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# International Union of Basic and Clinical Pharmacology. XCV. Recent Advances in the Understanding of the Pharmacology and Biological Roles of Relaxin Family Peptide Receptors 1–4, the Receptors for Relaxin Family Peptides

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Abstract.....	391
I. Introduction.....	391
A. Receptors for Relaxins and Insulin-Like Peptides.....	392
1. Relaxin Family Peptide Receptors 1 and 2—The Leucine-Rich Repeat-Containing Receptors for Relaxin and Insulin-Like Peptide 3.....	392
2. Relaxin Family Peptide Receptors 3 and 4—Receptors for Relaxin-3 and Insulin-Like Peptide 5 That Resemble G Protein-Coupled Receptors Activated by Small Peptides Like Somatostatin or Angiotensin.....	393
B. Structural Features of Relaxin Family Peptide Receptors.....	393
1. Functional Domains of Relaxin Family Peptide Receptors 1 and 2.....	393
a. Low-density lipoprotein receptor type a module.....	394
b. Leucine-rich repeat region.....	395
c. Transmembrane domains and dimerization.....	396
d. Intracellular receptor domains.....	396
2. Functional Domains of Relaxin Family Peptide Receptors 3 and 4.....	397
a. Extracellular regions.....	397
b. Transmembrane spanning regions.....	398
II. Ligands That Act at Relaxin Family Peptide Receptors.....	398
A. Ligands That Act at Relaxin Family Peptide Receptor 1.....	399
1. Relaxin Family Peptide Agonists.....	399
2. C1q-Tumor Necrosis Factor-Related Protein 8: An Additional Relaxin Family Peptide Receptor 1 Ligand?.....	399
3. Small-Molecular-Weight Agonists.....	400
4. Relaxin Family Peptide Receptor 1 Antagonists.....	401
B. Ligands That Act at Relaxin Family Peptide Receptor 2.....	402
1. Relaxin Family Peptide Agonists.....	402
2. Relaxin Family Peptide Antagonists.....	402

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C.	Ligands That Act at Relaxin Family Peptide Receptor 3	403
1.	Relaxin Family Peptide Agonists	403
2.	Biased Agonists Acting at Relaxin Family Peptide Receptor 3	403
3.	Allosteric Modulators	403
4.	Relaxin Family Peptide Receptor 3 Antagonists	403
D.	Ligands That Act at Relaxin Family Peptide Receptor 4	404
1.	Relaxin Family Peptide Agonists	404
2.	Modified Peptide Agonists	405
3.	Relaxin Family Peptide Receptor 4 Antagonists	405
III.	Signal Transduction Pathways	405
A.	Relaxin Family Peptide Receptor 1 Signaling	405
1.	Canonical Signaling Pathways	405
2.	Noncanonical Signaling Pathways	408
3.	The Relaxin Family Peptide Receptor 1 Signalingosome—A Protein Complex That Facilitates High-Sensitivity Signaling in Response to Relaxin	409
4.	Homo- and Hetero-Oligomerization of RXFP1 and Its Functional Consequences	410
5.	Allosteric Agonist Signaling at Relaxin Family Peptide Receptor 1	411
B.	Relaxin Family Peptide Receptor 2 Signaling	411
1.	Canonical Signaling Pathways	411
C.	Relaxin Family Peptide Receptor 3 Signaling	411
1.	Canonical Signaling Pathways	411
2.	Ligand-Directed Signaling Bias at Relaxin Family Peptide Receptor 3	412
3.	Allosteric Modulation of Relaxin Family Peptide Receptor 3	414
D.	Relaxin Family Peptide Receptor 4 Signaling	414
1.	Canonical Signaling Pathways	414
IV.	Receptor-Protein Signaling and Regulatory Complexes	414
A.	Relaxin Family Peptide Receptor 1	414
1.	G Proteins That Couple to Relaxin Family Peptide Receptor 1	414
2.	Interactions between Relaxin Family Peptide Receptor 1 and $\beta$ -Arrestins	415
B.	Relaxin Family Peptide Receptor 2	415
1.	G Proteins That Couple to Relaxin Family Peptide Receptor 2	415
C.	Relaxin Family Peptide Receptor 3	415
1.	G Proteins That Couple to Relaxin Family Peptide Receptor 3 after Activation by Human Relaxin-3	415
2.	G Proteins That Couple to Relaxin Family Peptide Receptor 3 after Activation by the Biased Ligands Human Relaxin and R3(B $\Delta$ 23–27)R/I5	415
3.	Interactions between Relaxin Family Peptide Receptor 3 and $\beta$ -Arrestins	416
D.	Relaxin Family Peptide Receptor 4	416
1.	G Proteins That Couple to Relaxin Family Peptide Receptor 4	416
V.	Physiologic Roles of Relaxin Family Peptide Receptors and Their Cognate Ligands	416
A.	Relaxin and Relaxin Family Peptide Receptor 1	416
1.	Reproduction	416
2.	Central Nervous System	417
3.	Blood Vessels	417
4.	Heart	419
5.	Kidney	421
6.	Fibrosis	421
7.	Organ Protection	422
8.	Formation and Spread of Tumors	422
9.	Diabetes	423
B.	Insulin-Like Peptide 3 and Relaxin Family Peptide Receptor 2	423
1.	Reproduction	423
2.	Brain	424
3.	Bone and Other Tissues	424
C.	Relaxin-3 and Relaxin Family Peptide Receptor 3	425
1.	Stress	425
2.	Feeding and Metabolism	425

3. Behavioral Activation and Arousal. . . . .	426
D. Insulin-Like Peptide 5 and Relaxin Family Peptide Receptor 4 . . . . .	427
1. Metabolism. . . . .	427
VI. Unresolved Pharmacological and Therapeutic Issues and Future Directions. . . . .	428
A. Signaling Paradigms. . . . .	428
1. Heterodimerization of Relaxin Family Peptide Receptor 1: A Possible Explanation for the Selective Actions of Relaxin in Diseased or Pathologic States? . . . . .	428
2. The Development of ML290, a Small-Molecular-Weight Allosteric Agonist at Relaxin Family Peptide Receptor 1. . . . .	429
3. Novel Activators of RXFP Receptors. . . . .	429
B. Therapeutic Applications . . . . .	430
1. Is There Therapeutic Potential in Small-Molecule Targeting of Relaxin Family Peptide Receptor 1 Heterodimers? . . . . .	430
2. Relaxin Family Peptide Receptor 3 as a Drug Target. . . . .	430
3. Relaxin Family Peptide Receptors and Metabolism. . . . .	431
References . . . . .	431

**Abstract**—Relaxin, insulin-like peptide 3 (INSL3), relaxin-3, and INSL5 are the cognate ligands for the relaxin family peptide (RXFP) receptors 1–4, respectively. RXFP1 activates pleiotropic signaling pathways including the signalosome protein complex that facilitates high-sensitivity signaling; coupling to  $G\alpha_s$ ,  $G\alpha_i$ , and  $G\alpha_o$  proteins; interaction with glucocorticoid receptors; and the formation of hetero-oligomers with distinctive pharmacological properties. In addition to relaxin-related ligands, RXFP1 is activated by Clq-tumor necrosis factor-related protein 8 and by small-molecular-weight agonists, such as ML290 [2-isopropoxy-*N*-(2-(3-(trifluoromethylsulfonyl)phenylcarbamoyl)phenyl)benzamide], that act allosterically. RXFP2 activates only the  $G\alpha_s$ - and  $G\alpha_o$ -coupled pathways. Relaxin-3 is primarily

a neuropeptide, and its cognate receptor RXFP3 is a target for the treatment of depression, anxiety, and autism. A variety of peptide agonists, antagonists, biased agonists, and an allosteric modulator target RXFP3. Both RXFP3 and the related RXFP4 couple to  $G\alpha_i/G\alpha_o$  proteins. INSL5 has the properties of an incretin; it is secreted from the gut and is orexigenic. The expression of RXFP4 in gut, adipose tissue, and  $\beta$ -islets together with compromised glucose tolerance in INSL5 or RXFP4 knockout mice suggests a metabolic role. This review focuses on the many advances in our understanding of RXFP receptors in the last 5 years, their signal transduction mechanisms, the development of novel compounds that target RXFP1–4, the challenges facing the field, and current prospects for new therapeutics.

## I. Introduction

Relaxin family peptides are heterodimeric and closely related structurally to insulin. Four of these peptides, relaxin, insulin-like peptide 3 (INSL3), relaxin-3, and insulin-like peptide 5 (INSL5) are the cognate ligands for the relaxin family peptide receptors RXFP1–4, respectively

(Bathgate et al., 2006a). Humans and higher primates have two (*RLN1* and *RLN2*) relaxin genes, whereas other mammals have only one (*Rln1*). The peptide encoded by the human *RLN2* gene and the *Rln1* gene from other mammals generates the circulating relaxin peptide originally detected during pregnancy (referred to herein

**ABBREVIATIONS:** 135PAM1, 3-[3,5-bis(trifluoromethyl)phenyl]-1-(3,4-dichlorobenzyl)-1-[2-(5-methoxy-1*H*-indol-3-yl)ethyl]urea; AC, adenylyl cyclase; AKAP79, A-kinase anchoring protein 79; AT<sub>2</sub>R, angiotensin type 2 receptor; BQ123, 2-[(3*R*,6*R*,9*S*,12*R*,15*S*)-6-(1*H*-indol-3-ylmethyl)-9-(2-methylpropyl)-2,5,8,11,14-pentaoxo-12-propan-2-yl-1,4,7,10,13-pentazabicyclo[1.3.3.0]octadecan-3-yl]acetic acid; BRET, bioluminescence resonance energy transfer; CNS, central nervous system; CRF, corticotropin releasing factor; CTRP8, Clq-tumor necrosis factor-related protein 8; D06, bis(4-*N,N*-dimethylaminophenyl)(2-chloro-5-nitrophenyl) methane; ECL, extracellular loop; ELISA, enzyme-linked immunosorbant assay; eNOS, endothelial nitric oxide synthase; ERK1/2, extracellular signal-regulated kinase; ET, endothelin; GLP-1, glucagon-like peptide 1; GM6001, (2*R*)-*N'*-hydroxy-*N*-[(2*S*)-3-(5*H*-indol-3-yl)-1-methylamino-1-oxopropan-2-yl]-2-(2-methylpropyl)butanediamide; GPCR, G protein-coupled receptor; GR, glucocorticoid receptor; 5-HT, 5-hydroxytryptamine; ICL, intracellular loop; IL, interleukin; iNOS, inducible nitric oxide synthase; INSL3, insulin-like peptide 3; INSL5, insulin-like peptide 5; IR, ischemia-reperfusion; LDLA, low-density lipoprotein receptor type A module; LRR, leucine-rich repeat; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride; MAPK, mitogen-activated protein kinase; ML290, 2-isopropoxy-*N*-(2-(3-(trifluoromethylsulfonyl)phenylcarbamoyl)phenyl)benzamide; MMP, matrix metalloproteinase; MS, medial septum; NF449, 4,4',4'',4'''-[carbonylbis(imino-5,1,3-benzenetriyl-bis(carbonylimino))]tetrakis-1,3-benzenedisulfonic acid, octasodium salt; NF $\kappa$ B, nuclear factor- $\kappa$ B; NI, nucleus incertus; NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal nitric oxide synthase; OVL1, organum vasculosum of the lamina terminalis; PAM, positive allosteric modulator; pERK1/2, phosphorylated ERK1/2; PD123319, *S*-(+)-1-[(4-(dimethylamino)-3-methylphenyl)methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridine-6-carboxylic acid di(trifluoroacetate); PD98059, 2'-amino-3'-methoxyflavone; PDE, phosphodiesterase; PGF, placental growth factor; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PTX, pertussis toxin; PVN, paraventricular nucleus of the hypothalamus; RU-486, 17 $\beta$ -hydroxy-11 $\beta$ -(4-dimethylamino-phenyl)-17 $\alpha$ -(1-propenyl)estra-4,9-dien-3-on; RWJ67657, 4-[4-(4-fluorophenyl)-1-(3-phenylpropyl)-5-(4-pyridinyl)-1*H*-imidazol-2-yl]-3-butyn-1-ol; RXFP1–4, relaxin family peptide receptor 1–4; SB209670, (+)-(1*S*,2*R*,3*S*)-3-(2-carboxymethoxy-4-methoxyphenyl)-1-(3,4-methylenedioxyphenyl)-5-(prop-1-yloxy)indane-2-carboxylic acid; SFO, subfornical organ; SOD, superoxide dismutase; SP600125, anthra[1,9-*cd*]pyrazol-6(2*H*)-one; SU5416, (3*Z*)-3-[(3,5-dimethyl-1*H*-pyrrol-2-yl)methylidene]-1,3-dihydro-2*H*-indol-2-one; T2DM, type-2 diabetes mellitus; TGF, transforming growth factor; TIMP, tissue inhibitor of metalloproteinase; TM, transmembrane; VEGF, vascular endothelial growth factor; WT, wild type.

as relaxin). The function of the *RLN1* gene in humans and higher primates is unknown (relaxin-1). In humans, the relaxin family peptide genes have a similar structure and all synthesize prepropeptides (Hsu, 2003) that are processed by convertases to produce the mature two-chain peptides; an A-chain linked to a B-chain by two disulfide bonds, and an additional intrachain disulfide in the A-chain.

Relaxin circulates in the blood of all mammals during pregnancy, although the levels and roles vary widely in different species. In humans, relaxin levels are highest in the first trimester and are probably associated with implantation and initiation of the cardiovascular changes that accompany pregnancy. However, relaxin is also produced in many tissues in both male and female mammals as a paracrine or autocrine factor to exert other physiologic roles (Sherwood, 2004; Bathgate et al., 2006a,c, 2013a).

Relaxin-3 is the most recently identified relaxin family peptide; it was named as a “relaxin” peptide because of the presence of the characteristic RxxxRxxI/V relaxin-binding motif in the B-chain but otherwise has relatively low sequence homology to other relaxin peptides. In contrast to other relaxins, the sequence of relaxin-3 is well conserved across species (Wilkinson et al., 2005b; Yegorov et al., 2009). Relaxin-3 is believed to be the ancestral peptide of the family (Wilkinson et al., 2005b) and in mammals is primarily a neuropeptide (Bathgate et al., 2002) involved in stress, memory, and appetite regulation (McGowan et al., 2005; Tanaka et al., 2005; Ma et al., 2007a; Banerjee et al., 2010; Ganella et al., 2013a,b; Ryan et al., 2013a,b; Smith et al., 2014).

INSL3 (formerly Leydig insulin-like peptide) was discovered in the Leydig cells of the testis (Adham et al., 1993) where it is highly expressed in all species that have the *INSL3* gene (Bathgate et al., 2006c). *INSL3* expression in other tissues occurs at much lower levels. *INSL3* has a critical role in testis descent, and *INSL3* knockout mice are cryptorchid and infertile (Nef and Parada, 1999; Zimmermann et al., 1999). It plays an important role in gubernaculum development, which is involved in the first stage of testis descent, and also appears to have a role in the maintenance of ovarian function (Spanel-Borowski et al., 2001; Kawamura et al., 2004; Glister et al., 2013).

*INSL5* is widely distributed with high expression in the gastrointestinal tract (Conklin et al., 1999) particularly in L cells isolated from mouse colon/rectum but also in ascending, transverse, and descending colon and proximal rectum, with lower levels in the cecum and distal rectum (Grosse et al., 2014). Low levels of *Insl5* mRNA were found in the pancreas, thymus, and eye (Grosse et al., 2014). *INSL5* knockout mice display dysfunctional glucose homeostasis (Burnicka-Turek et al., 2012). *INSL5* activates RXFP4, but not RXFP1 or RXFP2, with high potency and is a weak antagonist at RXFP3 (Liu et al., 2005b).

Thus, although relaxin peptides resemble each other closely in structure, each is the cognate ligand for a specific G protein-coupled receptor (GPCR) and each possesses a wide variety of physiologic functions. Relaxin has roles in reproduction, cardiovascular system, organ protection, metabolism, and as a neuropeptide in the brain; *INSL3*, although acting on a similar receptor, has highly specialized roles in reproduction; relaxin-3 is a neuropeptide, and *INSL5* acts as an incretin.

#### A. Receptors for Relaxins and Insulin-Like Peptides

1. *Relaxin Family Peptide Receptors 1 and 2—The Leucine-Rich Repeat-Containing Receptors for Relaxin and Insulin-Like Peptide 3.* Early studies showed an increase in tyrosine phosphorylation of a 220-kDa protein in response to relaxin (Palejwala et al., 1998), suggesting that relaxin receptors, like those that respond to insulin, were tyrosine kinases. However, *Insl3* knockout mice (Nef and Parada, 1999; Zimmermann et al., 1999) displayed abnormal testis descent as did mice with disruptions in the GPCR encoded by the *GREAT* gene (later shown to be the mouse ortholog of human LGR8 or RXFP2) (Overbeek et al., 2001). This led to the deorphanization of LGR7 (RXFP1) and LGR8 (RXFP2) (Hsu et al., 2002), two family A GPCRs.

