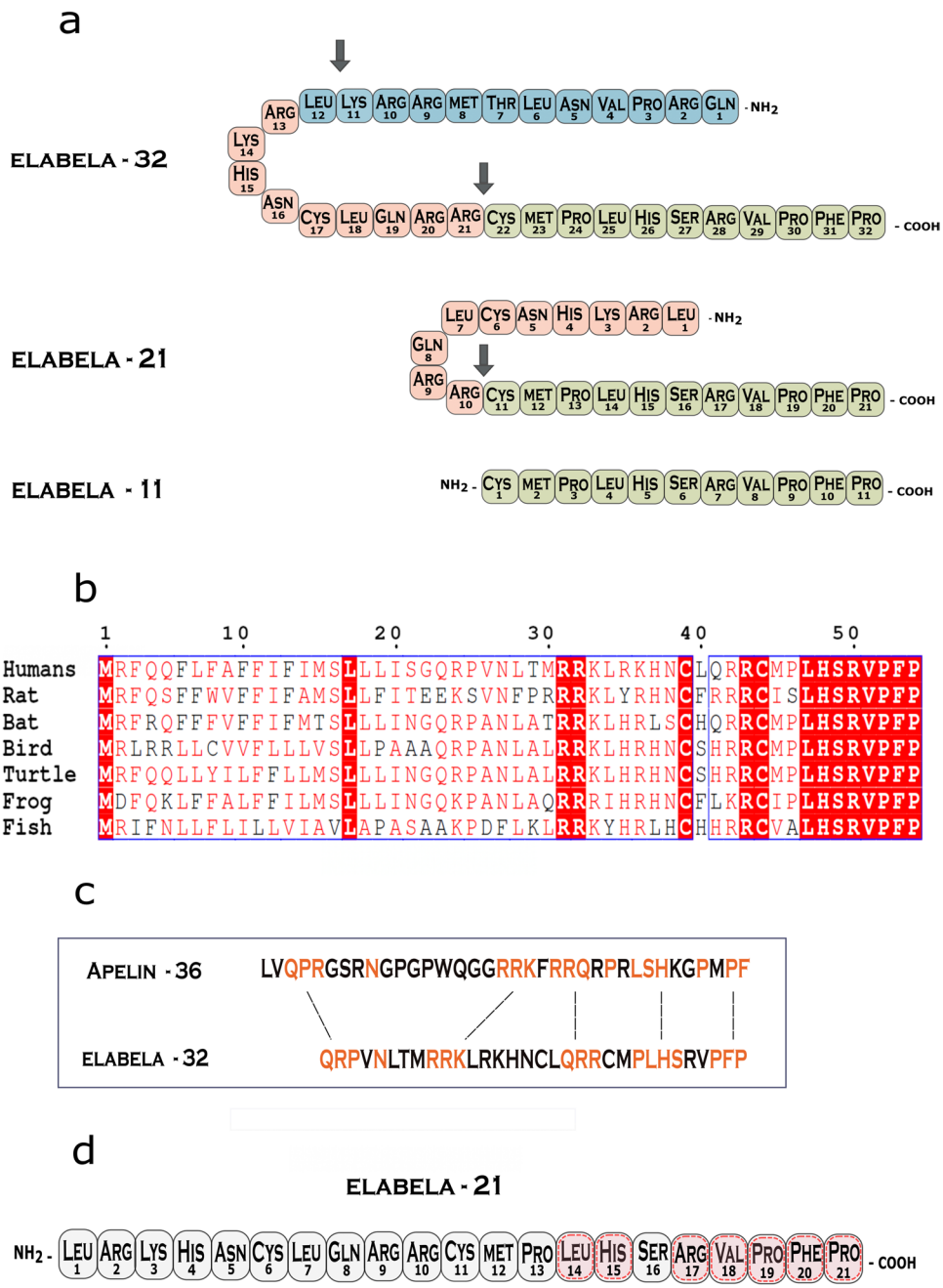

Fig. 2 Conserved sequence of elabela isoforms. **a** Amino acid sequence of elabela-32, elabela-21 and elabela-11. Grey arrows depict the conserved cleavage site by PCSK3/Furin enzyme on larger isoforms elabela-32 and elabela-21. **b** Sequence alignment of elabela-54 pre-peptide in various organisms. The eight amino acids of the C-terminal region and the two cysteine residues show complete conservation, as highlighted in red. The alignment was performed using Clustal Omega (Sievers et al. 2011). **c** Comparison of Apelin-36 and Elabela-32 shows conserved residues (highlighted in orange) at similar positions in both the N- and C-terminal of the peptides. This further emphasizes their shared structure–function relationship inactivation of the receptor. **d** Structure–function relationship of elabela. The highlighted residues that predominantly fall in the C-terminal region are considered vital in the peptide's function

M^{11} with unnatural amino acids. All of them showed an increase in binding affinity, plasma stability, and bias towards $G_{\alpha 12}$ signaling (Tran et al. 2022). The details of modified agonists and antagonists for APJR are summarized in Table 2.