In humans, RXFP1 is the cognate receptor for human relaxin; it has the classic seven-transmembrane (TM) spanning regions of a GPCR as well as a large extracellular domain containing 10 leucine-rich repeats (LRR) and a unique N-terminal low-density lipoprotein receptor type A (LDLa) module (Hsu et al., 2002). *RXFP1* mRNA and protein is found in ovary, uterus, placenta, mammary gland, prostate, and testis but also in the heart, arteries, kidney, lung, liver, and blood cells as well as in a number of areas of the brain, such as cortex, hippocampus, arcuate nucleus, organum vasculosum of the lamina terminalis (OVLT), and subfornical organ (SFO) (for details, see Novak et al., 2006, and Bathgate et al., 2013a). Thus, relaxin, in addition to autocrine and paracrine roles, also acts as a neuropeptide. Interaction of relaxin with RXFP1 to trigger cell signaling involves at least three stages: high-affinity binding between the B-chain of relaxin and the RXFP1 LRR region, lower affinity binding to the TM extracellular loops (ECLs), and finally an essential interaction involving the LDLa module (Sudo et al., 2003; Halls et al., 2005b). Although RXFP1 couples to numerous signal transduction pathways, many early studies in reproductive tissues indicated that relaxin caused increases in cAMP levels (Braddon, 1978; Cheah and Sherwood, 1980; Sanborn et al., 1980; Chen et al., 1988), and a constitutively active receptor mutant also generates cAMP (Hsu et al., 2002). The pattern of cAMP production after stimulation of HEK293 cells expressing RXFP1 is complex and involves at least three G proteins (Halls et al., 2006, 2009a,b). RXFP1 also activates extracellular signal-regulated kinase 1/2 (ERK1/2), tyrosine kinase(s), gene transcription,

and nitric oxide (NO) signaling, and relaxin interacts with the glucocorticoid receptor (GR). The full implications of the pleiotropic effects of relaxin are still to be elucidated.

RXFP2 is the cognate receptor for INSL3 and is structurally similar to RXFP1 (Hsu et al., 2002; Kumagai et al., 2002). It is primarily expressed in the ovary, testis, and gubernaculum (for details, see Bathgate et al., 2013a). It has a specialized role in the gubernaculum during testicular descent, it is expressed in the testis and ovary affecting gonadal function (Kawamura et al., 2004) and it influences bone metabolism (Ferlin et al., 2009). RXFP2 signaling also involves adenylyl cyclase (AC) activation and cAMP generation, utilizing a subset of the G proteins used by RXFP1. Although in vitro cell systems that are often used to study RXFP2 show increases in cAMP levels, in endogenously expressing systems both increases and decreases in cAMP may be seen. Thus, in gubernacular cells (Kumagai et al., 2002) or osteoblasts (Ferlin et al., 2009), activation of RXFP2 causes increased cAMP, but in male germ cells and oocytes, decreased cAMP is observed (Kawamura et al., 2004), perhaps reflecting expression patterns of signaling proteins in different cells (Halls et al., 2009a). Although the relaxins of some species activate RXFP2 in vitro (Hsu et al., 2002; Kumagai et al., 2002; Scott et al., 2005a; Bathgate et al., 2006b), there is no evidence that relaxin activates RXFP2 in vivo.

Thus, RXFP1 and RXFP2 are structurally similar receptors that use some common signaling mechanisms. However, current evidence suggests that the relaxin/RXFP1 system has a much wider range of distribution and functions than the INSL3/RXFP2 system.

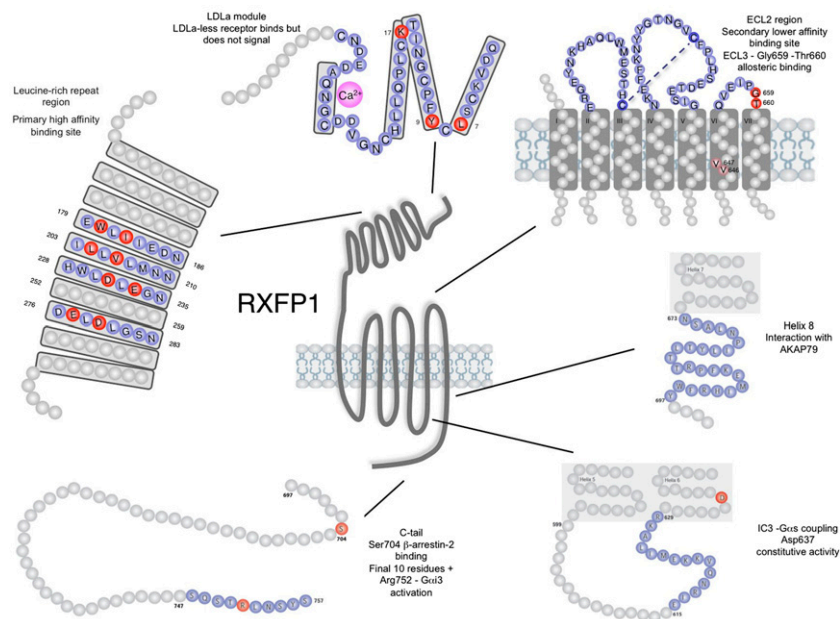
**2. Relaxin Family Peptide Receptors 3 and 4—Receptors for Relaxin-3 and Insulin-Like Peptide 5 That Resemble G Protein-Coupled Receptors Activated by Small Peptides Like Somatostatin or Angiotensin.** RXFP3 and RXFP4 are the targets for relaxin-3 and INSL5, respectively (Liu et al., 2003a,b). They are distinctly different in structure from RXFP1 and RXFP2 and instead resemble the class A small peptide receptors. RXFP3 was originally named SALPR or somatostatin and angiotensin-like receptor (Matsumoto et al., 2000), whereas RXFP4 was thought to be a bradykinin receptor (Boels and Schaller, 2003) before pairing with their cognate ligands relaxin-3 (Liu et al., 2003b) and INSL5 (Liu et al., 2005b). The receptor expression profiles suggest that RXFP3 is a neuropeptide receptor and RXFP4 is a gut hormone receptor (Bathgate et al., 2013a; Grosse et al., 2014). RXFP3 couples to  $G\alpha_{i10}$  to inhibit AC (Liu et al., 2003b; van der Westhuizen et al., 2007) and also causes ERK1/2 phosphorylation (van der Westhuizen et al., 2007). Based on binding and signaling studies, relaxin can also bind to a subtly different region on RXFP3 to activate a subset of the pathways activated by relaxin-3 (van der Westhuizen et al., 2010; Kocan et al., 2014). Relatively little is known about RXFP4 signaling, but like RXFP3 it couples to inhibitory G proteins.

RXFP3 and RXFP4 are closely related receptors with structurally similar cognate ligands. However, RXFP3 and relaxin-3 are predominantly involved in central nervous system neurotransmission, whereas INSL5 is an incretin that targets RXFP4 located in the gastrointestinal tract and tissues controlling metabolism.

### *B. Structural Features of Relaxin Family Peptide Receptors*

The relaxin family peptide receptors are four highly conserved family A GPCRs that can be considered as two distinct pairs based on their architecture and signaling properties. Genes encoding RXFP1 and RXFP2 have introns giving rise to a large number of splice variants with functions that are mostly yet to be established (Muda et al., 2005; Kern et al., 2008), whereas genes encoding RXFP3 and RXFP4 are intronless. RXFP1 and RXFP2 have a large extracellular N terminus with an LRR domain containing a high-affinity ligand binding site complemented by a low-affinity binding interaction within ECL2 and uniquely, an N-terminal LDLa module that is essential for signaling, and a role in trafficking (Fig. 1). RXFP1 and RXFP2 show similarities in ligand binding and signaling, but only RXFP1 has a C terminus that contains motifs that induce the formation of protein signaling complexes termed signalosomes (see section III.A.3) (Halls and Cooper, 2010; Halls, 2012) (Fig. 1). The third intracellular loop (ICL3) is likely essential for G protein coupling in all RXFP receptors. Both RXFP1 and RXFP2 couple to  $G\alpha_s$  and to inhibitory  $G\alpha_{oB}$ , which modulates the effect of  $G\alpha_s$  coupling, but only RXFP1 is able to couple to  $G\alpha_{i3}$  to produce a delayed surge in cAMP accumulation that occurs in many but not all cell types (Halls et al., 2009a). In contrast, RXFP3 and RXFP4 have a very small N-terminal domain and couple solely to  $G\alpha_i$  and  $G\alpha_o$  proteins. For all receptors, the pattern of G protein coupling is dependent on the cell type in which the receptor is expressed.

**1. Functional Domains of Relaxin Family Peptide Receptors 1 and 2.** The similarity between RXFP1 and RXFP2 has helped the identification of the functional domains of the two receptors. Initial work used chimeric receptors and relaxin-3, because it is selective for RXFP1 over RXFP2, and revealed that the peptide interacts with both the LRR domain and ECL2 of the TM domain of RXFP1 to produce the full binding and cAMP signaling profile (Sudo et al., 2003) (Fig. 1). This mechanism was later shown to be a feature of both receptors (Halls et al., 2005b). Support for two binding sites also came from functional assays, with high-affinity LRR binding producing cAMP accumulation more effectively than the lower affinity ECL site (Halls et al., 2005b). In addition to the LRR region, the N-terminal LDLa module is essential for signaling (Fig. 1) but has no role in ligand binding (Scott et al., 2006; Hopkins et al., 2007; Kern et al., 2007). The LDLa module likely interacts with other receptor domains (potentially the ECLs and TM domains) in a manner analogous to a tethered ligand (Fig. 1).



**Fig. 1.** Functional domains of RXFP1. The N-terminal region of RXFP1 consists of an LDLa module that is essential for signaling promoted by relaxin but not the allosteric modulator ML290. Residues L29, Y31, and K39 are important for receptor activation by relaxin. The LDLa module is connected to the leucine-rich repeat region that contains the primary high-affinity binding site for relaxin. The relaxin B-chain residues R13 and R17 are believed to bind to E277 and D279 in LRR8 and D231 and E233 in LRR6, whereas I20 is thought to interact with W180 and I182 in LRR4 and L204 and V206 in LRR5. The ECL2 region contains the secondary low-affinity binding site for relaxin, whereas ECL3 contains two residues, G659 and T660, that are essential for activation of RXFP1 by ML290. Helix 8 contains binding motifs that are essential for interaction with AKAP79 that is required for signalosome formation. The distal region of ICL3 is required for coupling to  $G_{\alpha_s}$  and the receptor containing D637 displays constitutive activity. In the C-terminal tail region S704 is required for  $\beta$ -arrestin binding and signalosome formation, whereas the final 10 residues and in particular R752 are required for coupling of RXFP1 to  $G_{\alpha_{13}}$ .

#### *a. Low-density lipoprotein receptor type a module.*

A unique feature of RXFP1 and RXFP2 is the LDLa module, originally defined by the structure of the LDL receptor (Yamamoto et al., 1984; Daly et al., 1995) but now recognized in a number of other proteins including the very-low-density lipoprotein receptor (Gafvels et al., 1993), the LDL receptor-related protein (Herz et al., 1988), renal glycoprotein gp330 (Lundgren et al., 1994; Hjalm et al., 1996), the C9 component of complement (DiScipio et al., 1984; Stanley et al., 1985), the Tva receptor for Rous sarcoma virus (Bates et al., 1993), and retina- and brain-specific neuropilin and tolloid-like protein 1 transmembrane protein (Stohr et al., 2002). RXFP1 and RXFP2 are the only known human GPCRs to contain this module (Bathgate et al., 2006a). The NMR solution structure of the RXFP1 LDLa module has been solved and reveals a typical fold generated by six essential cysteine residues and the incorporation of a calcium ion by a largely conserved motif of acidic residues (Hopkins et al., 2007). The role of the LDLa module was first discovered during characterization of a splice variant of RXFP2 that is missing the LDLa module. This receptor and a similar RXFP1 mutant were expressed at the cell surface and bound their native ligands normally but were not able to signal through cAMP (Scott et al., 2006). More recent studies used a panel of GPCR-relevant signaling reporter genes to demonstrate that the RXFP1 receptor lacking the LDLa module does not signal through any pathway tested (Kong et al., 2013). Importantly, a soluble

recombinant form of the RXFP1 LDLa module acts as an RXFP1 antagonist when introduced into cells expressing RXFP1 (Scott et al., 2005c, 2006). These data suggest that the LDLa module may act as an RXFP1 tethered ligand and its actions can be blocked by the addition of recombinant LDLa. Further mutagenesis studies have provided evidence of a specific LDLa-RXFP1 interaction that drives receptor activation. Mutation of residues involved in the folding of the LDLa module produce mutants that are unable to signal but maintain intact ligand binding profiles (Scott et al., 2006; Hopkins et al., 2007; Kern et al., 2007) (Fig. 1). Specifically, mutation of cysteines (C47A and C53A) involved in folding or of D58E involved in calcium-binding produces RXFP1 receptors that are unable to increase cAMP accumulation in response to relaxin (Kern et al., 2007). Other mutations that affect calcium ligation and hence folding (corresponding to C27S and C40S within the full-length RXFP1 sequence) also abolish signaling (Hopkins et al., 2007) (Fig. 1). Similar studies at human RXFP2 with mutations of the calcium ligating residue D70Y or conserved cysteine C71Y also resulted in a loss of signaling, again highlighting the common mechanism of activation of the two receptors (Bogatcheva et al., 2007).

Further evidence for a specific interaction between the LDLa module and RXFP1 that drives receptor activation comes from studies on chimeric and mutant RXFP1 receptors (Hopkins et al., 2007; Kong et al., 2013). A chimera where the RXFP1 LDLa module is swapped

for a homologous LDLa module from the second ligand-binding domain of the LDL receptor produces a receptor (LB2-RXFP1) that is unable to signal in response to relaxin but binds ligand normally (Hopkins et al., 2007). Mutagenesis of specific conserved residues in the RXFP1 LDLa module highlighted potential roles for L29 and Y31 in receptor activation (Hopkins et al., 2007). Further studies have used the signaling-deficient LB2-RXFP1 receptor to define the potential “signaling surface” of the LDLa module. Within the signaling-deficient LB2-RXFP1, gain of function mutations, loss of function mutations, and judicious amino acid substitutions were able to confirm the key role of L29 and Y31 and to additionally identify K39 as important residues in the RXFP1 LDLa module that are involved in the hydrophobic interactions that drive the active receptor conformation (Kong et al., 2013) (Fig. 1). Studies using chimeric RXFP1 and RXFP2 receptors with their LDLa modules swapped C-terminally to the final cysteine residue of the module demonstrate that RXFP2 likely uses a similar mechanism to drive receptor activation. Importantly the chimeric receptors were still able to bind ligand normally and were also able to signal, albeit with altered activity compared with wild-type receptors (Bruell et al., 2013). Furthermore, when the TM domains of the RXFP1 chimera with an RXFP2 LDLa (RXFP211) were swapped to match the LDLa module (RXFP212) the activity of the receptor approached wild type, suggesting a specific interaction between the LDLa module and the receptor TM domains. It also highlights that the RXFP1 and RXFP2 LDLa modules function by similar but distinct mechanisms.

The LDLa module may also be involved in receptor maturation and translocation to the cell surface. When expressed recombinantly in HEK293 cells, a large proportion of wild-type RXFP1 receptors exist in an immature form containing high mannose-type N-linked oligosaccharides within the endoplasmic reticulum (Kern et al., 2007). In the case of other glycoprotein hormone receptors, such as the leutenizing hormone receptor (Ascoli et al., 2002; Tao et al., 2004; Pietila et al., 2005) and follicle-stimulating hormone receptor (Quintana et al., 1993; Davis et al., 1995), this has been suggested to be a mechanism controlling cell surface expression. In the same study it was demonstrated that an RXFP1 mutant lacking the LDLa module was expressed as the mature form only, as was a chimeric RXFP1 receptor containing the LDLa module of RXFP2 (Kern et al., 2007). In addition, mutation of a conserved glycosylation site within the LDLa module results in a receptor with a reduced ability to generate cAMP, attributed to a decrease in cell surface expression (Kern et al., 2007; Yan et al., 2008). However, similar studies on RXFP1 mutants with a disruption of the LDLa glycosylation site show only a minor (Yan et al., 2008) or no effect (Kong et al., 2013) on signaling and cell surface expression. Additionally, studies using LDLa-less RXFP1 and RXFP2 (Scott et al., 2006), LB2-RXFP1 mutants, RXFP1 LDLa loss of function mutants (Kong et al., 2013), or misfolded RXFP1 LDLa mutants (Hopkins

et al., 2007) demonstrated no effect on cell surface expression. In RXFP2, mutations of amino acid residues that form the disulfide bond or coordinate calcium binding in the LDLa module (C70Y and D71Y) did reduce cell-surface expression (Bogatcheva et al., 2007).

The LDLa module clearly plays an important and specific role in protein maturation, cell surface expression, and in the activation of both RXFP1 and RXFP2.

*b. Leucine-rich repeat region.* Glycosylation, a post-translational modification common to many GPCRs, is important for receptor delivery to the cell surface, ligand binding, and signal transduction. In addition to sites within the LDLa module, the LRR region is also glycosylated at a number of residues (N105, N250, N303, and N346), all of which are important for the translocation of receptors to the cell surface and full signaling efficacy but not ligand binding (Yan et al., 2008). The glycosylation status of RXFP2 has yet to be studied in detail.

In addition to a role in cell surface delivery, the LRR region is essential for ligand binding and signal transduction. Similar to other members of the LGR family, the primary high-affinity ligand binding site for relaxin and INSL3 is in the LRR region of RXFP1 and RXFP2 (Sudo et al., 2003; Halls et al., 2005b). Studies based on peptide mutagenesis and radioligand binding for both relaxin and INSL3 (outlined in sections II.A.1 and II.B.1) highlight that B-chain residues of the peptides contribute most to the binding affinity. Subsequently, two independent studies used homology modeling of the LRRs and mutagenesis to define the relaxin and INSL3 binding sites in RXFP1 and RXFP2, respectively. The RXFP1 LRRs were modeled based on the crystal structure of the porcine ribonuclease inhibitor (a protein with LRRs) together with in silico peptide docking and targeted receptor mutagenesis. Relaxin used the well characterized RxxxRxxI binding motif within the peptide B-chain at a 45° angle across five of the parallel LRRs (Bullesbach and Schwabe, 2005b). Hence the relaxin B-chain residues R13 and R17 were predicted to interact with acidic groups within the concave face of the LRRs (E277 and D279 in LRR8 and D231 and E233 in LRR6) (Bullesbach and Schwabe, 2005b) (Fig. 1). The B-chain I20 was predicted to form a hydrophobic interaction with W180 and I182 within LRR4 and L204 and V206 within LRR5 (Bullesbach and Schwabe, 2005b) (Fig. 1).

The interaction of INSL3 with RXFP2 uses different residues in the B-chain as determined from peptide studies (see section II.B.1). Modeling of the INSL3 interaction with the RXFP2 LRRs used the NMR solution structure of INSL3 (Rosengren et al., 2006b) and a molecular model of the RXFP2 LRR based upon the crystal structure of the Nogo receptor (which shares higher amino acid sequence homology than the porcine ribonuclease inhibitor; Scott et al., 2007). With the use of a combination of peptide and LRR mutants in conjunction with in silico docking of the INSL3 B-chain to the LRR of RXFP2, seven residues within RXFP2 that potentially make contact with INSL3



were defined. The B-chain residue R16 was predicted to interact with RXFP2 D227, H12 with RXFP2 W177, V19 with RXFP2 I179, R20 with RXFP2 E229 and D181, and W27 with RXFP2 F131 and Q133 (Scott et al., 2007). Interestingly, although five of the RXFP2 residues that potentially interact with INSL3 are also found in RXFP1, the affinity of INSL3 for RXFP1 is very low (Sudo et al., 2003; Halls et al., 2005b). Another study that investigated this apparent contradiction used the RXFP1 extracellular domain construct linked to a single CD8 TM domain, termed 7BP (Hsu et al., 2002). This construct has a high affinity for INSL3 (Halls et al., 2005b) and was used as a template to further explore the interaction of INSL3 and the RXFP1 LRRs (Scott et al., 2012). Only four amino acid changes were necessary to obtain the equivalent RXFP2 affinity for INSL3 in the RXFP1 LRRs, but when these mutations were inserted into full-length RXFP1 there was only a modest gain of function for INSL3. This, in conjunction with previous studies (see section I.B.1.c), confirmed an important role for the TM domains in modulating ligand binding. Molecular modeling studies demonstrated that relaxin and INSL3 have distinct orientations of binding to the LRRs. Taken together the results suggest that there are critical differences both in the extracellular domain binding and the coordination of this binding with the TM binding site between RXFP1 and RXFP2 (Scott et al., 2012). Relaxin clearly binds to RXFP2 in a different manner from RXFP1 and also in a different manner from INSL3 binding to RXFP2.

*c. Transmembrane domains and dimerization.* The information above highlights the essential coordination of the LRR binding site with the binding site in the TM domains. As mentioned earlier, studies using chimeric receptors and the selectivity of relaxin-3 for RXFP1 versus RXFP2 suggest that this additional binding site is in ECL2 (Sudo et al., 2003) (Fig. 1). The presence of this additional binding site within the TM was confirmed using the same chimeric receptors together with relaxin peptides that have selectivity for RXFP1 or RXFP2 (rat relaxin, RXFP1 > RXFP2; INSL3, RXFP2 > RXFP1) (Halls et al., 2005b). Modeling studies suggest that A-chain residues in relaxin and INSL3 interact with this potential binding site (Hartley et al., 2009), but this has yet to be fully characterized. The current model of activation of RXFP1 and RXFP2 suggests that ligand binding to the LRRs and TM ECLs directs the LDL $\alpha$  to interact with the TM domain of a receptor homodimer partner to drive receptor activation (Kong et al., 2010; Bruell et al., 2013). However this model still requires experimental verification using site-directed mutagenesis or other methods that prevent dimer formation.

As with many other class A GPCRs, RXFP1 and RXFP2 form homo- and heterodimers (Kern et al., 2008; Svendsen et al., 2008a,b). Dimerization occurs in the absence of, and is independent, of ligand occupation of the receptor (Svendsen et al., 2008a,b). Heterodimers also form between the haloreceptor and a number of

splice variants and are present at all stages of receptor translocation from the endoplasmic reticulum to the plasma membrane (Kern et al., 2008). Dimerization can also occur with a TM-only domain receptor (Svendsen et al., 2008a,b), highlighting that the TM domains likely drive dimerization as with other GPCRs. The authors suggested that the receptor ectodomains could be required for stabilization of the dimer because of the lower dimerization efficiency observed for the TM-only receptors. However, a relatively poor expression of the TM-only receptors could also explain these results (R. A. D. Bathgate, unpublished data).

There is evidence to suggest that dimerization is associated with negative cooperativity (Svendsen et al., 2008a,b), whereby the affinity of unoccupied receptor binding sites progressively decreases as receptor occupancy increases. Two consequences of this are an increase in the functional concentration range of the ligand and a decrease in ligand residence time corresponding to an increase in free ligand concentration, potentially allowing selective activation of different signaling pathways (Shymko et al., 1997). This may provide one explanation for the observation that in many experimental and clinical situations the concentration-response curves for relaxin acting at RXFP1 are bell shaped (also see section III.A.4).

*d. Intracellular receptor domains.* There is evidence that ICL3 and the C-terminal tail have important roles in signaling. ICL3 has an important role in coupling RXFP1 to  $G\alpha_s$  and is therefore essential for activation of AC and cAMP signaling (Fig. 1). Peptide fragments based on the N-terminal region of ICL3 [residues 615–629, and 619–629-Lys(Palm)] increased AC activity (Shpakov et al., 2007). These peptides functionally "antagonized" the cAMP response to relaxin activation of RXFP1 endogenously expressed in rat striatum and rat cardiac muscle (Shpakov et al., 2007). A synthetic peptide based on the C terminus of  $G\alpha_s$  (residues 385–394) inhibited AC activity that was stimulated by relaxin or the ICL3 peptides (Shpakov et al., 2007), suggesting an interaction between  $G\alpha_s$  and ICL3. This is a region that in many GPCRs interacts with G proteins (Kjelsberg et al., 1992; Ren et al., 1993; Herrick-Davis et al., 1997; Egan et al., 1998). ICL3 may also direct coupling to  $G\alpha_{oB}$  for both RXFP1 and RXFP2, although direct evidence is lacking; however, truncation of the C-terminal tail of either receptor did not affect the  $G\alpha_s$  or the  $G\alpha_{oB}$  components of the cAMP signaling response, suggesting a common interaction site. Constitutive activation of RXFP1 involving  $G\alpha_s$  (and  $G\beta\gamma$ ) activation of AC2 (Halls and Cooper, 2010) is also likely to depend upon  $G\alpha_s$  coupling to ICL3 (see section III.A.3).

The C-terminal tail of RXFP1 has two clear roles in signaling; it controls cAMP accumulation through the  $G\alpha_{i3}$  pathway and is essential for signalosome formation (see sections III.A.1 and III.A.3). Only RXFP1 increases cAMP accumulation by coupling to  $G\alpha_{i3}$  (Halls et al.,



2006) and activation of AC5, utilizing a G $\beta$  $\gamma$ -phosphatidylinositol 3-kinase (PI3K)-protein kinase C (PKC) $\zeta$  pathway. Coupling of RXFP1 to this pathway involves the final 10 amino acids of the C terminus and absolutely requires R752; truncation of the C terminus or substitution of R752 selectively removes coupling to G $\alpha_{i3}$  (Halls et al., 2009b) (Fig. 1). As yet the precise mechanism by which the terminal 10 residues of RXFP1 direct G $\alpha_{i3}$  coupling is unclear. The possibilities include direct G $\alpha_{i3}$  coupling (although not observed for any other GPCR), G $\alpha_{i3}$  coupling after receptor phosphorylation, or recruitment of scaffolding proteins for colocalization of the receptor with G $\alpha_{i3}$ .

The C-terminal tail of RXFP1 also controls a small degree of constitutive activity (Halls and Cooper, 2010), and the receptor constitutively couples to AC2 via an association between helix 8 and A-kinase anchoring protein 79 (AKAP79). The protein complex facilitates G $\alpha_s$ - and G $\beta$  $\gamma$ -mediated stimulation of AC2 to cause cAMP accumulation in response to subpicomolar concentrations of relaxin. The amount of cAMP generated by this complex is tightly controlled by protein kinase A (PKA)-stimulated phosphodiesterase (PDE) 4D3, which is scaffolded to the receptor C terminus by an association between  $\beta$ -arrestin-2 and S704 of RXFP1 (Fig. 1). This protein complex, termed a signalosome, directed and maintained by the RXFP1 C-terminal tail, mediates a cAMP response to low concentrations of peptide and may provide a novel cellular response to low levels of relaxin in some physiologic situations.

The RXFP1 C-terminal tail also contains a number of potential consensus sequences for phosphorylation and protein-protein interactions and is the region of most variation between RXFP1 and the highly-related RXFP2 (Halls et al., 2007b). As such, it represents an area of functional divergence between the two receptors that may relate to the more varied physiologic roles of relaxin in relation to INSL3.

RXFP1 and RXFP2 therefore have similar structures, binding sites, and signaling mechanisms. Both receptors possess a high-affinity binding site in the LRR region, a lower affinity site in ECL2 of the TM domain, and require an intact LDLa module for signaling. RXFP1 has a more complex C-terminal region that contains residues essential for signaling involving G $\alpha_{i3}$  and interactions with signalosomes. Both receptors form homo- and heterodimers, but the functional significance of this is unclear at present.

**2. Functional Domains of Relaxin Family Peptide Receptors 3 and 4.** RXFP3 and RXFP4 are classic peptide receptors of the Rhodopsin family of class A GPCRs and possess small N-terminal domains that are markedly different from those described for RXFP1 and RXFP2. Additionally, the relaxin-3 B-chain alone can bind and activate RXFP3, although with lower potency than the two-chain peptide, whereas both chains are required for interaction with RXFP1 (Kuei et al., 2007;

Hossain et al., 2008). Thus the mode of binding and activation differs from the relaxin/RXFP1 activation mechanism. Alanine scans show that R8, R12, R16, I15, and F20 in the B-chain central helix are required for human relaxin-3 binding, whereas R26 and W27 are essential for receptor activation (Kuei et al., 2007). The first study to investigate the interaction between relaxin-3 and the RXFP3 receptor used peptide selectivity for RXFP3 and RXFP4: relaxin-3 binds to and activates both RXFP3 and RXFP4, whereas INSL5 only activates RXFP4. Chimeric RXFP3 and RXFP4 receptors were used to identify potential functional roles for the extracellular domains and TM helices (Zhu et al., 2008; Bathgate et al., 2013a). Chimeras with swapped N-terminal domains suggested that the N terminus of RXFP4 is required for INSL5 and possibly relaxin-3 binding. Chimeras of RXFP3 with RXFP4 ECL1 or ECL3 domains demonstrated that these ECLs were not important for INSL5 binding to either receptor (Zhu et al., 2008). However, chimeras with swapped ECL2 domains revealed that ECL2 in RXFP3 and RXFP4 are required for ligand binding and possibly receptor activation. Furthermore, insertion of the N terminus and ECL2 of RXFP4 into RXFP3 produced a chimera with full INSL5 binding, demonstrating that these domains are necessary for INSL5 binding in RXFP4 (Zhu et al., 2008). However, this chimera did not increase GTP $\gamma$ S binding, showing that these domains are not sufficient for receptor activation by INSL5.

*a. Extracellular regions.* A recent study performed comparative sequence analysis of multiple mammalian RXFP3 sequences, molecular modeling, and mutagenesis to map the relaxin-3 binding site in RXFP3 (Bathgate et al., 2013b). The truncation of the first 33 amino acids of the RXFP3 N terminus demonstrated that none of these residues were involved in relaxin-3 binding (Fig. 2). Rather, relaxin-3 used multiple arginine residues across a large peptide surface to interact with RXFP3, leading to a focus on potential glutamic and aspartic acid residues in the ECLs that may form electrostatic interactions with these critical arginine residues. Mutagenesis experiments demonstrated that E141 and D145 in ECL1 and E244 in ECL2 were essential for relaxin-3 binding (Fig. 2). These data were then used to dock the NMR solution structure of relaxin-3 (Rosengren et al., 2006b) into a homology model of RXFP3 based on the CXCR4 crystal structure bound to a peptide antagonist (Wu et al., 2010). The subsequent model describing the relaxin-3/RXFP3 interaction demonstrated that the three acidic residues E141, D145, and E244 likely coordinate binding to the three arginines in relaxin-3. Hence, R12 and R16 on the B-chain helical segment of relaxin-3 interact with RXFP3 residues E244 and D145, respectively. This model allows for the relaxin-3 C-terminal tail that was shown to be flexible in the NMR solution structure to insert into the classic "GPCR binding pocket" within the TM domains where relaxin-3 R26 can potentially form a salt bridge with RXFP3 E141 (Bathgate

et al., 2013b). This model is consistent with the data showing that relaxin-3 residues in the core  $\beta$ -helix are involved in RXFP3 binding, whereas the relaxin-3 C-terminal RW residues likely drive activation by interactions in the RXFP3 TM core. A slightly different relaxin-3/RXFP3 interaction model was recently described that is nonetheless still consistent with the activation mode described above (Zhang et al., 2014) (Fig. 2).

*b. Transmembrane spanning regions.* Chimeras were used to investigate the role of the TM spanning regions of RXFP3 and RXFP4. Replacement of TM3 or TM5 of RXFP4 with those of RXFP3 decreased affinity and abolished INSL5 activity (Zhu et al., 2008; Bathgate et al., 2013a), suggesting that these regions are necessary for INSL5 binding and activation. However, the reverse chimera of RXFP3 with TM3 and TM5 from RXFP4 had increased INSL5 binding affinity but did not activate GTP $\gamma$ S binding (Zhu et al., 2008), demonstrating that TM3 and TM5 alone are not sufficient for INSL5 activation of RXFP3. A chimera of RXFP3 with TM2, TM3, TM5, and ECL2 of RXFP4 displayed similar affinity for both relaxin-3 and INSL5 to wild-type RXFP4 (Zhu et al., 2008), suggesting that all regions influence binding. Relaxin-3 activated GTP $\gamma$ S binding at this chimera similarly to wild-type RXFP4, and although INSL5 showed increased potency, it was still slightly lower than at wild-type RXFP4 (Zhu et al., 2008). This suggests that TM2, TM3, TM5, and ECL2 are all involved in ligand binding and activation of RXFP3 and RXFP4.

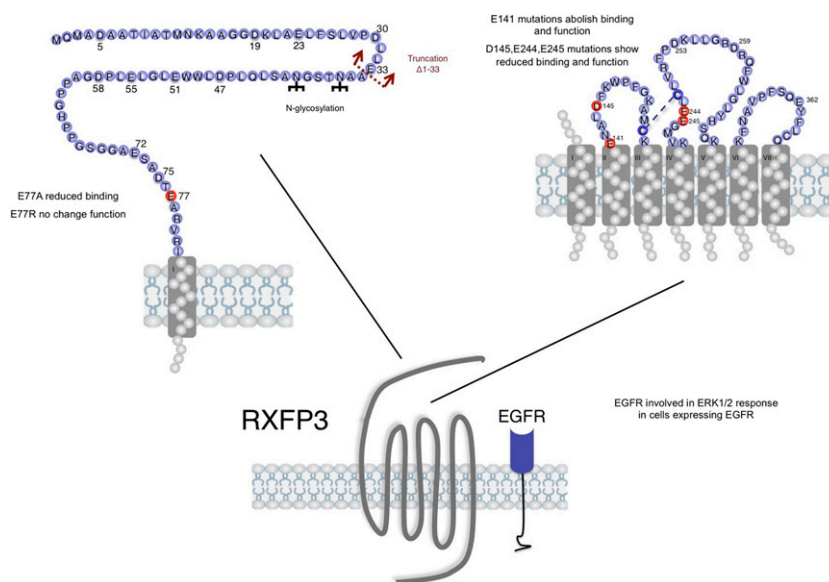
The mode of activation of RXFP3 and RXFP4 by their cognate ligands is more conventional than that observed for RXFP1 and RXFP2 and involves an interaction of the ligand with just the receptor N terminus and ECL2.