Structural determinants of apelin receptor

Role of extracellular loop regions

The combination of results from structural and mutational studies has revealed how the GPCRs respond to ligand binding. The extracellular loops (ECL) of GPCRs are highly flexible, structurally disordered, thereby enabling ligand recognition and initiation of a cascade of conformational changes (Pal and Chattopadhyay 2019). Numerous studies have investigated the structural features of the ligand-binding and activation modes of the APJ receptor. The N-terminal chain and ECL form disulfide bonds that help stabilize the receptor's structure in the membrane, a characteristic feature of many class A GPCRs (Wu et al. 2017). In APJR, two disulfide bonds are formed between C^{19} (N-term)-C^{281} (ECL3) and C^{102}(TM3)-C^{181}(ECL2) (Fig. 3) (Ma et al. 2017). Since APJR recognizes multiple isoforms of apelin and elabela peptides, many studies have been performed

Table 2 List of modified apelin agonist and antagonists

Name	Sequence and structure information	Signaling details	References
MM07	Cyclic peptide; cysteine on either side of RPRL used to cyclize the peptide. CRPRLCHKGPMPF	Potent agonist, preferential $G\alpha_i$ signaling over β-arrestin signaling	(Brame et al. 2015)
MM54	Cyclic peptide; Two cyclized RPRL motif separated by -KH spacer CRPRLCKHCRPRLC	Potent competitive antagonist	(Macaluso et al. 2011)
P92	Apelin-17 analog with chemically modifications	Agonists with increased affinity and stability.	(Gerbier et al. 2017)
LIT01–196	Fluorocarbon chain added at the N terminus of modified apelin-17 analog		
E339-3D6	Non-peptide compound	Agonist; $G\alpha_i$ signaling and internalization; causes vasorelaxation in muscles.	(Iturrioz et al. 2010a, p. 339)
CMF-019	Benzoimidazole-carboxylic acid amide derivatives	Biased agonist towards $G\alpha_i$ signaling; Increases cardiac contractility.	(Read et al. 2016)
Compound 15a	Biphenyl Acid derivative	Agonist; Increased cardiac output in rats	(Su et al. 2019)
ML233	Cyclohexa-2,5-dienone backbone	Agonist	(Khan et al. 2010)
ML211	Kojic acid-based compound	Antagonist	(Maloney et al. 2010)

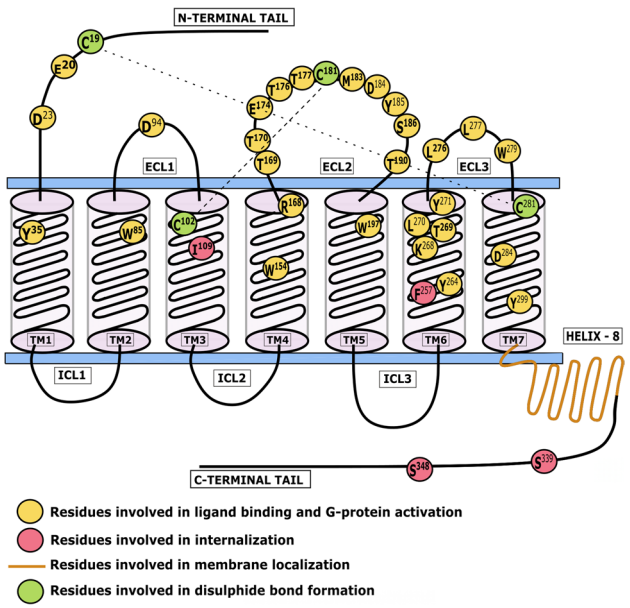

Fig. 3 Snake plot of APJR depicting the structure–function relationship of critical residues. The conserved amino acids of APJR are presented here as their single letter code with the amino acid position in superscript. They are highlighted based on their roles in ligand binding, G-protein activation, and structural stability. The disulfide bond formed between conserved cysteines is represented as dotted lines. ECL, extracellular loop; TM, transmembrane; ICL, intracellular loop

to explore the structural determinants of the ECL regions. Interestingly, mutational analyses in ECL regions of APJR elicit a similar response as they do in other class A GPCRs. Most such mutations in ECL regions of other class A GPCRs are associated with diseases (such as retinitis pigmentosa and nephrogenic diabetes insipidus), exhibiting their importance in pathophysiological problems in signaling (Schöneberg et al. 2004).

In the crystal structure of APJR, reported by Ma et al., the N-terminal region of the cyclic peptide AMG3054 interacts with the N-terminal and ECL2 regions of the receptor (Fig. 1) (Ma et al. 2017). Residues E^{174} (ECL2) and T^{176} (ECL2) interact with H^6 of the peptide (apelin residues number based on Fig. 1), while T^{177} (ECL2) forms hydrogen bonds with Q of AMG3054 peptide. Additionally, the ^{2}RPRL^5 motif of AMG3054 forms a short antiparallel β-sheet with amino acids of the N–terminal chain of the receptor. These interactions stabilize the peptide in its orthosteric binding site, identified as site 2 in the two-state binding model (Ma et al. 2017). The agonist antibody JN241 raised against APJR also makes extensive contacts with the extracellular loops of APJR, where one of the loops of the single-domain antibody occupies the orthosteric binding site. Mutation of E^{174}(ECL2) residue was observed to abolish the binding of the agonist antibody, indicating the vital role of conserved amino acids in this loop in recognizing agonist ligands (Ma et al. 2020). Previous studies by