## II. Ligands That Act at Relaxin Family Peptide Receptors

All of the relaxin family peptides that are the cognate ligands for relaxin family peptide receptors are synthesized and processed in a manner similar to insulin and have a conserved prohormone structure (signal peptide, B-chain, C-chain, and C-terminal A-chain) that necessitates modification to produce the mature, active peptide. The signal peptide is removed first, followed by the C peptide, to form a mature heterodimeric peptide with two disulfide bonds between the A- and B-chains and an additional intra-A-chain bond. However, cleavage of the C peptide has been directly demonstrated for only some of the peptides.

Relaxin has been isolated and characterized from many species, and all show the two-chain, three disulfide-bonded structure similar to insulin (Bathgate et al., 2006a) (Fig. 3). Similar processing has been demonstrated for porcine relaxin-3 (Liu et al., 2003b) but not yet for INSL5. INSL3 has been isolated as both a prohormone form in the goat (Siqin et al., 2013) and boar (Minagawa et al., 2012) and as a processed form in the bovine (Bullesbach and Schwabe, 2002). Importantly, and as for relaxin, both processed and unprocessed forms of INSL3 are bioactive, although relaxin-3 must be processed to be active at RXFP3 (Ganella et al., 2013a). Structural studies on recombinant human relaxin (Eigenbrot et al., 1991), synthetic INSL3 (Rosengren et al., 2006b), human relaxin-3 (Rosengren et al., 2006a), and INSL5 (Haugaard-Jonsson et al., 2008) have determined the three-dimensional structures of the peptides.

The crystal structure of the human relaxin peptide reveals an overall fold similar to insulin (Eigenbrot



**Fig. 2.** Functional domains of RXFP3. The first 33 residues from the N terminus do not appear to be involved in binding of relaxin-3. R12 and R16 in the relaxin-3 B-chain interact with RXFP3 E244 and D145, and relaxin-3 R26 can potentially form a salt bridge with RXFP3 E141. Transmembrane regions also determine ligand affinity and specificity, although precise residues have yet to be identified. There is evidence for transactivation of EGFR in the ERK1/2 response in those cells that express the EGFR.

et al., 1991) as well as the formation of an asymmetric dimer, albeit in a different orientation. The main differences between relaxin family peptides occur around their termini (Rosengren et al., 2009). NMR studies of human relaxin-3 show similarities to human relaxin and insulin, although relaxin-3 has a more hydrophobic core with a condensed B-chain  $\alpha$ -helix, allowing interaction with W27 (Rosengren et al., 2006a). In human relaxin, the B-chain  $\alpha$ -helix is one turn longer, forcing the tryptophan to face away from the core of the molecule and making it solvent exposed (Rosengren et al., 2006a). NMR studies suggest that this is similar in INSL5 (Haugaard-Jonsson et al., 2009). In INSL3, the C-terminal orientation is superficially similar to that observed for human relaxin-3, with the C terminus contacting the hydrophobic core (Rosengren et al., 2006a). However, the C termini show disparity in their precise orientation that has direct ramifications for the key role of the C-terminal tryptophan in the activity of these peptides.

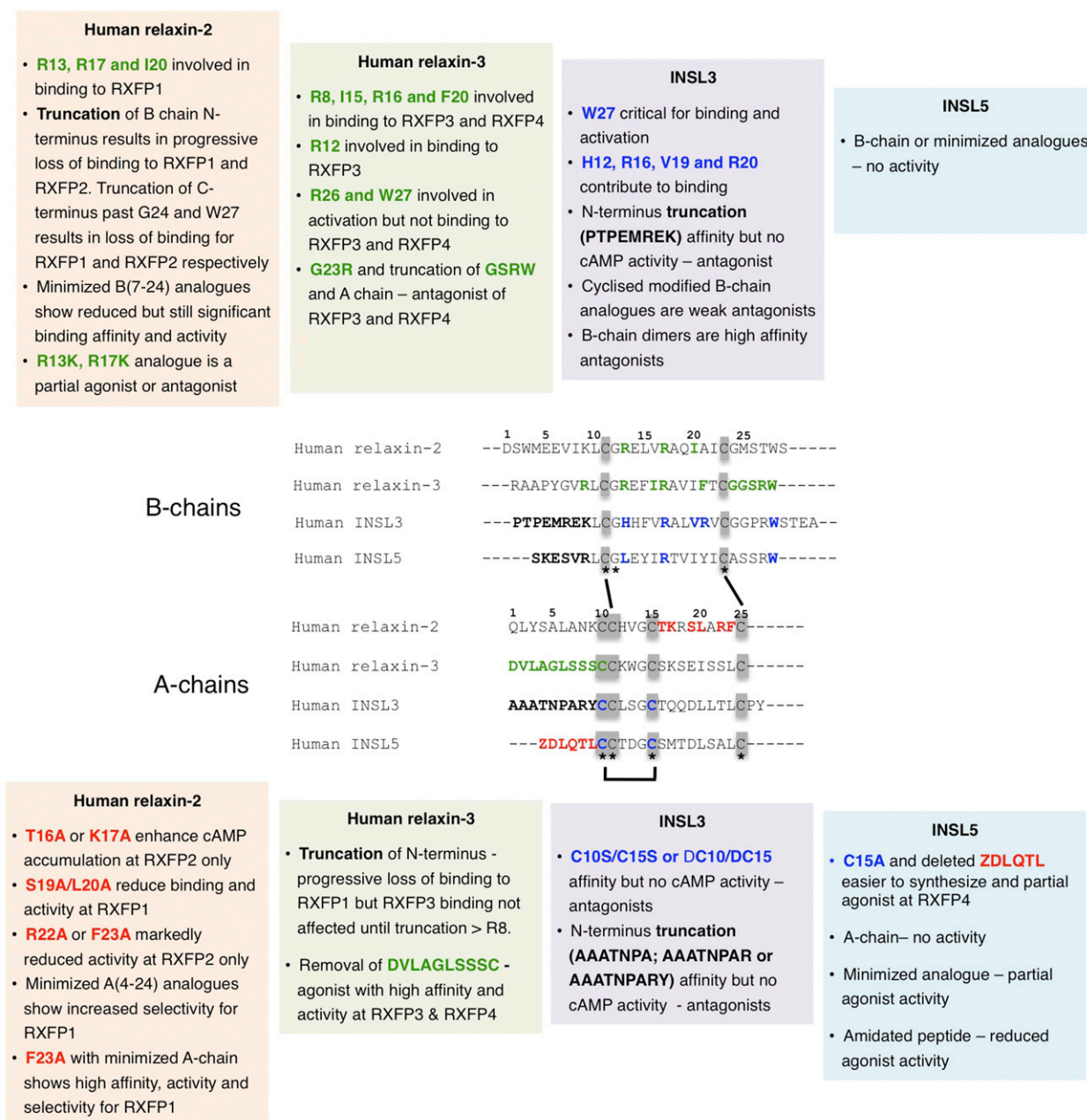
#### A. Ligands That Act at Relaxin Family Peptide Receptor 1

**1. Relaxin Family Peptide Agonists.** Although the relaxin peptides show considerable variation across species, alignment of amino acid sequences showed that in addition to the cysteines necessary for the two-chain structure there was a conserved RxxxRxxI/V motif in the B-chain. Examination of the X-ray crystal structure of relaxin indicates that the arginines are outward facing and located on the first and second loop of the  $\alpha$ -helix (Eigenbrot et al., 1991) where together with an isoleucine or valine residue they form the receptor binding site for relaxin (Schwabe and Bullesbach, 1994; Tan et al., 2002). Replacement of the arginine, isoleucine, or valine residues in this motif (RxxxRxxI/V) markedly reduces or abolishes activity (Schwabe and Bullesbach, 1994; Tan et al., 2002). The relaxin A-chain shows greater sequence variation than the B-chain across species (Sherwood, 1994). Other than the conserved cysteine residues, G14 is also highly conserved and necessary for chain flexibility and structure (Bullesbach and Schwabe, 1994). Truncation of the A-chain of human relaxin produces peptides that progressively lose the ability to bind to and activate RXFP1 and RXFP2 (Hossain et al., 2008) (Fig. 3). Studies using targeted point mutations within the A-chain show an influence of the A-chain on receptor binding and activation. T16A and K17A mutations had little effect on the cAMP response to RXFP1 activation but enhanced cAMP responses after activation of RXFP2; conversely, the R22A and F23A mutations markedly reduced the activity of human relaxin at RXFP2 without reducing binding or activity at RXFP1 (Park et al., 2008); mutations S19A and L20A are also known to reduce peptide binding and activity at RXFP1 (Bullesbach and Schwabe, 1994) (Fig. 3). More recent detailed studies of the key A-chain residues (Chan et al., 2012) show that no single amino

acid drives the interactions between RXFP1 and human relaxin, although Y3, L20, and F23 all have some role. These studies also identify differences in the mechanisms by which relaxin binds to and activates RXFP1 compared with RXFP2, leading to the development of a potent and selective peptide agonist for RXFP1, H2:A (4–24) (F23A) that has similar potency and biologic activity to human relaxin but no significant activity at RXFP2 (Chan et al., 2012) (Table 1). The contention that human relaxin binds to RXFP1 and RXFP2 by distinct mechanisms is further supported by the species-specific nature of these structure activity relationships: neither mouse nor rat relaxin is able to bind to or activate RXFP2 (Scott et al., 2005b), and although human relaxin-3 binds with good affinity to RXFP1, it has only a poor affinity for RXFP2 (Bathgate et al., 2006b). The importance of the peptide A-chain is also emphasized by the structure activity relationships displayed by relaxin-3. Replacement of the relaxin-3 A-chain with that of INSL5 produces a chimeric peptide (R3/I5) that no longer binds to or activates RXFP1 but has unchanged activity at RXFP3 (Liu et al., 2005a) (Fig. 3). A similar profile is attained after truncation of the relaxin-3 A-chain (Hossain et al., 2008).

Examination of the role of the B-chain in activation of RXFP1 demonstrates that it is possible to truncate both ends while still retaining binding and functional activity (Hossain et al., 2011). Peptides with a reduced B-chain [H2:(B7–24)] or reduced A- and B-chains [H2:(A4–24) (B7–24)] show some reduction in binding activity and ability to generate cAMP but have similar antifibrotic activity to human relaxin and less activity at RXFP2 (Hossain et al., 2011) (Table 1).

**2. C1q-Tumor Necrosis Factor-Related Protein 8: An Additional Relaxin Family Peptide Receptor 1 Ligand?** Short linear peptides derived from a naturally occurring protein containing a collagen-like repeat have been reported to act at RXFP1 (Shemesh et al., 2009). Although the effects produced by the peptides CGEN-25009 and CGEN-25010 in several systems were extremely variable and the effects of human relaxin in these systems unusual (Shemesh et al., 2008, 2009), there is some evidence to suggest relaxin-like activity of these peptides in THP-1 cells and in a fibrosis model (Pini et al., 2010). In the latter study, CGEN-25009 and human relaxin increased cAMP, cGMP, and nitrite and decreased collagen deposition and increased matrix metalloproteinase-2 (MMP-2) activity in human dermal fibroblasts (Pini et al., 2010). More recent studies with these peptides and the precursor protein C1q-tumor necrosis factor-related protein 8 (CTRP8) demonstrated activation of RXFP1 (Glogowska et al., 2013) with cAMP production and a PI3K-mediated promigratory phenotype in glioblastoma cell lines and primary cells. Coimmunoprecipitation studies demonstrated a direct interaction between human CTRP8 and RXFP1. Although these studies suggest that CTRP8 or peptide fragments are able to activate RXFP1, it remains to be seen whether



**Fig. 3.** Structure-activity relationships for the relaxin family peptides relaxin, relaxin-3, INSL3, and INSL5. All four peptides share common structural features including the two intrachain disulfide bonds and the interchain disulfide bond. However, the interaction sites between the peptides and their cognate receptors show distinct characteristics. Binding of relaxin to RXFP1 involves the RxxxRxxI/V motif in the B-chain, whereas alterations in A-chain length influence activity at RXFP1 and RXFP2. Binding of INSL3 to RXFP2 involves a similar motif but displaced one turn along the  $\alpha$ -helix of the B-chain. N terminus truncation of either the A- or the B-chain reduces efficacy but not affinity and has been used as a strategy for producing antagonists. Disruption of intrachain disulfide bonds in INSL3 reduces binding to a minor extent but destroys agonist activity (C10S/C15S or C10del/C15del). Binding of relaxin-3 to RXFP3 involves predominantly the B-chain, and reduction of the A-chain removes activity at RXFP1 without affecting activity at RXFP3. Relaxin-3 R8, R16, I5, and F20 are required for binding to RXFP3 and RXFP4, with R12 required for RXFP3 but not RXFP4. Relaxin-3 R26 and W27 are required for activation of RXFP3 and RXFP4. Truncation of the B-chain and G23R produces an antagonist, and truncation of the A-chain retains high-affinity agonist activity. Binding of INSL5 to RXFP4 shows a number of differences from the relaxin-3/RXFP3 interaction. Unlike relaxin-3, the B-chain of INSL5 is inactive and minimized analogs generally show reduced affinity and efficacy. Many of the agonist and antagonist peptides active at RXFP3 are also active at RXFP4.

they are native ligands and/or whether activation of RXFP1 by CTRP8 occurs specifically in glioblastomas.

3. *Small-Molecular-Weight Agonists.* The recent success of the phase III clinical trial of relaxin for acute heart failure (see below) encouraged a screening campaign to identify small molecule agonists of RXFP1. By

using HEK293 cells stably expressing RXFP1, 365,677 compounds were screened for their ability to cause cAMP accumulation. Both of the hits obtained contained a 2-acetamido-*N*-phenylbenzamide structure that was used as a basis for medicinal chemistry optimization (Chen et al., 2013; <http://www.ncbi.nlm.nih.gov/books/NBK153218/>). Of

TABLE 1  
Ligands that interact with RXFP1

Ligand		Binding Affinity $pK_i$ , $pK_D$	cAMP $pEC_{50}$	pERK1/2 $pEC_{50}$	Reference
Human relaxin-1	AG	8.84	9.1		Bathgate et al. (2006b)
Relaxin	AG	9.24	10.37		Hossain et al. (2008)
Relaxin	AG	9.75	9.39		Bathgate et al. (2013a)
Relaxin	AG		9.71	9.60	Siwek et al., unpublished
Relaxin	AG		16.96, 9.46		Halls and Cooper (2010)
A(4–24)(B7–24)H2	AG	6.99	8.22		Hossain et al. (2011)
A(4–24)(B7–24)H2	AG		8.36	8.24	Siwek et al., unpublished
Relaxin-3	AG	7.69	9.36		Hossain et al. (2008)
Relaxin-3	AG	8.6	8.74		Shabanpoor et al. (2012)
Relaxin-3	AG		8.85	8.57	Siwek et al., unpublished
INSL3	AG	5.68	N.A.		Bathgate et al. (2013a)
B–R13/17KH2	PA	6.29	6.67		Hossain et al. (2010)
B–R13/17KH2 (AT-001)	PA	5.03	5.66		Silvertown et al. (2007), Neschadim et al. (2014)
B–R13/17KH2	PA		7.59	8.41 (7.97 ant)	Siwek et al., unpublished
A(4–24)(F23A)H2	AG	9.17	9.82		Chan et al. (2012)
Minimized relaxin-3 analog 2	AG	<5	N.A.		Shabanpoor et al. (2012)
Minimized relaxin-3 analog 3	AG	<5	N.D.		Shabanpoor et al. (2012)
ML290	AA	N.A.	7.03	N.A.	Xiao et al. (2013)

AA, allosteric agonist; AG, agonist; BA, biased agonist; N.A., no activity; N.D., not determined; PA, partial agonist.

these compounds, a number showed reasonable potency for cAMP generation in both HEK293-RXFP1 and THP-1 cells (that endogenously express RXFP1) and had good specificity for RXFP1 versus RXFP2 (Xiao et al., 2013) (Table 1). Several of the compounds increased vascular endothelial growth factor (VEGF) gene expression in THP-1 cells with similar efficacy to relaxin, and compound 8 [ML290; 2-isopropoxy-*N*-(2-(3-(trifluoromethylsulfonyl)phenylcarbonyl)phenyl)benzamide] was also shown to increase cellular impedance in a label-free system albeit with at least 500-fold lower potency than relaxin (Xiao et al., 2013). In addition, comparison of the binding mode of action of ML290 with that of relaxin suggests that the small-molecule agonist acts as an allosteric agonist at RXFP1 (Xiao et al., 2013; Christopoulos et al., 2014). Relaxin binds with high affinity to the LRR region of RXFP1 followed by a lower affinity interaction with ECL2 (Sudo et al., 2003; Halls et al., 2005a,b). This triggers an interaction involving the LDLa module that is essential for signaling (Scott et al., 2006; Kern et al., 2007; Kong et al., 2013). Mutations of the calcium binding asparagine within the LDLa module (D58E), produce a receptor where the cAMP response to relaxin is completely abolished but that to ML290 (compound 8) is unaffected (Xiao et al., 2013). Because, unlike the human receptor, mouse RXFP1 does not respond to ML290, chimeric constructs of human and mouse RXFP1 were used to identify the region of RXFP1 that interacts with ML290. Examination of the chimeras narrowed the region of interest down to ECL3, and two pairs of amino acids divergent between human and mouse RXFP1 were identified. The substitution of the N-terminal IL within mouse ECL3 to the VV of human RXFP1 did not rescue function (Xiao et al., 2013). C-terminal substitution of GT within human RXFP1 to the DS of mouse RXFP1 produced a chimeric human RXFP1 that did not respond to ML290. The converse substitution of DS to GT in mouse RXFP1 created a partially active receptor. The

mouse receptor with a double substitution of the mouse to the human residues, IL to VV and DS to GT, produced a fully active mouse RXFP1 that responded to ML290 in a similar fashion to the human receptor (Xiao et al., 2013). The 2-acetamido-*N*-phenylbenzamide derivatives represent the first synthetic small-molecule agonists at RXFP1. They show good potency, selectivity, and a number of relaxin-like functions in cell-based assays. They appear to behave as allosteric agonists acting at the ECL3 loop of RXFP1 (Fig. 1). It remains to be demonstrated whether the profile of activity of ML290 and similar compounds matches that of relaxin in a variety of cellular and animal disease models.