Table 3 Critical residues of APJR that mediate the various aspects of the receptor signaling. Amino acids are represented in their single letter codes

Amino acid	Region in the receptor	Mutation	Function				References
			Ga_i mediated	β-arrestin mediated	Ligand binding	Others	
E^{20}	N-Terminal	E20A	Reduced $G\alpha_q$-mediated Ca^{2+} signaling	Slightly lowered internalization	Lowered binding affinity	NMR studies show contacts with the membrane; possibly help in capturing the ligand	(Zhou et al. 2003)
D^{23}	N-Terminal	D23A	Abolished $G\alpha_q$-mediated Ca^{2+} signaling	Abolished internalization	Lowered binding affinity		
D^{94}	ECL1	D94A	Diminished $G\alpha_i$-mediated cAMP signaling	Abolished internalization upon apelin-13	Demonstrated no binding	Part of β-turn of ECL2 demonstrated in APJR-AMG3054 structure	(Gerbier et al. 2015; Ma et al. 2017)
		D94N	Did not alter signaling	Did not affect internalization	Did not affect the binding		
M^{183}, D^{184}, Y^{185}, S^{186}	ECL2	MDYS to AAAA	Abolished $G\alpha_q$-mediated Ca^{2+} signaling	Abolished internalization		Showed ERK1/2 phosphorylation; indicative of bias signaling towards $G\alpha_i$ signaling	(Kumar et al. 2016)
T^{169}, T^{170}	ECL2	T169A, T170A	Did not affect $G\alpha_q$-mediated Ca^{2+} signaling	Did not affect β-arrestin 2 recruitment	Showed mild decrease in binding to apelin-13		(Ashokan et al. 2021)
E^{174}	ECL2	E174A	Diminished $G\alpha_i$-mediated cAMP signaling	Abolished internalization	Diminished binding to apelin-13		(Gerbier et al. 2015)
T^{177}; T^{176}	ECL2	T176A, T177A				Observed to form hydrogen bond with His of AMG3054 (His 7 of apelin-13)	(Ma et al. 2017)
T^{190}	ECL2	T190A	Did not affect $G\alpha_q$-mediated Ca^{2+} signaling	Did not affect β-arrestin 2 recruitment	Showed mild decrease in binding to apelin-13		(Ashokan et al. 2021)
W^{197}	ECL2	W197A	Decreased $G\alpha_q$-mediated Ca^{2+} signaling	Decreased β-arrestin 2 recruitment	Showed mild decrease in binding to apelin-13		
K^{268}, T^{269}, L^{270}	ECL3	KTL to AAA	Did not alter $G\alpha_q$-mediated Ca^{2+} signaling	Did not alter internalization			(Kumar et al. 2016)
L^{276}, L^{277}, W^{279}	ECL3	LLW to AAA	Abolished $G\alpha_q$-mediated Ca^{2+} signaling	Abolished β-arrestin 2 recruitment	Abolished binding to apelin -13		(Ashokan et al. 2021)

Table 3 (continued)

Amino acid	Region in the receptor	Mutation	Function				References
			Ga_i mediated	β-arrestin mediated	Ligand binding	Others	
Y^{35}	TM1	Y35A	Abolished $G\alpha_q$-mediated Ca^{2+} signaling	Abolished internalization	Disrupted binding of apelin-13 ligand		(Ban et al. 2018)
W^{85}	TM2	W85A	Abolished $G\alpha_q$-mediated Ca^{2+} signaling	Abolished internalization	Disrupted binding of apelin-13 ligand		
I^{109}	TM3	I109A	Retained $G\alpha_i$-mediated cAMP signaling	Abolished internalization		Causes bias toward β-arrestin-mediated signaling	
N^{112}	TM3	N112G	Higher baseline cAMP activation without ligand addition	Spontaneous internalization without ligand addition		Constitutive active mutant	(Liu et al. 2022)
W^{154}	TM4	W154A				Caused misfolding of protein and no cell surface expression	(Iturrioz et al. 2010b)
R^{168}	TM4	R168A	Abolished $G\alpha_q$-mediated Ca^{2+} signaling	Abolished internalization	Disrupted binding of apelin-13 ligand		(Ban et al. 2018)
F^{257}	TM6	F257W			Abolished internalization	Phe is part of the aromatic pocket that is known to accommodate Phe13 of apelin-13	(Iturrioz et al. 2010b)
Y^{264}	TM6	Y264F	Did not alter $G\alpha_i$-mediated cAMP signaling	Did not alter β-arrestin-mediated signaling	Slightly lowered binding affinity		(Ban et al. 2018)
Y^{271}	TM6	Y271A	Did not alter $G\alpha_i$-mediated cAMP signaling	Abolished internalization	Disrupted binding of apelin-13 ligand		
C^{281}	TM7	C281A	Did not alter $G\alpha_q$-mediated Ca^{2+} signaling	Did not alter internalization			(Kumar et al. 2016)
D^{284}	TM7	D284A/D284N	Diminished $G\alpha_i$-mediated cAMP signaling	Abolished alter internalization	Diminished binding to apelin-13		(Gerbier et al. 2015)