**4. Relaxin Family Peptide Receptor 1 Antagonists.** In contrast to the other members of the RXFP receptor family there have been few examples of peptides with antagonist properties at RXFP1. Peptide AT-001 (Neschadim et al., 2014) contains mutations of the B-chain residues R13 and R17 [that are essential for relaxin-like activity (Büllesbach et al., 1992)] to K13 and K17 (B-R13/17K relaxin). Initially, antagonism was demonstrated by lentiviral expression of the B-R13/17K relaxin, which decreased prostate cancer xenograft growth (Silvertown et al., 2007). In addition, medium from cells infected with the lentivirus showed human relaxin immunoreactivity and antagonized cAMP production from THP-1 cells in response to relaxin (Silvertown et al., 2007). However, a subsequent synthesis of the B-R13/17K peptide showed that although it competed for relaxin binding, the peptide was actually a partial agonist for cAMP production in cells overexpressing RXFP1 (Hossain et al., 2010; Neschadim et al., 2014) (Table 1). In cells expressing RXFP1 at lower (more physiologic) levels, B-R13/17K relaxin displayed functional antagonism, inhibited MCF-7 cell invasion, and prevented the inhibitory effect of relaxin on renal myofibroblast differentiation (Hossain et al., 2010). In androgen-independent PC3 prostate cancer xenografts,



B-R13/17K suppresses growth and has antiangiogenic effects and synergizes with the chemotherapeutic agent docetaxel (Neschadim et al., 2014). The activity of B-R13/17K at other RXFP receptors has not been examined.

The detailed understanding of the mode of interaction of peptide ligands at RXFP1 has led to the development of potent and selective agonists and also partial agonists that have antagonist properties in systems with low levels of receptor expression. An exciting recent development is the small molecular weight allosteric agonists, typified by ML290, that are currently being characterized in terms of their ability to activate the full repertoire of RXFP1 signaling.

### B. Ligands That Act at Relaxin Family Peptide Receptor 2

**1. Relaxin Family Peptide Agonists.** INSL3 is the cognate ligand for RXFP2 that interacts with RXFP1 only at extremely high concentrations (Kumagai et al., 2002; Halls et al., 2005b), suggesting that it does not bind to or activate RXFP1 in a physiologic setting. In contrast, the cognate ligand for RXFP1, human relaxin, potently activates RXFP2, although the mode of interaction of both human relaxin and INSL3 with RXFP2 shows significant differences compared with the interaction of human relaxin with RXFP1 (see section II.B.1) (Table 2). Mutated INSL3 peptides demonstrate the utilization of distinct amino acids in the B-chain to bind to and activate RXFP2. W27 within INSL3 is critical for RXFP2 binding and activation (Bullesbach and Schwabe, 2004, 2005a), although other single amino acid replacements with either alanine or valine suggest that there are additional influences within the B-chain. Although individual substitutions only slightly reduce INSL3 binding to RXFP2, the combined mutation of H12A, R16A, V19A, R20A, and W27A abolishes binding (Rosengren et al., 2006b).

Truncation of the A-chain of human relaxin results in a peptide that progressively loses the ability to bind to and activate both RXFP1 and RXFP2 (Hossain et al., 2008), possibly because of a loss of structure in the

B-chain. However, truncation of the A-chain of INSL3 completely abolishes activation with no effect on binding (Bullesbach and Schwabe, 2005a) (Table 2). Additionally, targeted point mutations within the A-chain selectively alter the receptor binding and activation profile: the T16A and K17A mutations do not markedly alter cAMP responses to RXFP1 activation but enhance cAMP responses at RXFP2; the R22A and F23A mutations reduce human relaxin activity at RXFP2 but not at RXFP1 (Park et al., 2008). The double S19A and L20A mutations reduce peptide binding and activity at RXFP1, again suggesting a role of the A-chain in receptor binding and activation (Bullesbach and Schwabe, 1994) (Table 2). Differences in the mechanism of binding and activation between RXFP1 and RXFP2 are further supported by the species-specific nature of these structure activity relationships; thus neither mouse nor rat relaxin can bind to or activate human, rat, or mouse RXFP2 (Scott et al., 2005b), and human relaxin-3 has only a poor affinity for RXFP2 (Bathgate et al., 2006b). All of these studies highlight the importance of both the A- and B-chains for INSL3 activation of RXFP2 and show that the mechanism of action is different from the relaxin-RXFP1 interaction (Hossain et al., 2008).

**2. Relaxin Family Peptide Antagonists.** In contrast to RXFP1, there are numerous peptide antagonists of RXFP2 due to the difference in the mode of activation of RXFP2 by INSL3. Thus deletion of 10 residues from the N terminus of the INSL3 A-chain produces a peptide that still binds to RXFP2 but no longer increases cAMP accumulation (Bullesbach and Schwabe, 2005a). This truncated peptide is a specific competitive inhibitor of INSL3 at RXFP2 (Bullesbach and Schwabe, 2005a) (Table 2). In addition, deletion of eight N-terminal residues of the B-chain produces an INSL3 peptide that retains binding affinity but loses agonist activity (Bullesbach and Schwabe, 2005a). Substitution of eight A- or B-chain residues with alanine does not affect receptor signaling (Bullesbach and Schwabe, 2005a). Disruption of the intra-A-chain disulfide bond (C10S/C15S; or C10del/C15del) produces peptides that retain RXFP2

TABLE 2  
Ligands acting at RXFP2

Ligand		Binding Affinity pK <sub>i</sub> , pK <sub>D</sub>	cAMP pEC <sub>50</sub>	Reference
Relaxin	AG	8.48	9.13	Hossain et al. (2008)
Relaxin	AG	8.96	7.88	Bathgate et al. (2013a)
INSL3	AG	9.34	10.35	Hossain et al. (2008)
INSL3	AG	9.71	8.27	Bathgate et al. (2013a)
A(4–24)(B7–24)H2	PA	>6	>6	Hossain et al. (2011)
A(4–24)(F23A)H2	AG	<6	<6	Chan et al. (2012)
A(9–26)INSL3	ANT	9.14	N.A.	Bullesbach and Schwabe (2005a); Hossain et al. (2008)
A(10–24)INSL3	ANT	8.67	N.A.	Hossain et al. (2008)
A(C10/15S)INSL3	ANT	8.59	N.A.	Zhang et al. (2010)
A(Δ10/15C)INSL3	ANT	8.32	N.A.	Zhang et al. (2010)
Cyclic INSL3 B-chain analog 6	ANT	6.65	N.A.	Shabanpoor et al. (2007)
Dimeric INSL3 B-chain analog 8	ANT	8.50	N.A.	Shabanpoor et al. (2011)
ML290	AA		N.A.	Xiao et al. (2013)

AA, allosteric agonist; AG, agonist; ANT, antagonist; N.A., no activity; PA, partial agonist.

binding but do not activate cAMP signaling (Zhang et al., 2010) (Table 2). The INSL3 B-chain alone is also a RXFP2 antagonist, although it only has low affinity for the receptor (Del Borgo et al., 2006; Shabanpoor et al., 2007). Modifications of the single-chain structure result in modest gains in affinity, but larger gains are obtained by linking two B-chains using the native cysteine residues (Shabanpoor et al., 2010) (Table 2). Modifications of this B-chain dimer resulted in antagonists with affinities only slightly lower than INSL3 (Shabanpoor et al., 2011).

Despite the similarity between RXFP1 and RXFP2 and their cognate ligands relaxin and INSL3, the precise mode of peptide activation of the receptors is quite different. This has facilitated the successful development of a number of antagonists for RXFP2.

### C. Ligands That Act at Relaxin Family Peptide Receptor 3

**1. Relaxin Family Peptide Agonists.** Human relaxin-3 has a tertiary structure, determined by solution NMR spectroscopy, that is similar to insulin and other relaxin family peptides (Rosengren et al., 2006a). However, unlike the interaction of human relaxin with RXFP1 that requires both A- and B-chains for binding and activation, the human relaxin-3 B-chain alone can bind to and activate both RXFP3 and RXFP4 (Liu et al., 2003b). Replacement of the human relaxin-3 A-chain with the INSL5 A-chain (R3/I5) does not influence RXFP3 binding or activation but markedly reduces the ability of the peptide to bind to and activate RXFP1 (Liu et al., 2005a) (Table 3). Similarly, truncation of the human relaxin-3 A-chain reduces binding to and activation of RXFP1 but retains full activity at RXFP3 (Hossain et al., 2008). The additional deletion of the A-chain disulfide bond results in the complete loss of RXFP1 binding and activation with little effect on RXFP3 binding and activation (Shabanpoor et al., 2012). Further development of this analog with a B23–27 deletion and addition of an arginine at B23 led to a high-affinity, RXFP3-selective, competitive antagonist (analog 3) (Table 3).

Site-directed mutagenesis of B-chain residues reveals that R8, R16, I5, and F20 are important for human relaxin-3 binding to RXFP3 and RXFP4 (Kuei et al., 2007), with R12 also required for binding to RXFP3 but not RXFP4 (Kuei et al., 2007). R26 and W27 toward the C terminus of the B-chain are required for activation of RXFP3 (Kuei et al., 2007). Truncation of the C terminus of the B-chain to C22 and the serendipitous addition of an additional arginine residue at the N terminus in the recombinant production system, when combined with the A-chain of INSL5, led to the discovery of a high-affinity RXFP3-selective antagonist R3(B $\Delta$ 23–27)R/I5 (Kuei et al., 2007) (see section II.C.4; Table 3). A single-chain antagonist has since been developed based on the B-chain of R3(B $\Delta$ 23–27)R/I5 with the cysteine residues mutated to serine (H3 B1–22R) (Haugaard-Kedstrom et al., 2011) (Table 3).

**2. Biased Agonists Acting at Relaxin Family Peptide Receptor 3.** Ligand-directed signaling bias is increasingly common in GPCR pharmacology and describes stabilization of distinct receptor conformations by ligands, resulting in selective activation of downstream signal transduction pathways (Baker and Hill, 2007; Evans et al., 2010; Kenakin and Miller, 2010). Although the original studies suggested that only relaxin-3 and its B-chain bound to and activated RXFP3, it is now clear that several relaxin peptides interact with RXFP3 to activate distinct signaling profiles through different, although sometimes overlapping pathways (Fig. 5). Initial studies showed that human relaxin-3 was selective for RXFP3 in both binding and AC inhibition assays, with no receptor activation by human relaxin or INSL3 (Liu et al., 2003b). However, cross-reactivity with other relaxin peptides was not examined over a wider range of signal transduction pathways, and the sensitivity of inhibitory cAMP assays can be influenced by the degree of activation of AC by forskolin and the time of stimulation (for detailed description see section III.C.2).

**3. Allosteric Modulators.** A novel selective allosteric modulator of RXFP3 has been described (Alvarez-Jaimes et al., 2012). 135PAM1 (3-[3,5-bis(trifluoromethyl)phenyl]-1-(3,4-dichlorobenzyl)-1-[2-(5-methoxy-1*H*-indol-3-yl)ethyl] urea) is a positive allosteric modulator (PAM) that curiously only displays activity with C-terminal amidated relaxin-3 or R3/I5 (probe selectivity). Binding studies conducted in HEK293-RXFP3 cell membranes showed that 135PAM1 does not compete with [<sup>125</sup>I]R3/I5(amide) binding at up to 1  $\mu$ M but at higher concentrations enhances binding consistent with activity as a PAM. The R3/I5(amide) peptide itself competes for [<sup>125</sup>I]R3/I5(amide) binding in a conventional manner (Alvarez-Jaimes et al., 2012). In HEK293-RXFP3 cells coexpressing G $\alpha$ q15, 135PAM1 increased calcium responses to EC<sub>20</sub> concentrations of relaxin-3(amide) or R3/I5(amide) but not the free-acid (OH) peptides. Concentration-response curves to the amidated but not the free-acid form of the peptides were shifted in a limiting manner by 135PAM1. Similar specificity of 135PAM1 was also displayed in a reporter gene assay that measured inhibition of CRE activity in cells expressing RXFP3 (Table 3). This is the only published example to date of a small molecule compound with activity at RXFP3. The discovery of 135PAM1 identifies an allosteric site on RXFP3 that can be modulated by small molecules.

**4. Relaxin Family Peptide Receptor 3 Antagonists.** The synthetic peptide R3(B $\Delta$ 23–27)R/I5 that was discovered as a RXFP3 antagonist has been instrumental in defining the physiologic functions of the receptor. Specificity for RXFP3 was initially achieved by synthesizing a chimeric peptide featuring the A-chain of INSL5 in combination with the B-chain of relaxin-3. The RXFP3 agonist properties of this peptide chimera were then markedly reduced by truncation of the relaxin-3 B-chain (Kuei et al., 2007; Hossain et al., 2009). Serendipitously,



TABLE 3  
Ligands acting at RXFP3

Ligand		Binding Affinity pK <sub>i</sub> , pK <sub>D</sub>	cAMP pEC <sub>50</sub>	GTP-γS pEC <sub>50</sub>	pERK 1/2 pEC <sub>50</sub>	P38 MAPK pEC <sub>50</sub>	Reference
Relaxin-3	AG	8.48	8.26				Hossain et al. (2008)
Relaxin-3	AG	7.78	9.0				Shabanpoor et al. (2012)
Relaxin-3	AG	9.29		9.48			Zhu et al. (2008)
Relaxin-3	AG	8.92	9.17		9.23		van der Westhuizen et al. (2007)
Relaxin-3	AG		9.13		9.18		van der Westhuizen et al. (2010)
Relaxin-3	AG				9.93	8.9	Kocan et al. (2014)
R3/I5	AG	9.28	9.35				Liu et al. (2005a)
R3/I5	AG	9.31	9.33				Kuei et al. (2007)
Relaxin	BA	10.0	7.05		7.99		van der Westhuizen et al. (2010)
Relaxin	BA, PA				7.15	8.35	Kocan et al. (2014)
INSL5	ANT	7.01		N.A.			Zhu et al. (2008)
Minimized relaxin-3 analog 2	AG	7.87	8.43		10.4		Shabanpoor et al. (2012)
Minimized relaxin-3 analog 3	ANT	7.6	N.A.				Shabanpoor et al. (2012)
Relaxin-3 B-chain dimer	AG	6.61					van der Westhuizen et al. (2007)
R3(BΔ23–27)R/I5	ANT	8.49	N.A.~8				Haugaard-Kedstrom et al. (2011)
R3(BΔ23–27)R/I5	ANT	9.17	N.A.				Kuei et al. (2007)
R3(BΔ23–27)R/I5	ANT, PA, BA				8.87	9.02	Kocan et al. (2014)
R3 B1–22R	ANT	7.44	N.A.				Haugaard-Kedstrom et al. (2011)
135PAM1	PAM		6.12				Alvarez-Jaimes et al. (2012)
Relaxin-3 B-chain	AG		7.06	~7.0			Liu et al. (2003b) Liu et al. (2005a)

AA, allosteric agonist; AG, agonist; ANT, antagonist; BA, biased agonist; N.A., no activity; PA, partial agonist .

upon recombinant production of R3(BΔ23–27)/I5 an extra arginine remained at the N terminus due to incomplete peptidase “trimming of the RR from the RRRR furin cleavage domain” to produce the peptide R3(BΔ23–27)R/I5. This peptide binds to RXFP3 and RXFP4 with high affinity and is a potent antagonist at RXFP3 in both rats and in human in vitro systems. Synthetic R3(BΔ23–27)/I5 (lacking R23) has lower affinity at RXFP3 and has weak agonist properties (Hossain et al., 2009), highlighting that the extra R23 creates an additional interaction with RXFP3 (Table 3). Administration of R3(BΔ23–27)R/I5 intracerebroventricularly to rats blocks food intake stimulated by the RXFP3 agonist R3/I5. It recently became apparent that R3(BΔ23–27)R/I5 has a complex antagonist profile and blocks some but not all pathways activated by RXFP3. It also has weak biased agonist properties (see section III.C.2). In addition, studies have revealed that the intra-A-chain disulfide bond in relaxin-3 is not important for interaction with RXFP3 but is necessary for the interaction of relaxin-3 with RXFP1. Analogs without this A-chain disulfide bond together with truncation of 10 amino acids from the N terminus of the A-chain remain potent RXFP3 agonists. If in addition, GGSRW is removed from the B-chain and replaced with R, the peptide becomes a high-affinity antagonist with similar biologic activity to R3(BΔ23–27)R/I5 (Shabanpoor et al., 2012) (Table 3).