Table 3 (continued)

Amino acid	Region in the receptor	Mutation	Function			Others	References
			$G\alpha_i$ mediated	β-arrestin mediated	Ligand binding		
Y^{299}	TM7	Y299F	Did not alter $G\alpha_i$-mediated cAMP signaling	Did not alter β-arrestin-mediated signaling		Tyr is part of the aromatic pocket that is known to accommodate Phe13 of apelin-13 in APJR structure	(Ban et al. 2018)
S^{348}	C-terminal	S348A	Did not alter $G\alpha_i$-mediated cAMP signaling	Abolished internalization	Did not affect the binding	C-terminal phosphorylation of Ser348 needed for recruitment of β-arrestin	(Chen et al. 2014)
S^{339}	C-terminal	S339A	Did not alter $G\alpha_i$-mediated cAMP signaling	Prevented β-arrestin1/2 recruitment upon apelin-36 addition	Did not affect the binding to apelin and ELABELA isoforms	Mutation impairs the receptor's interaction with GRK2	(Chen et al. 2020)

our group have revealed that mutation of conserved residues in ECL2 and ECL3 regions of APJR caused biased signaling favoring G-protein mediated signaling ^{183}MDYS^{186} (ECL2) mutated to AAAA caused APJR to prefer $G_{\alpha i}$-mediated signaling, while ^{268}KTL^{270} (TM6) to AAA mutation resulted in the receptor favoring $G_{\alpha q}$-mediated signaling over β-arrestin pathways (Kumar et al. 2016; Ashokan and Aradhyam 2017a, b). The extracellular region of APJ involved in ligand binding and activation are summarized in Table 3.

Structural determinants in the transmembrane domains

The transmembrane regions (TM) of class A GPCRs undergo characteristic conformational changes following ligand dependent activation (Cherezov et al. 2007). Upon ligand binding, the TM helices undergo dramatic rigid body movements with respect to the membrane axis that allows subsequent G-protein binding and signaling. The most notable movement in TM domains, initially observed in rhodopsin, is the external rotation of TM6 that creates a binding pocket for Gα binding (Nygaard et al. 2009). Interestingly, APJR-AMG3054 complex was observed to be in an inactive conformation owing to the mutations in conserved residues that are necessary for activation of the receptor (Ma et al. 2017).

The orthosteric binding site of APJR involves the transmembrane regions in a deep binding pocket, relative to the membrane surface. This feature is observed in many class A GPCRs with peptide ligands such as κ- and μ-opioid (Wu et al. 2012; Koehl et al. 2018) and CXCR4 receptors (Wu et al. 2010). In APJR, AMG3054 peptide makes contact with TM 1, 2, 6, and 7 as part of the binding site (summarized in Table 1). The conserved C-terminal region of the peptide penetrates the ligand-binding pocket where F^{13} interacts with critical residues of the transmembrane region such as R^{168} (TM4), Y^{264} (TM6) and triggers activation of the receptor (Ma et al. 2017). It is interesting to note that in molecular dynamics (MD) simulations of APJR and agonist antibody JN241-9, a tyrosine residue introduced into the binding site of JN241 interacted with aromatic amino acids of TM2 and TM7 that led to outward movement of TM6, indicating active state conformation of the receptor (Ma et al. 2020). A recent study has revealed that N112G mutation (TM3) leads to constitutive activation of the receptor. The mutant APJR showed increased basal cAMP levels and internalization in the absence of the ligand (Liu et al. 2022). Molecular modeling suggested that other critical and notable interactions observed between the AMG3054 peptide and the transmembrane region of the receptor involve M^{113} (TM3), W^{261} (TM6), D^{284} (TM7), Y^{271} (TM6), and Y^{264} (TM6). These residues of APJR are conserved in class A GPCRs and are known to form inter-helical contacts that

constitute a ligand-binding cradle critical for its activation and downstream signaling (Venkatakrishnan et al. 2013). For example, mutation of conserved residues in helix 6 (F^{255}, W^{259}) retained $G\alpha_i$-mediated signaling of the receptor upon ligand binding but led to a loss of internalization, indicating activation of specific conformational state of the receptor towards biased signaling (Iturrioz et al. 2010b).

Oligomeric status of APJR

Class A GPCRs form hetero- or homo-dimers, increasing the complexity of their signaling profile in cells (Milligan 2009). Class A GPCRs could therefore be allosterically modulated by each other based on the ligand and the physiological response required from the cells. The interaction between TM helices of two receptors is the likely basis for forming heterodimers, and many studies have attempted to establish this principle (González-Maeso 2011). Towards this point, A2A adenosine and D2 dopamine receptors were observed to form heterodimers in regions of the central nervous systems, and studies involving peptides of individual helices revealed that TM-4 and TM-5 form the interface of their interaction (Borroto-Escuela et al. 2010).

Similarly, APJR has also been found to form homo-dimers in an agonist independent manner, and the TM helices 1–4 are likely involved in forming the interfaces for dimerization. APJR and neurotensin-1 receptors form heterodimers leading to increased ERK signaling and preferential $G_{\alpha q}$ coupling upon agonist stimulation compared to their individual signaling profiles (Bai et al. 2014). APJR was also found to form heterodimers with the κ-opioid receptor enhancing the proliferative signaling in HUVEC cells (Li et al. 2012). However, the transmembrane domains involved in the dimer interface are yet to be determined in detail.