Studies with R3(BΔ23–27)R/I5 suggested that the additional RXFP3 affinity provided by the R23 residue might result in the B-chain only R3(BΔ23–27)R having significant affinity for RXFP3 (Table 3). This has been proved experimentally and H3 B1–22R is a high-affinity antagonist of RXFP3 that is far easier to produce than

the two-chain peptides (Haugaard-Kedstrom et al., 2011). Importantly this peptide is also specific for RXFP3 and has no activity at RXFP4. The peptide was shown to have efficacy in vivo and blocks increases in feeding produced by intracerebroventricular injection of R3/I5 in rats (Haugaard-Kedstrom et al., 2011). It was subsequently used to demonstrate a role for the endogenous relaxin-3 system in addiction in rats (Ryan et al., 2013b) and in motivated food seeking and consumption in mice (Smith et al., 2014).

The structure-activity relationships for the interaction of relaxin peptides with RXFP3 are now well understood. Unlike RXFP1 and RXFP2, RXFP3 can be activated by peptides comprising only the B-chain, and a number of selective agonists and antagonists have been developed. Both relaxin and some RXFP3 antagonists display biased agonist properties at RXFP3. However, the utility of a small molecule allosteric modulator in vivo is currently limited by probe selectivity.

#### D. Ligands That Act at Relaxin Family Peptide Receptor 4

1. *Relaxin Family Peptide Agonists.* INSL5 is the cognate ligand for RXFP4 (Liu et al., 2005b). INSL5 has many of the characteristics of an incretin being secreted from enteroendocrine L cells and regulating insulin secretion and glucose homeostasis (Burnicka-Turek et al., 2012). Synthesis of human INSL5 represents considerable challenges so that some of the characterization of the peptide has been performed with mouse INSL5 that has 71% homology (Belgi et al., 2011). Compared with human INSL5, mouse INSL5 displays an 8-fold increase in affinity in binding assays and a 5-fold greater potency in

cAMP inhibition assays, possibly related to the overall greater positive charge associated with the nonconserved residues (Belgi et al., 2011) (Table 4). In addition, the amidated peptides show a considerable reduction in potency compared with the free acids (Belgi et al., 2011). Although relaxin-3 binds both RXFP3 and RXFP4 with high affinity, INSL5 is actually a weak antagonist at RXFP3 (Zhu et al., 2008) (Table 4). Furthermore, although the relaxin-3 B-chain alone was sufficient to bind and activate RXFP3, the INSL5 A- and B-chains alone are inactive at RXFP4 (Belgi et al., 2013).

The relaxin-3 B-chain alone is also a weak agonist at RXFP4 (Liu et al., 2003a, 2005a) (Table 4). Up to seven residues of the N terminus of the B-chain of relaxin-3 can be deleted with little effect on binding or activation of RXFP4 (Kuei et al., 2007; Hossain and Wade, 2010), whereas the  $\alpha$ -helical region of the B-chain is important for activity at RXFP4 (Hossain and Wade, 2010). R12 and R16 are important for binding to both RXFP1 and RXFP3, whereas only R16 is important for RXFP4 binding (Kuei et al., 2007). A critical residue for RXFP4 and RXFP3 binding appears to be F20, and mutations of this residue are associated with a marked loss of affinity (Kuei et al., 2007; Hossain and Wade, 2010). In addition, the C terminus of the relaxin-3 B-chain appears to be important for activation of RXFP4, and mutation of these residues blocks agonist activity without influencing affinity (Hossain and Wade, 2010).

**2. Modified Peptide Agonists.** The potential of RXFP4 as a therapeutic target and the difficulty involved in the synthesis of human INSL5 has led to the search for more easily synthesized and selective analogs. Minimized analogs of relaxin-3 display some interesting properties. Removal of the first eight residues of the A-chain of relaxin-3 produces an analog (analog 2) with similar binding affinity and potency to relaxin-3 at RXFP3 and RXFP4 but no activity at RXFP1 (Shabanpoor et al., 2012) (Table 4). This activity

is retained in a peptide where the B-chain is truncated by nine residues (Shabanpoor et al., 2012). These studies indicate that the C terminus of the B-chain and the interchain disulfide bonds are the major structural features required for activity at RXFP4.

**3. Relaxin Family Peptide Receptor 4 Antagonists.** Several of the compounds developed as antagonists of RXFP3 also have antagonist properties at RXFP4. These peptides include the minimized relaxin-3 analog 3 (minimized A-chain and truncated B-chain) that has no agonist activity at RXFP4 but only a slightly reduced binding affinity (Shabanpoor et al., 2012) (Table 4). Likewise the R3(B $\Delta$ 23–27)R/I5 RXFP3 antagonist also is a high-affinity antagonist at RXFP4 (Kuei et al., 2007). Interestingly, the single-chain variant of this peptide H3 B1–22R does not bind to RXFP4 (Haugaard-Kedstrom et al., 2011) (Table 4).

Much less information is available on RXFP4 compared with the other family members, but this has been accelerating due to the identification of INSL5 as a likely incretin. There is now a good understanding of the mode of interaction of INSL5 with RXFP4 and an increasing range of synthetic peptide agonists and antagonists have been developed.

### III. Signal Transduction Pathways

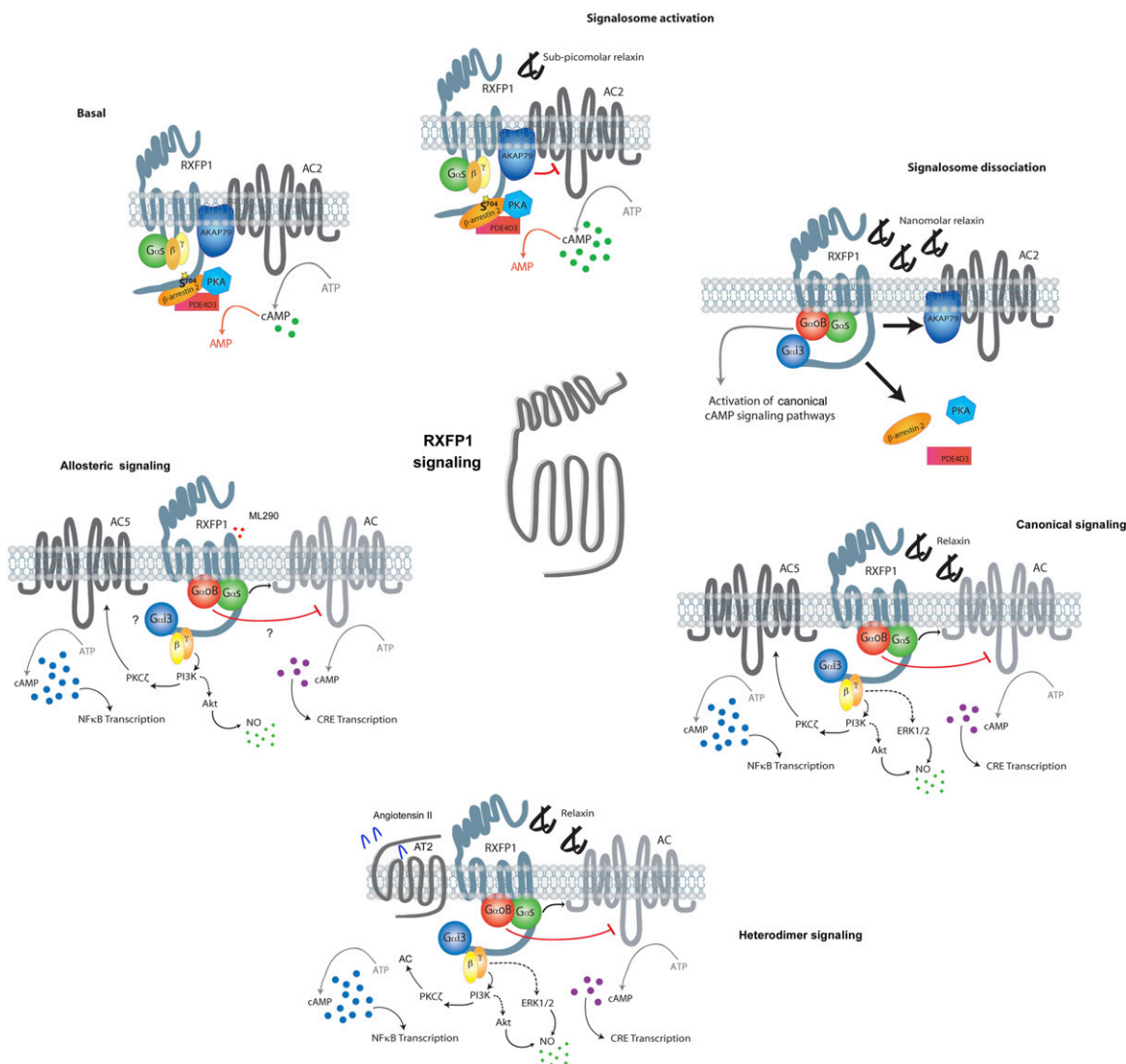
#### A. Relaxin Family Peptide Receptor 1 Signaling

**1. Canonical Signaling Pathways.** RXFP1 displays pleiotropic signaling to a variety of pathways including cAMP accumulation, activation of MAP kinases, tyrosine kinases, and NO as well as pathways associated with connective tissue metabolism (Fig. 4). Early studies before receptor identification, showed that treatment with relaxin increased cAMP accumulation in THP-1 cells (Parsell et al., 1996), MCF-7 cells (Bigazzi et al., 1992), the mouse pubic symphysis (Braddon, 1978), uterine strips (Sanborn et al.,

TABLE 4  
Ligands acting at RXFP4

Ligand		Binding Affinity pK <sub>i</sub> , pK <sub>D</sub>	cAMP pEC <sub>50</sub>	GTP $\gamma$ S pEC <sub>50</sub>	Reference
hINSL5	AG	7.33	8.51		Shabanpoor et al. (2012)
hINSL5	AG	7.59	8.62		Belgi et al. (2013)
hINSL5	AG	8.66		8.94	Zhu et al. (2008)
hINSL5	AG	8.8	8.92	8.89	Liu et al. (2005b)
hINSL5 amide	AG	6.94	7.48		Belgi et al. (2011)
mINSL5	AG	8.47	9.29		Belgi et al. (2013)
mINSL5 amide	AG	7.07	6.85		Belgi et al. (2011)
Relaxin-3	AG	8.81		9.01	Zhu et al. (2008)
Relaxin-3	AG	8.83	9.02		Kuei et al. (2007)
Relaxin-3 B-chain	AG	7.07	7.06	~7.0	Liu et al. (2003b)
Relaxin-3 B-chain	AG		6.9		Liu et al. (2005a)
R3/I5	AG	8.92	9.04		Liu et al. (2005a)
R3/I5	AG	8.88	8.94		Kuei et al. (2007)
Minimized hINSL5 analog 7	PA	6.28	7.40		Belgi et al. (2013)
Relaxin-3	AG		8.94		Shabanpoor et al. (2012)
Minimized relaxin-3 analog 2	AG	7.1	7.7		Shabanpoor et al. (2012)
Minimized relaxin-3 analog 3	ANT	6.6	N.A.		Shabanpoor et al. (2012)
R3(B $\Delta$ 23–27)R/I5	ANT	~8	N.D.		Haugaard-Kedstrom et al. (2011)
R3(B $\Delta$ 23–27)R/I5	ANT	8.64	N.A.		Kuei et al. (2007)
R3 B1–22R		N.A.	N.D.		Haugaard-Kedstrom et al. (2011)

AG, agonist; ANT, antagonist; N.A., no activity; PA, partial agonist.



**Fig. 4.** Signal transduction pathways activated by RXFP1. In the resting state at least some RXFP1 receptors are bound to AKAP79 and adenylyl cyclase in the signalosome complex. Low, subpicomolar concentrations of relaxin activate the signalosome to produce tightly controlled cAMP signals. When the relaxin concentration rises to nanomolar the signalosome dissociates and RXFP1 activates canonical pathways to increase cAMP, ERK1/2, and NO and to activate NFκB. In pathologic conditions, the increased expression of the AT<sub>2</sub>R promotes formation of RXFP1/AT<sub>2</sub>R heterodimers that are essential for the antifibrotic actions of relaxin. The allosteric agonist ML290 interacts with RXFP1 to produce a different signaling pattern to relaxin.

1980), uterine longitudinal muscle (Osa et al., 1991) from estrogen-primed rats, and in cultures of human endometrial cells (Fei et al., 1990), human endometrial glandular epithelial cells (Chen et al., 1988), newborn rhesus monkey uterine cells (Kramer et al., 1990), rat myometrial cells (Hsu et al., 1985), and rat anterior pituitary cells (Cronin et al., 1987). The importance of cAMP as a signaling pathway for relaxin was confirmed on RXFP1 deorphanization, because constitutively active mutants of RXFP1 (TM6: D637Y) increased cAMP accumulation in a ligand-independent manner (Hsu et al., 2000, 2002). RXFP1 couples to G<sub>α<sub>s</sub></sub> to increase cAMP (Hsu et al., 2000, 2002; Halls et al., 2006), an effect that is negatively modulated by coupling to G<sub>α<sub>oB</sub></sub> (Halls et al., 2006) (Fig. 4). RXFP1 also couples to G<sub>α<sub>13</sub></sub> to activate a delayed surge of cAMP accumulation via a Gβγ-PI3K-PKCζ pathway that activates

AC5 (Nguyen et al., 2003; Nguyen and Dessauer, 2005b; Halls et al., 2006, 2009b). The delayed pathway was initially identified in THP-1 cells that endogenously express RXFP1 where relaxin causes a biphasic increase in cAMP accumulation, with the later phase partially blocked by the PI3K inhibitors LY294002 [2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride] and Wortmannin. This was confirmed when relaxin stimulation of RXFP1 was also shown to increase PI3K activity (Nguyen et al., 2003). PKCζ was proposed as the candidate protein that linked PI3K activation to cAMP formation and is an atypical isoform that is insensitive to diacylglycerol and calcium but activated by phosphatidylinositol 3,4,5-trisphosphate and other lipids (Ono et al., 1989; Nakanishi et al., 1993; Standaert et al., 2001). Treatment with porcine relaxin causes

a concentration-dependent translocation of PKC $\zeta$  to the cell membrane in MCF-7 (human breast cancer), PHM1-31 (pregnant human myometrial), MMC (mouse mesangial), and THP-1 cells (Nguyen and Dessauer, 2005b), with translocation dependent upon PI3K activation by relaxin but independent of cAMP accumulation. cAMP increases mediated by relaxin were also dependent upon PKC $\zeta$  expression (Nguyen and Dessauer, 2005b). Although in MCF-7 cells, relaxin stimulation activates PI3K and causes translocation of PKC $\zeta$  without increasing cAMP accumulation, transfection of AC5, but not AC2 or AC4, into these cells produces a cAMP response to relaxin (Nguyen and Dessauer, 2005a). Thus RXFP1 activates PI3K and causes translocation of PKC $\zeta$  to the cell membrane, which in turn stimulates AC5 (Fig. 4).

This pathway also occurs in HEK293 cells transiently or stably expressing RXFP1 (Halls et al., 2006, 2009b) and is downstream of G $\alpha$  and G $\beta\gamma$  subunits, because transfection of cells with G $\alpha_{i/o}$  mutants that are insensitive to ADP ribosylation by pertussis toxin (PTX) show coupling of RXFP1 to G $\alpha_{i3}$  (in addition to G $\alpha_s$  and inhibitory G $\alpha_{oB}$ ) as the mediator of the G $\beta\gamma$ -PI3K-PKC $\zeta$ -AC5 pathway. This also occurs in endogenous settings: in rat left atria, relaxin increases cAMP accumulation that is reduced by PTX pretreatment, which also reduced the inotropic and chronotropic responses to relaxin (Kompa et al., 2002); in the failing human heart there is increased G $\alpha_{i/o}$  expression (Eschenhagen et al., 1992; Bohm et al., 1994), and the positive inotropic effects of relaxin in humans are preserved in this condition (Dschietzig et al., 2011).

The final 10 amino acids of the RXFP1 C terminus, particularly R752, are essential for activation of the G $\alpha_{i3}$  pathway (Halls et al., 2009b) (Fig. 1), as is the presence of lipid-rich membrane domains, suggesting compartmentalization of the RXFP1-stimulated cAMP response (Halls et al., 2009b). GTP $\gamma$ S-immunoprecipitation studies show that G $\alpha_{i3}$  is activated immediately after RXFP1 stimulation, suggesting that the delay observed is downstream of the G protein and probably involves the translocation of PKC $\zeta$  (Halls et al., 2009b). In HEK293 cells, only activation of G $\alpha_s$ - and G $\alpha_{oB}$ -dependent cAMP signaling pathways increases CRE-mediated gene transcription, whereas G $\alpha_{i3}$ -mediated signaling appears to selectively regulate nuclear factor- $\kappa$ B (NF $\kappa$ B)-dependent gene transcription (Halls et al., 2007a), again suggesting that there is compartmentalization of signaling events and inferring that distinct physiologic outcomes can be anticipated downstream of different cAMP signaling branches (Halls et al., 2007a). cAMP accumulation may also occur in response to relaxin by a G protein-independent mechanism, and in some cells may be downstream of a tyrosine kinase. In THP-1 cells and cultures of primary human myometrial or endometrial stromal cells, porcine relaxin increased cAMP accumulation that was blocked by inhibition of tyrosine kinase activity (Kuznetsova et al., 1999; Bartsch et al., 2001; Anand-Ivell et al., 2007; Heng

et al., 2008). This response could be potentiated by the phosphotyrosine phosphatase inhibitors [bpV(phen) and mpV(pic)] that mimic tyrosine kinase activation (Bartsch et al., 2001). In human lower uterine segment fibroblasts, relaxin stimulation caused tyrosine phosphorylation of cellular extracts, with no effect upon cAMP accumulation (Palejwala et al., 1998). The tyrosine kinase-dependent increase in cAMP accumulation may occur by inhibition of a PDE, thereby preventing cAMP hydrolysis and thus reflexively increasing cAMP levels. However, the same tyrosine kinase inhibitors do not affect relaxin-stimulated cAMP accumulation in HEK293 cells expressing RXFP1 (Anand-Ivell et al., 2007), emphasizing the variation in cellular responses between different cell types. In cells using the tyrosine kinase pathway, there was some degree of cAMP inhibition by the PI3K inhibitor LY294002 (Anand-Ivell et al., 2007; Heng et al., 2008) and evidence of a negative feedback loop involving PKA (Anand-Ivell et al., 2007).

The stimulation of NO production, cGMP generation, and PKG activation provides another crucial pathway by which relaxin exerts its effects. Depending on the cell type under investigation, relaxin may activate endothelial nitric oxide synthase (eNOS) (Baccari et al., 2007; Dschietzig et al., 2012) and neuronal NOS (nNOS) (Baccari et al., 2004; Mookerjee et al., 2009) or stimulate the expression of inducible NOS (iNOS) (Bani et al., 1998a; Alexiou et al., 2013) (Fig. 4). In rat isolated lungs, the relaxin-mediated iNOS upregulation depends on a subtle balance between stimulatory ERK1/2 activation and counterregulatory PI3K stimulation (Alexiou et al., 2013).

In addition to cAMP accumulation, many cells that express RXFP1 such as human endometrial stromal cells (Zhang et al., 2002), THP-1 cells and primary cultures of human coronary artery cells, pulmonary artery smooth muscle cells, renal myofibroblasts (Mookerjee et al., 2009), and fibrochondrocytes (Ahmad et al., 2012) respond to relaxin with a rapid activation of ERK1/2 (Fig. 4). In normal human endometrial cells relaxin causes rapid but transient phosphorylation of ERK1/2, with a peak response between 5 and 10 minutes (Zhang et al., 2002). The same time course was observed for phosphorylation of MEK and CREB, but relaxin treatment did not affect Akt or JNK phosphorylation and treatment with a MEK inhibitor blocked phosphorylation of ERK1/2 in response to relaxin, suggesting that MEK is activated upstream of ERK1/2 (Zhang et al., 2002). Increased phosphorylation of ERK1/2 (pERK1/2) in response to relaxin is also observed in THP-1 cells and cultures of human coronary artery and pulmonary artery smooth muscle cells and is associated with increased transcription of VEGF (Zhang et al., 2002).

In HeLa cells and primary human umbilical vein endothelial cells, the pERK1/2 response is more prolonged (Dschietzig et al., 2003). In HeLa, EAhy926 (an endothelial cell line), HT-29 (a colonic cell line), and in primary

fibrochondrocyte cells, relaxin increased the phosphorylation of both ERK1/2 and Akt after 30 minutes (Dschietzig et al., 2009a; Ahmad et al., 2012). In primary fibrochondrocytes, treatment with relaxin also activates PI3K, PKC $\zeta$ , NF $\kappa$ B, c-fos, and Elk-1, all of which influence the expression of MMP-9 (Ahmad et al., 2012). Relaxin also produces a sustained increase in pERK1/2 in rat renal myofibroblasts, potentiated by inhibition of G $\alpha_{i/o}$  by PTX, suggesting that phosphorylation of ERK1/2 may be downstream of G protein coupling (Mookerjee et al., 2009). In contrast, in human vascular smooth muscle cells, relaxin stimulation did not affect pERK1/2 levels but instead increased the phosphorylation of p38 mitogen-activated protein kinase (MAPK) (Dschietzig et al., 2003). In rat pulmonary arterial endothelial and smooth muscle cells, relaxin increased iNOS activity and expression dependent on a balance of pERK1/2 and PI3K pathway activation (Alexiou et al., 2013). Thus, although relaxin increases phosphorylation of a number of kinases in multiple cell types, the precise isoform and mode of activation appears to vary, and the physiologic consequences of activation of these pathways are as yet unclear.

Several of the aforementioned canonical pathways are involved in the well established antifibrotic actions of relaxin. Transforming growth factor (TGF)- $\beta_1$  is profibrotic, causing Smad2/3 phosphorylation, translocation of the Smad complex to the nucleus, and activation of profibrotic genes. Inhibition of this process by relaxin involves activation of PI3K and NOS-NO-cGMP signaling (Ahmad et al., 2012; Chow et al., 2012). There is also recent evidence that suggests that relaxin influences differentiation of neonatal fibroblasts into myofibroblasts by preventing the inhibition of Notch-1 signaling by TGF- $\beta_1$ . Notch-1 signaling controls cell differentiation and fate and is involved in fibrosis (Fan et al., 2011). The Notch-1 pathway comprises Notch-1 receptors that, when activated by the TM ligands Jagged1/2, release the Notch intracellular domain that migrates to the nucleus to inhibit transcription (Chillakuri et al., 2012). Downregulation of Notch-1 is a necessary step in the differentiation of rat cardiac fibroblasts into myofibroblasts and is induced by TGF- $\beta_1$  (Fan et al., 2011). A recent study demonstrates that relaxin prevents the enhancement by TGF- $\beta_1$  of cytoskeletal assembly in 3T3 cells and primary neonatal fibroblasts as measured by F-actin and vinculin staining (Sassoli et al., 2013). In addition, relaxin prevented the TGF- $\beta_1$ -mediated increases in  $\alpha$ -SMA, type 1 collagen, and tissue inhibitor of metalloproteinase (TIMP)-2, while preventing the TGF- $\beta_1$ -mediated decreases in MMP-2 and MMP-9. Relaxin treatment also prevented the downregulation of Notch-1 expression by TGF- $\beta_1$  as well as the inhibition of Notch intracellular domain and Jagged-1 release (Sassoli et al., 2013).

**2. Noncanonical Signaling Pathways.** Relaxin activates the GR, a nuclear receptor that acts as a ligand-dependent transcription factor (Dschietzig et al., 2004).

The activation, and the subsequent changes in gene transcription, may account for the many effects of relaxin upon the expression levels of a variety of proteins, including those involved in connective tissue metabolism (see above). Relaxin treatment of THP-1 cells that have differentiated into a macrophage phenotype blunts the stimulated production of cytokines including interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)- $\alpha$ , and this effect is abolished by the GR antagonists RU-486 [mifepristone; 17 $\beta$ -hydroxy-11 $\beta$ -(4-dimethylamino-phenyl)-17 $\alpha$ -(1-propinyl)estra-4,9-dien-3-on] and D06 [bis(4-*N,N*-dimethylaminophenyl)(2-chloro-5-nitrophenyl) methane] (Dschietzig et al., 2004, 2009a). Relaxin coimmunoprecipitates with the GR, and the amount of the GR within the nucleus increases after 30-minute stimulation with relaxin. In whole cell and in fluorescence polarization assays, relaxin displaces classic glucocorticoids from the GR (Dschietzig et al., 2004, 2009a). The region of relaxin that binds to the GR differs from that involved in relaxin binding to RXFP1, because a modified relaxin that was unable to activate RXFP1 could still interact with the GR, and relaxin was found to cause phosphorylation of S211 of the GR, which is used as a biomarker of agonist-related receptor activation (Dschietzig et al., 2009a). Indeed relaxin, via its interaction with the GR, is also able to autoregulate its own expression by binding to half-sites of glucocorticoid response elements located 160–200 bp upstream of transcription start at the human RLN2 promoter (Dschietzig et al., 2009b). Actions at the GR are also involved in the vasodilator effects of relaxin. In rat aortic rings, relaxation responses to acetylcholine are impaired by TNF- $\alpha$  in a model of endothelial dysfunction, and the effect is reversed by relaxin (Dschietzig et al., 2012). The reversal of the effect of TNF- $\alpha$  by relaxin is blocked by the PI3K inhibitor Wortmannin but also by the GR and progesterone receptor antagonist RU-486. In rat primary aortic endothelial cells TNF- $\alpha$  treatment increased endothelin-1 (ET-1) and arginase II expression, decreased superoxide dismutase (SOD)-1 expression, and stimulated superoxide and nitrotyrosine formation (Dschietzig et al., 2012). All of these effects were restored or attenuated by relaxin treatment acting at the GR (Dschietzig et al., 2012). The findings suggest that relaxin has a protective effect on endothelial dysfunction mediated not only by actions on the canonical PI3K-Akt-eNOS pathway but also by its actions at the GR, with SOD-1 upregulation being dependent on relaxin-GR-c/EBP- $\beta$  signaling (Dschietzig et al., 2012). Reports that appear to confirm the principal relaxin-GR finding include relaxin-related protection in a rodent model of severe acute pancreatitis that is markedly attenuated after cotreatment with RU-486 (Cosen-Binker et al., 2006), relaxin-induced GR activation in reporter gene experiments (Halls et al., 2007a), and blockade of relaxin-mediated decreases in granulocyte macrophage colony-stimulating factor and IL-8 secretion from primary decidual macrophages by RU-486 (Horton et al., 2011).

Hitherto, the precise mode of interaction between relaxin and GR has been unclear and there are contradictory findings regarding RXFP1 involvement in the relaxin-GR pathway. Although relaxin seems to interact with GR in spleen cells that do not express RXFP1 (Dschietzig et al., 2004) and also after chemical modification to destroy RXFP1 affinity (Dschietzig et al., 2009a), RXFP1 was necessary for relaxin to activate the GR in an in vitro study using reporter genes (Halls et al., 2007a).

**3. The Relaxin Family Peptide Receptor 1 Signalosome—A Protein Complex That Facilitates High-Sensitivity Signaling in Response to Relaxin.** Responses to relaxin produced by activation of canonical signaling pathways occur in the nanomolar concentration range (Bathgate et al., 2013a). However, circulating concentrations of relaxin are usually lower than those required to produce cellular responses by canonical mechanisms (Sherwood, 2004). Recently, a constitutive RXFP1-dependent cAMP response has been identified using Förster resonance energy transfer-based cAMP biosensors in single rat cardiac fibroblasts, HeLa cells, and HEK293 cells expressing RXFP1 (Halls and Cooper, 2010). The response is dependent upon a protein complex, or signalosome, linked to the relaxin receptor, and the signalosome is highly sensitive to attomolar concentrations of relaxin. Importantly, this may provide the basis for some of the physiologic responses to relaxin when it is present in the circulation at levels below those necessary to activate canonical signaling pathways. The signalosome consists of RXFP1 that is scaffolded to AC2 by AKAP79, facilitating efficient activation of the AC by  $G\alpha_s$  and  $G\beta\gamma$  subunits (Fig. 4). The levels of cAMP produced are tightly regulated by the activity of PKA-activated PDE4D3 that itself is scaffolded to the receptor C terminus (specifically requiring S704) by  $\beta$ -arrestin-2 (Halls and Cooper, 2010). The signalosome possesses stimulatory (AKAP79 and AC2) and regulatory ( $\beta$ -arrestin-2, PKA, and PDE4D3) arms that are both spatially and functionally distinct. Knockdown of AKAP79 has no effect on the regulatory arm, and knockdown of  $\beta$ -arrestin-2 does not influence the stimulatory arm of the signalosome. In addition, there is no effect of inhibitors of classic pathway specific proteins (including  $G\alpha_{i/o}$ , PI3K, and PKC) on cAMP generated in response to subpicomolar concentrations of relaxin and no effect of inhibition of signalosome-specific proteins (including AC2, AKAP79, and  $\beta$ -arrestin-2) upon classic relaxin cAMP signaling. The pathways also generate cAMP in quite distinct regions of the cell. Thus signalosome-specific AC2 is known to be preferentially excluded from lipid-rich domains (Willoughby et al., 2007), whereas activation of the  $G\alpha_{i3}$  pathway with nanomolar relaxin concentrations depends upon lipid-rich domains in HEK293 cells (Halls et al., 2009b). Because AC2 expression occurs predominantly in brain, lung, skeletal muscle, heart, and uterine myometrium (Defer et al., 2000; Willoughby et al., 2007; Sadana and Dessauer, 2009), it is likely that some tissues display

RXFP1 signalosome signaling, whereas others do not, which may help to determine the physiologic role of signalosome-localized RXFP1.

Targeted protein knockdown or overexpression of dominant negative mutants (Halls and Cooper, 2010) suggests that the regulatory arm is isoform-specific (i.e., interacts only with PDE4D3) and that assembly of the components depends upon constitutive association between the receptor and  $\beta$ -arrestin-2 (not  $\beta$ -arrestin-1) (Halls and Cooper, 2010). The regulatory arm also has some unusual features that call into question some of the current paradigms surrounding  $\beta$ -arrestins and receptor desensitization and internalization (DeFea, 2011). For example, the  $\beta_2$ -adrenoceptor is phosphorylated by G protein receptor kinase (GRK)-2 after receptor activation, and subsequently becomes a substrate for  $\beta$ -arrestin-2 that triggers formation of clathrin-coated vesicles and internalization (for review, see Luttrell and Gesty-Palmer, 2010). However, and in contrast, the interaction between RXFP1 and  $\beta$ -arrestin-2 is constitutive and does not involve receptor activation or phosphorylation and does not appear to be involved in desensitization (Halls and Cooper, 2010). Although the interaction involves S704 of RXFP1 (Halls and Cooper, 2010), there is no significant increase in RXFP1 phosphorylation after stimulation with high concentrations of relaxin nor any effect of receptor activation on desensitization or internalization (Tan et al., 1998; Callander et al., 2009). Thus  $\beta$ -arrestin-2 appears to act solely as a scaffold for the formation of the regulatory complex and has no role in receptor desensitization or internalization. On the other hand, the stimulatory arm of the signalosome depends upon a specific and constitutive association between helix 8 of the receptor and AKAP79 (Fig. 4). However, AKAP79 is also known to scaffold many other proteins including other GPCRs, PKC, several ion channels, and a number of AC isoforms (see Baillie et al., 2005; Dessauer, 2009; Skroblin et al., 2010; Halls, 2012). The activity of AC isoforms may be variably influenced by interaction with AKAP79 and AC2 activity is inhibited (Efendiev et al., 2010), although this appears to be primarily offset in RXFP1 signalosomes by the scaffolding of RXFP1 and AC2. Signalosome signaling occurs quite separately from canonical signaling, and the signalosome complex dissociates when exposed to nanomolar concentrations of relaxin (Halls and Cooper, 2010).

High-sensitivity signaling such as that exhibited by the RXFP1 signalosome has been previously shown for suppression of proinflammatory cytokine production by IL-15 (Alleva et al., 1997), proliferation of helper T cells by IL-1 (Orencole and Dinarello, 1989), the effects of neuropeptides and neurosteroids in nociception (Sanchez-Blazquez and Garzon, 1995; Ueda et al., 2001), and the long-term effects of TGF- $\beta$  on basal follicle-stimulating hormone levels (Ying et al., 1986). Thus the RXFP1-signalosome represents a highly sensitive signaling platform that produces tightly regulated cAMP responses over a wide range of subpicomolar relaxin concentrations.

Although this represents a mechanism whereby cells and tissues could respond to low circulating levels of relaxin, a precise physiologic role for the mechanism has yet to be determined.

**4. Homo- and Hetero-Oligomerization of RXFP1 and Its Functional Consequences.** Like many other class A GPCRs, there is evidence from bioluminescence resonance energy transfer (BRET) studies that RXFP1 receptors form homo- and heterodimers (Kern et al., 2008; Svendsen et al., 2008a,b) in the absence of, and independent of, ligand occupation of the receptor (Svendsen et al., 2008a,b). It has been suggested that dimer formation is necessary for signal transduction, with ligand binding occurring at the LRR region of one dimer partner, followed by interaction of the bound ligand with the ECL2 of the second partner and initiation of signaling (Kong et al., 2010). However, at present there are no experiments with two inactive mutant receptor dimer partners that show complementation and rescue of function by dimerization to support this concept. One functional consequence of dimerization is negative cooperativity (Svendsen et al., 2008a,b), where the two binding sites do not have a fixed affinity and the affinity of each remaining unoccupied receptor binding site decreases as occupancy increases. The evidence for negative cooperativity at RXFP1 comprises receptor kinetic studies where the rate of dissociation after incubation of cells expressing RXFP1 with [<sup>125</sup>I]human relaxin was determined after equilibration followed by infinite dilution in the absence or presence of unlabeled ligand. The presence of unlabeled ligand was associated with modest increases in the rate of dissociation, suggesting negative cooperativity. It should be borne in mind, however, that such behavior does not necessarily reflect the formation of dimers (Chabre et al., 2009), and studies are required using receptors that retain function but are unable to form dimers or on receptors expressed in model phospholipid bilayers that allow examination of their functional characteristics when in monomeric form (Whorton et al., 2007; Velez-Ruiz and Sunahara, 2011). Nevertheless, there are two intriguing, functionally relevant consequences of negative cooperativity: an increased functional range of the ligand over a wider concentration range and a decrease in ligand residence time at the receptor as the free ligand concentration increases, potentially allowing selective activation of different signaling pathways (Shymko et al., 1997). The negative cooperativity concentration-response curve for relaxin binding to RXFP1 is linear, although the absence of a protein structure precludes conclusions regarding the functional consequences of this observation (Svendsen et al., 2008b).