Structural determinants in the intracellular loop regions

The intracellular loop (ICL) regions of GPCRs interact with G-proteins and the downstream signaling molecules that include the scaffolding protein, β-arrestin. Upon ligand binding, ICL regions such as the "DRY" motif in TM3 and "NPXXY" motif in TM7 play a vital role in orchestrating recruitment of the downstream effector molecules. It is known that ICL2 and ICL3 directly make contacts with the Gα subunit, thereby activating the signaling cascade (Nygaard et al. 2009). The "DRY" motif present in APJR and most class A GPCRs holds the receptor in an inactive state by forming an "ionic lock" with TM6. The "NPXXY" motif and the C-terminal Helix 8 region are essential for G-protein binding and internalization in class A GPCRs. Helix 8 (H8) is an alpha-helical motif of the C-terminal region, and many reports have proved that the positive residues of H8 are essential in the folding of the receptor and aid in cell surface localization. (Faussner et al. 2005; Ahn et al. 2010; Kaye et al. 2011). A recent study explored the structure of H8 and its role in the recruitment of APJR to the membrane (Pandey et al. 2019). NMR analysis showed that the C-terminal region of APJR formed an α-helix structure in the H8 region, consisting of 13–16 amino acids. The disruption of the amphipathic nature of the C-terminal tail by mutations led to reduced cell surface expression compared to the wild type receptor (Pandey et al. 2019).

Another essential role of intracellular loops is their interaction with the β-arrestin protein. Receptors recruit arrestins, consequent to their activation, followed by the endocytosis machinery which leads to their internalization. It has been speculated that phosphorylation of C-terminal serine residues in GPCRs acts as a barcode to be recognized by β-arrestin proteins (DeWire et al. 2007). In APJR, S^{348} (C-terminal tail position) was shown to be one of the critical amino acids that mediate the recruitment of β-arrestins and mutation of the same leads to biased signaling towards G-protein signaling (Chen et al. 2014). Another study reported that mutations of S^{335} and S^{339} positions decreased the ability of APJR to recruit β-arrestin1/2 upon apelin-36 peptide stimulation (Chen et al. 2020).

Binding of APJR ligands

The GPCR family of receptors accommodates many ligands with varying sizes and chemical properties (Wu et al. 2017). In peptide-binding GPCRs such as neurotensin-1, histamine, k-OR and CXC4, the ligand-binding pockets differ significantly in terms of their depth and the interactions between the ligand and the receptor (Davenport et al. 2020). AMG3054, used in crystal studies of APJR, is a cyclic peptide with modified amino acids at positions 6 & 10–12 (Residues number based on Fig. 1). The orthosteric binding site for AMG3054 extends into the transmembrane region, forming a deep ligand-binding pocket (Fig. 1). The peptide binds to the receptor with two sets of interactions, described as a "two-site binding mode." The N-terminal amino acids (QRPRL) are anchored on the surface, interacting with residues of the N-terminal and ECL2 regions of the receptor, called "site 1" of the two-state binding model described (Ma et al. 2017). In contrast, the C-terminal end, containing the conserved –PMPF residues, interacts with the transmembrane region of the receptor (Ma et al. 2017). The N-terminal of the peptide and ECL2 regions orchestrate the ligand recognition process, while the C-terminal end of the peptide, probably hold the receptor in one of the active state conformations. This mechanism possibly allows for recognizing multiple isoforms of the apelin ligand whose C-terminal region is conserved and does not vary in length (Iturrioz et al. 2010b).

While apelin and elebela have some degree of sequence similarity (Fig. 2), the binding mode appears to be different. Residues of APJR involved in activation upon apelin binding were tested for their role in elabela binding. Mutation of D284^{TM7}, which is critical for activation of APJR in response to apelin, did not affect elabela binding or subsequent downstream signaling. However, the mutation of residues D92^{ECL2} and E172 ^{ECL2} abolishes signaling for apelin and elabela isoforms, indicating that these residues of APJR share a common function of activating both sets of ligand. (Couvineau et al. 2020). The complete mechanism of elabela binding is not currently fully understood and more work is required in this area.

Signalling profile of APJR/apelin

G-protein-mediated signaling

APJR bound to all apelin isoforms activates $G_{\alpha i}$- and $G_{\alpha q}$-mediated signaling in various cell types. Many studies have established that apelin induces extracellular signal-regulated kinase (ERKs), protein kinase B (PKB/Akt) and p70S in a PTX sensitive, $G_{\alpha i}$-mediated pathway (Chapman et al. 2014). Both in neurons and HEK293 cells apelin-13 and apelin-36 mediate $G_{\alpha q}$ signaling by PLCβ as measured by an increase in intracellular calcium release stimulation (Chaves-Almagro et al. 2015). The specificity to $G_{\alpha i}$

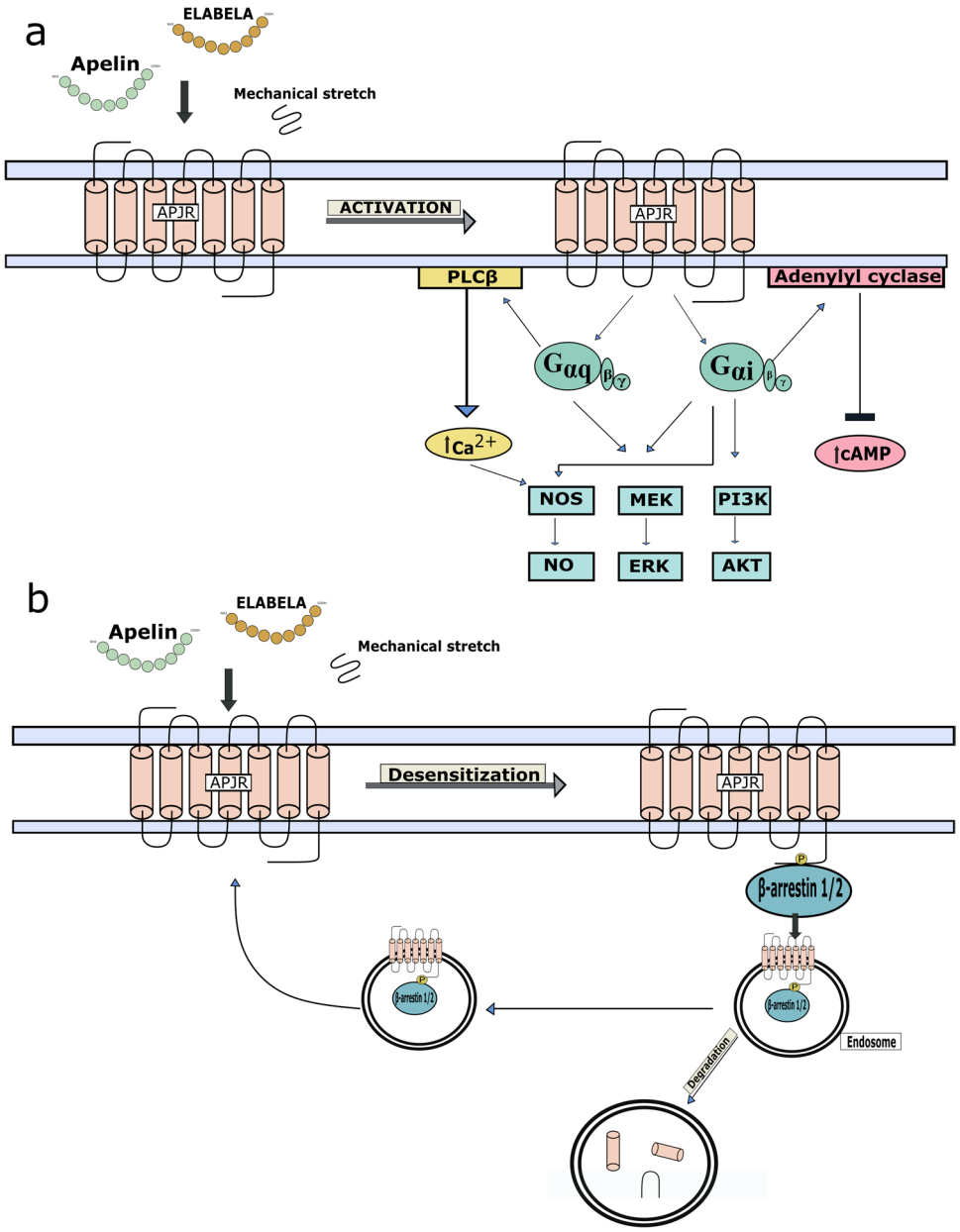

Fig. 4 Overall signaling pathways mediated by APJR and its various ligands. **a** Classic G-protein-mediated activation pathway upon ligand binding. $G_{\alpha q}$ coupling to the activated receptor leads to the activation of PLCβ, which increases intracellular calcium that drives the phosphorylation of ERK, AKT, and production of NO. $G_{\alpha i}$ coupling to the receptor also mediates the ERK, AKT, and NOS pathways and inhibits cAMP production. **b** Desensitization pathway of APJR. Subsequent to activation, phosphorylation of the C-terminal chain of APJR recruits β-arrestins that drive the receptor's endocytosis-mediated internalization drive the receptor's endocytosis-mediated internalization. The internalized receptor is either recycled back to the membrane or directed to proteolysis. ERK, extracellular-signal-regulated kinase; NOS, nitric oxide synthase; NO, nitric oxide; MEK, mitogen-activated protein kinase; PLCβ. phospholipase C; PI3K, phosphoinositide 3-kinases; cAMP, cyclic AMP

protein subtypes was observed to be cell line dependent. In CHO and HEK293 cells, APJR prefers association with $G_{\alpha i1}$ and $G_{\alpha i2}$ (Masri et al. 2006; Bai et al. 2008); whereas in HUVEC cells, APJR preferentially couples to $G_{\alpha i3}$ (Kang et al. 2013). On the other hand, in adipocytes and skeletal muscles, APJR activates the AMP-activated protein kinase pathway (AMPK) (Dray et al. 2010). The G-protein-coupled pathways of APJR are illustrated in Fig. 4.

β-arrestin-mediated signaling

Upon activation, GPCRs undergo internalization mediated by the scaffold protein, β-arrestin. They, in turn, recruit endocytosis machinery such as AP2 and clathrin proteins (DeWire et al. 2007). Following internalization, the receptors are either returned to the membrane or degraded by ubiquitination (Abrisqueta et al. 2018). Based on the rate of recycling to the membrane following internalization, GPCRs are divided into class A (less retention, fast recycling) and class B (longer retention, slow recycling) (Oakley et al. 2000) Interestingly, APJR falls into both classes, based on the ligand that binds to it. In APJR, internalization caused by the shorter isoforms apelin-13 and 17 is transient compared to apelin-36, which causes sustained internalization (Medhurst et al. 2003). Apelin-13 stimulated receptor, upon internalization, is rapidly released before endocytosis. Apelin-36-stimulated receptor follows a different endocytosis pathway, targeting it for degradation in the lysosome (Lee et al. 2010).

The recruitment of β-arrestin 1/2 to the receptor is driven by phosphorylation of the receptor's C-terminal tail, caused by G-protein-coupled receptor-related kinases (GRK) (Gurevich and Gurevich 2019). GRK-2 is known to be involved in GPCR phosphorylation, and after internalization, the endocytosed complex containing the receptor is known to mediate signaling of its own (Gurevich and Gurevich 2019). β-arrestin-mediated signaling goes beyond its role in receptor internalization and warrants further study to understand its role in downstream signaling and physiology.

Biased signaling

GPCRs activate many downstream pathways, and their ligands have been demonstrated to have the capability to activate a particular pathway selectively and preferentially. This phenomenon is known as functional selectivity or biased signaling and such a bias exists between G-protein and β-arrestin-mediated pathways (Davenport et al. 2020). Each pathway performs a distinct function for different physiological conditions, and biased ligands may be more effective in their therapeutic activity with decreased side effects, as seen in the case of many class A GPCRs (Luttrell 2014). Multiple studies of biased signaling have revealed how mutations in receptor residues or differences in ligands cause the receptor to activate either G-protein or β-arrestin-mediated pathways preferentially. In APJR, the I109A mutation in TM3 abolished the ability of the receptor to recruit β-arrestin while still being able to activate G-protein-mediated cAMP signaling (Ban et al. 2018). Synthetic analogue MM07 demonstrated functional selectivity towards the Gα pathway, compared to β-arrestin-mediated internalization (Brame et al. 2015). The synthetic analogue MM07 also demonstrated functional selectivity by favoring the Gα pathway over β-arrestin-mediated internalization (Murza et al. 2015). Apelin-36 analogues were created with L28A substitution and L28C linked with 30 kDa polyethylene glycol (PEG). These analogues were shown to compete with apelin-13 ligand in rat hearts and were observed to be biased towards G-protein-mediated signaling (Nyimanu et al. 2019). Thus, functional selectivity is a crucial aspect to consider while designing drugs targeting APJR.

Ligand-independent signaling

In addition to apelin isoforms and elabela, APJ receptor is also activated by mechanical stretch (Scimia et al. 2012). A study has shown that mechanical shear stress can activate β-arrestin mediated signaling in endothelial cells, consequently regulating polarization (Kwon et al. 2016). In endothelial cells, shear stress increased apelin/APJR expression and caused the release of nitric oxide, which was further amplified when exposed to apelin-12 peptide (Busch et al. 2015). Further studies have shown APJR regulates the cytoskeletal organization in human umbilical vein endothelial cells (HUVEC), and knockdown of APJR in these cells leads to disturbance in the cytoskeleton arrangement in response shear-flow (Strohbach et al. 2018). A study on mechanosensitive properties of histamine H1 receptors (H1R) revealed that helix 8 was involved in the signal transduction of mechanical signals in endothelial cells. H1R receptors lacking helix 8 were unable to respond to shear flow, indicating its essential role in this process (Erdogmus et al. 2019). A similar study on APJR would be very interesting in terms of the physiological phenomenon. The molecular mechanism of mechanical shear induced APJR signaling remains elusive and is a compelling area for future research.

Apelin/APJR Axis in the various physiological system

In the past decade, there has been a tremendous increase in studies that implicate the role of APJR and its ligands in various physiological systems. APJR/Apelin are widely

distributed in the body and are involved in a multitude of disorders such as heart failure (Goidescu and Vida-Simiti 2015), diabetes (Chaves-Almagro et al. 2015), and cancer (Liu et al. 2021). Characterizing the signaling mechanism of APJ receptor and apelin paves the way for developing drugs to treat multiple diseases. The role of APJR/Apelin in physiological systems is summarized in the following sections.

Cardiovascular system

Apelin/APJR system is one of the essential regulators of the heart and the vascular system, and extensive studies for the development of drugs targeting this system are underway. Apelin/APJR are highly expressed in the endothelial lining and play a vital role in angiogenesis. Apelin, one of the most potent endogenous positive inotropes, is implicated for its protective role in cardiovascular diseases such as heart failure, myocardial infarction, and hypertension (Wysocka et al. 2018). In mice, apelin-13 can promote angiogenesis after an ischemic stroke by increasing the expression of VEGF and MMP9 (Chen et al. 2015a). Apelin isoforms -13, 17 and 36 cause vasodilation, thereby regulating blood pressure and have the capacity to lower blood pressure through nitric oxide synthase-dependent mechanisms (He et al. 2015). In mice with myocardial infarction, apelin is seen to drive the recruitment of progenitor cells, promoting neovascularization and, therefore, increasing cardiac function in the diseased tissues (Xu et al. 2012). Apelin was also found to induce angiogenesis and maturation in retinal endothelial cells (Kasai et al. 2004, 2013) and treatment with antagonist ML221 reduced pathological angiogenesis (Ishimaru et al. 2017).

Adipo-insular axis

The physiological effects of apelin have been greatly characterized in adipocytes, where apelin treatment substantially inhibits adipogenesis, decreases free fatty acid release and increases brown adipogenesis (Yue et al. 2011; Than et al. 2015). Administration of apelin-13 increases myocardial glucose uptake and GLUT4 membrane translocation in mice, consequently increasing glucose transport in the cardio myoblast cell line. APJR was observed to mediate this through the AMPK pathway (Xu et al. 2012). Studies on the role of apelin in diabetic nephropathy show varied results. In mice models, apelin administration decreased renal hypertrophy and inflammation, thereby playing a protective role against diabetic nephropathy (Day et al. 2013). However, in humans and mice models, apelin was found to accelerate renal dysfunction by causing the proteasome-driven injury of podocytes (Guo et al. 2015). Several preliminary studies have demonstrated varied roles for apelin. Apelin has also been shown to decrease glycemia, possibly by controlling glucose uptake or gluconeogenesis pathways in skeletal muscles (Dray et al. 2008). In addition, apelin was observed to modulate both insulin and insulin receptor levels. Administration of apelin-13 to diabetic mouse models reduces glycated hemoglobin (HbA1c) and glucose levels in the blood and reduced cholesterol levels (Clarke et al. 2009). These aspects impart therapeutic importance to the apelin/APJ system with the possibility of novel diabetes treatment.

Cancer

The role of APJR/apelin in cancer progression has been a recent area of study, and it has been observed to be involved in critical hallmarks of cancer progression (Hanahan and Weinberg 2011). Apelin peptide has been demonstrated to have a protective role against apoptosis in various cancer cell types such as VSMC, pericytes, and glioblastoma (Cui et al. 2010; Chen et al. 2015b; Harford-Wright et al. 2017). Apelin also increases the proliferation of various cancer types, including lung, colon carcinoma, cholangiocarcinoma (Hall et al. 2017). Most importantly, apelin, a primary player in endothelial regulation and signaling, aids in angiogenesis in cancer cells. In hepatocellular carcinoma, apelin contributes to angiogenesis and dilation of the micro-vessels in tumors, affecting tumor growth (Muto et al. 2014). The apelin/APJ system has also been widely recorded to contribute to the multistep process of invasion and metastasis of cancer. In colon cancer cell lines, four apelin isoforms were able to increase the migration of cancer cells and increase metalloprotease MT1-MMP and these effects were reversed on the addition of antagonist ML221 (Podgórska et al. 2018). A thorough analysis of APJR/Apelin in various cancer types may provide a possible therapeutic target against cancer.

Reproductive physiology

Apelin peptide isoforms are expressed in the uterus and ovaries. Combined with their role in signaling in endothelial cells, APJ/apelin system may play a role in regulating reproductive physiology (Kurowska et al. 2018). Administration of apelin-17 leads to an increase in the levels of ACTH and MSH, respectively, demonstrating its ability to control the hormones from the pituitary and hypothalamus (Reaux-Le Goazigo et al. 2007, 2011). Apelin/APJR play a critical role in follicular development and maturation and possibly does so by activating the AKT pathway (Shimizu et al. 2009). Expression levels of the apelin/APJ receptor system increased during the estrous cycle in bovine ovary cells, seemingly regulated by progesterone and FSH (Schilffarth et al. 2009). The established role of apelin in regulating glucose and fat metabolism also highlights its potential role in disorders such as PCOS, where insulin resistance and obesity are characteristics of prognostic pathogenesis (Kiyak Caglayan et al. 2016). APJR/

apelin-driven regulation of the reproductive system is an emerging arena of research.

Conclusion

APJR/apelin signaling is complex and context dependent, its contribution being elicited by multiple ligand isoforms. Although the isoforms differ in length, they demonstrate similar modes of binding and activation of the receptor. The presence of two agonist bound APJR structures will serve as a guide for detailed studies into the activation mechanism of the receptor as well as the development of agonists/antagonists with increased precision. The signaling profile of APJR/apelin is also diverse, and non-canonical signaling pathways such as response to mechanical stretch and β-arrestin-mediated signaling following internalization are exciting avenues to be explored. Elabela-mediated signaling, although recently discovered, has shed light on the multifaceted nature of APJR. The APJR/apelin system potentially provides therapeutic opportunities to treat disorders such as diabetes, hypertension, cardiac myopathy, and cancer.

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