Receptor oligomerization has been suggested to explain one intriguing aspect of relaxin-RXFP1 pharmacology, the phenomenon of bell-shaped concentration-response curves that are observed in many bioassays from studies in recombinant and primary cell systems (Halls et al., 2006; Sarwar et al., 2015), animal studies (Danielson and Conrad, 2003; Debrah et al., 2005), and clinical trials

(Teerlink et al., 2009). Negative cooperativity could be a potential explanation, but currently available information does not appear to support this mechanism. Although both RXFP1 and RXFP2 display negative cooperativity, bell-shaped curves for cAMP accumulation are observed only in response to 3- or 30-minute activation of RXFP1 or 30-minute activation of RXFP2, but not 3-minute activation of RXFP2 (Halls et al., 2006). It could be argued that the RXFP2 system at 3 minutes is not in equilibrium and that the effect of increasing receptor occupation balances out the reduced receptor affinity, but this does not convincingly explain the difference between RXFP1 and RXFP2 at the same time point. In studies of human vascular cells that endogenously express RXFP1, venous endothelial and smooth muscle cells displayed pronounced bell-shaped concentration-response curves, yet arterial smooth muscle cells showed conventional sigmoidal concentration-response curves (Sarwar et al., 2015). This would appear to be incompatible with an explanation based on RXFP1 homodimer formation and negative cooperativity.

There is also evidence for dimer formation between the haloreceptor and a number of splice variants (encoding the LDLa module only and up to eight LRR) and dimers are present at all stages of receptor translocation from the endoplasmic reticulum to the plasma membrane (Kern et al., 2008). This suggests an important role of dimerization over the lifetime of the receptor. Dimers were also formed between the haloreceptor and a TM-only domain receptor, although the BRET<sub>2</sub> ratio was decreased compared with that obtained for haloreceptor dimers (Svendsen et al., 2008a,b). This suggests that although the TM domain is sufficient for dimerization, the ectodomains play an important role in stabilizing the oligomer. Examination of three of the splice variants cloned from human fetal membranes showed that although their expression in HEK293 cells produced no response to relaxin (Kern et al., 2008), coexpression with RXFP1 produced a parallel shift to the right of the cAMP concentration-response curve. Because the splice variants also markedly reduced cell surface expression of RXFP1, the reduced responses are likely due to a dominant negative effect of the splice variants (Kern et al., 2008).

Recent studies also suggest that there is heterodimer formation between RXFP1 and other GPCRs. The antifibrotic actions of relaxin are believed to be mediated through a RXFP1-pERK1/2-nNOS-NO-cGMP dependent pathway, leading to regulation of collagen-degrading MMPs (Chow et al., 2012). The effect of relaxin is completely absent in angiotensin type 2 receptor (AT<sub>2</sub>R)<sup>-/-</sup> mice or in mice treated with the AT<sub>2</sub>R antagonist PD123319 [S-(+)-1-[(4-(dimethylamino)-3-methylphenyl)methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridine-6-carboxylic acid di(trifluoroacetate)], suggesting that the AT<sub>2</sub>R is necessary for the antifibrotic actions of relaxin. Interestingly BRET studies show that



RXFP1 and AT<sub>2</sub>R form constitutive heterodimers, indicating that the receptor complex is responsible for the novel pharmacology observed (Fig. 4). There is no direct binding of relaxin to the AT<sub>2</sub>R, and the peptide does not affect the BRET signal from RXFP1-AT<sub>2</sub>R complexes (Chow et al., 2014). Importantly, the findings also explain why the antifibrotic actions of relaxin are only observed in pathologic states: under normal physiologic conditions AT<sub>2</sub>R are expressed at low levels but are dramatically increased with injury and disease (Siragy and Carey, 1997; Matsubara, 1998; Carey, 2005; Savoia et al., 2006; Jones et al., 2008).

It is interesting to note that the expression of relaxin-RXFP1 as measured by immunohistochemistry and expression of the renin-angiotensin-aldosterone system may both be increased during pregnancy (Ferreira et al., 2009; Cheung and Lafayette, 2013), suggesting another state where RXFP1-AT<sub>2</sub>R heterodimers may have a functional role. However, a study in rat primary mesangial cells did not corroborate this suggestion (Carvalho et al., 2012). Although RXFP1 and AT<sub>2</sub>R were increased in cells obtained from pregnant rats compared with virgin animals and the hyporesponsiveness of cells from pregnant animals to angiotensin II could be attributed to increased endogenous relaxin production, the AT<sub>2</sub>R antagonist PD123319 had no effect. Thus, the issue of RXFP1-AT<sub>2</sub>R interaction during pregnancy remains a hypothesis.

**5. Allosteric Agonist Signaling at Relaxin Family Peptide Receptor 1.** The small molecular weight ligand ML290 (see section II.A.3) is an allosteric agonist at RXFP1 (Xiao et al., 2013) that unlike relaxin does not require the LDLa module for signaling (Xiao et al., 2013). ML290 interacts with ECL3 of RXFP1, specifically requiring G659 and T660 within ECL3 for activity (Xiao et al., 2013) (Fig. 1). ML290 does not interact with the classic binding sites on RXFP1 used by relaxin and does not compete for <sup>125</sup>I relaxin binding (Table 1).

## B. Relaxin Family Peptide Receptor 2 Signaling

**1. Canonical Signaling Pathways.** INSL3 is the cognate ligand at RXFP2, although human relaxin but not rat or mouse relaxin, also activates the receptor. Despite the similar structure of relaxin and INSL3 and their receptors RXFP1 and RXFP2, the mode of interaction of these ligands with RXFP2 is different (see section I.B.1). Signaling pathways that are initiated after stimulation of RXFP2 are simpler than those observed for RXFP1. INSL3 (or relaxin) stimulation of HEK293-RXFP2 cells causes coupling to G $\alpha_s$  to increase cAMP accumulation and to G $\alpha_{oB}$  to negatively modulate this effect (Kumagai et al., 2002; Halls et al., 2006). This stage of signaling therefore closely resembles the first stage of RXFP1 signaling in response to relaxin (Halls et al., 2006). The contribution of G $\alpha_{oB}$  to the response is seen by the increase in cAMP after removal of G $\beta\gamma$  using  $\beta$ ARK-ct or inhibition of G $\alpha_{i/o}$  with PTX (Halls et al., 2006).

Interestingly, for RXFP2 there is no evidence for constitutive activity or a high-sensitivity response to INSL3 as seen with the RXFP1 signalosome (Halls and Cooper, 2010); neither is there activation of the G $\alpha_{i3}$ -cAMP signaling pathway that is unique to RXFP1 (Halls et al., 2006, 2009b). Activation of RXFP2 (by either relaxin or INSL3) also induces increased CRE-dependent gene transcription (Halls et al., 2007a) as observed with relaxin acting at RXFP1.

G $\alpha_s$  and G $\alpha_{oB}$  are also involved in cAMP accumulation in cells that endogenously express RXFP2. In rat gubernacular cells (Kumagai et al., 2002), in a human osteoblast cell line (MG-63) (Ferlin et al., 2008), and in mouse primary Leydig cells (Pathirana et al., 2012), INSL3 stimulation of RXFP2 leads to increased cAMP accumulation, probably involving G $\alpha_s$ . However, and in contrast, primary cultures of testicular germ cells and oocytes respond to INSL3 activation of RXFP2 with a PTX-sensitive *inhibition* of cAMP accumulation (Kawamura et al., 2004), consistent with RXFP2 coupling to G $\alpha_{oB}$ . Thus, as for RXFP1, the net signaling outcome of RXFP2 stimulation will depend upon the signaling components (especially G protein isoforms) that are expressed in a particular cell type.

In response to relaxin, RXFP1 activates a number of signaling pathways including cAMP, cGMP, and pERK1/2. Relaxin also activates the GR to alter gene expression. RXFP1 forms protein complexes or signalosomes that facilitate high-sensitivity responses to relaxin. In contrast, INSL3 activates RXFP2 to increase or decrease cAMP in a cell-dependent manner. INSL3 does not activate the GR and RXFP2 does not form signalosomes.

## C. Relaxin Family Peptide Receptor 3 Signaling

**1. Canonical Signaling Pathways.** Activation of RXFP3 by human relaxin-3 causes PTX-sensitive inhibition of forskolin-stimulated cAMP accumulation, suggesting that the receptor is coupled to inhibitory G $\alpha_{i/o}$  proteins (Liu et al., 2003b). Activation of G $\alpha_{i/o}$  proteins by RXFP3 is also associated with PI3K- and PKC-dependent phosphorylation of ERK1/2 and other MAPKs (van der Westhuizen et al., 2007, 2010) (Fig. 5). Both human relaxin-3 or its B-chain compete for [<sup>125</sup>I]human relaxin-3 binding and inhibit AC activity (Liu et al., 2003b), but more recent studies show that human relaxin also interacts with human RXFP3 to activate a unique signaling pattern (see below) (van der Westhuizen et al., 2010).

Forskolin-stimulated cAMP accumulation in CHO-K1 or HEK293 cells stably expressing RXFP3 or in mouse SN56 cells that endogenously express the receptor (Liu et al., 2003b; van der Westhuizen et al., 2007) is inhibited after stimulation with human relaxin-3, and this response is completely prevented by pretreatment with PTX. In CHO-K1 cells transiently transfected with PTX insensitive (C351I mutation) variants of G $\alpha_{i/o}$  proteins and treated with PTX (to remove the influence of endogenous G $\alpha_{i/o}$  proteins), G $\alpha_{i2}$  was the major G protein

involved in the inhibition of cAMP accumulation, whereas in HEK293 cells,  $G_{\alpha_{i3}}$ ,  $G_{\alpha_{oB}}$ , and  $G_{\alpha_{oA}}$  were all involved (van der Westhuizen, 2008). Thus, the G proteins involved, although broadly similar, can vary with the different cell types. Similar effects are observed with human relaxin-3 B-chain peptides or with human relaxin and porcine relaxin in the CHO-K1, HEK293, and SN56 cell backgrounds (Liu et al., 2003b; van der Westhuizen et al., 2007, 2010).

Human relaxin-3 causes a rapid and transient increase in ERK1/2 phosphorylation (peak response 2–5 minutes) in CHO-K1 and HEK293 cells stably expressing human RXFP3 (CHO-RXFP3 or HEK-RXFP3, respectively; van der Westhuizen et al., 2007, 2010) (Fig. 5). The human relaxin-3 B-chain dimer also activates ERK1/2, albeit with low potency and efficacy (van der Westhuizen et al., 2007), and there is also a weak response to human relaxin but not to porcine relaxin or INSL3 (van der Westhuizen et al., 2010) (Fig. 5). PTX pretreatment caused an approximately 90% inhibition of human relaxin-3 stimulated ERK1/2 phosphorylation in CHO-RXFP3 cells, whereas the signal was completely abolished in HEK-RXFP3 or SN56 cells (van der Westhuizen et al., 2007). Thus coupling of RXFP3 to PTX-sensitive  $G_{\alpha_{i/o}}$  proteins mediates ERK1/2 phosphorylation in both recombinant and endogenous systems (van der Westhuizen et al., 2007). There are two pathways downstream of  $G_{\alpha_{i/o}}$  involved in RXFP3-mediated ERK1/2 phosphorylation in CHO-K1, HEK293, and SN56 cells. About 50% of the MAPK response is blocked by the PI3K inhibitors LY294002 or Wortmannin, whereas the remainder is inhibited by general and isoform-selective PKC inhibitors (van der Westhuizen et al., 2007). The ERK1/2 response appears to be involved in central feeding responses in rats (Morikawa et al., 2004; Shen et al., 2004; Sasaguri et al., 2005), suggesting that it is physiologically relevant.

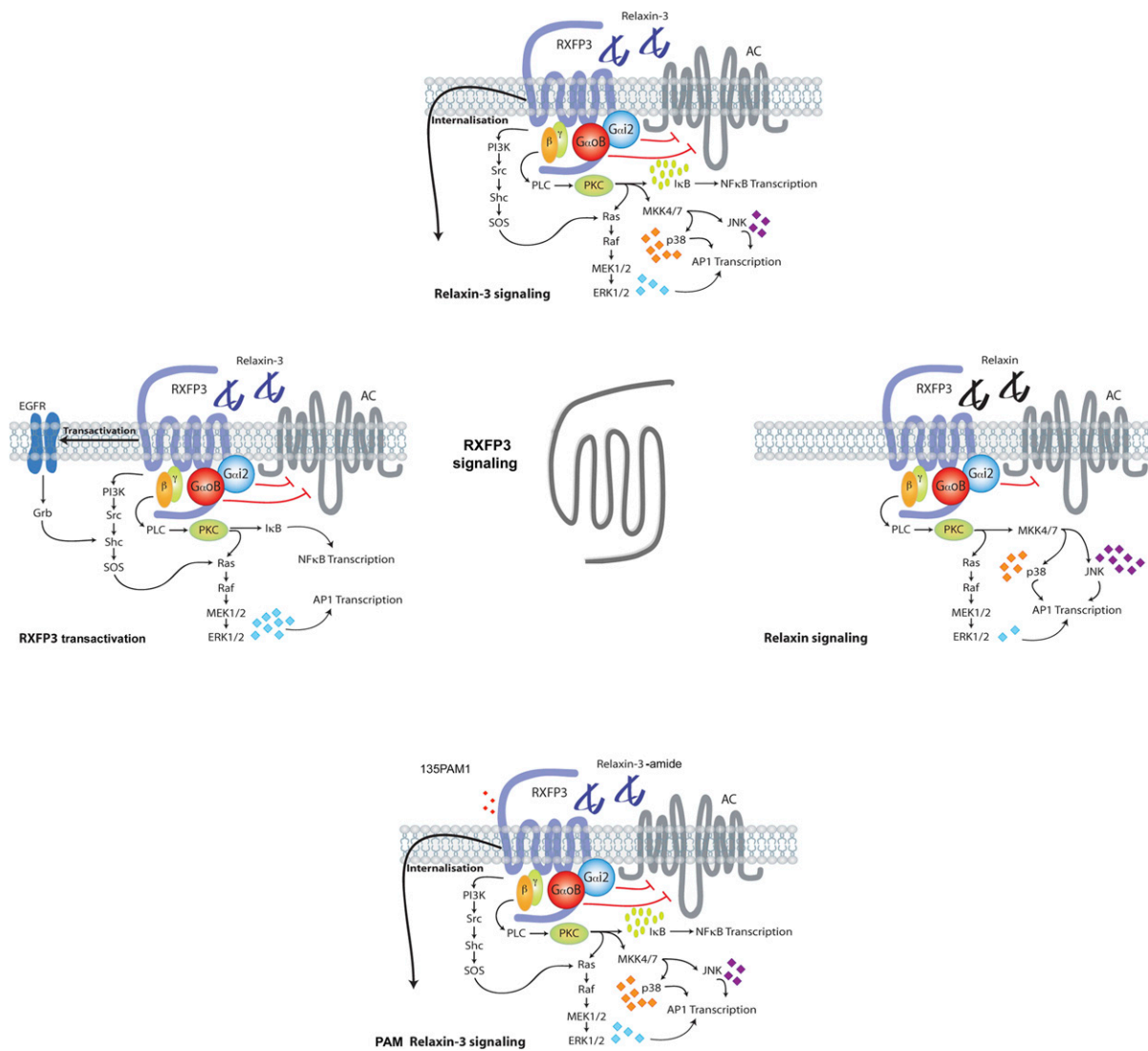
Signaling pathway analysis using reporter genes gives a broader view of the signal transduction mechanisms activated by RXFP3. Many MAPK signaling pathways [p38 MAPK (Roux and Blenis, 2004); JNK (Davis, 2000); and ERK1/2 (Price et al., 1996; Whitmarsh and Davis, 1996)] converge on AP-1 elements to increase gene transcription, therefore AP-1-linked reporter genes together with selective inhibitors provide useful information on signaling in different cellular backgrounds. Inhibition of  $G_{\alpha_{i/o}}$  proteins after pretreatment with PTX blocks RXFP3-mediated AP-1 reporter activation in SN56 cells but not in CHO-RXFP3 and HEK-RXFP3 cells (van der Westhuizen et al., 2010), suggesting that AP-1 reporter gene activation was downstream of  $G_{\alpha_{i/o}}$  in the mouse-derived cell line but not in the other two cell types (van der Westhuizen et al., 2010). Human relaxin-3-mediated AP-1 reporter gene activation in CHO-RXFP3 and SN56 cells is completely blocked by the p38 MAPK inhibitor (RWJ67657; 4-[4-(4-fluorophenyl)-1-(3-phenylpropyl)-5-(4-pyridinyl)-1H-imidazol-2-yl]-3-butyn-1-ol),

whereas MEK (PD98059; 2'-amino-3'-methoxyflavone) or JNK inhibition (SP600125; anthra[1,9-*cd*]pyrazol-6 (2*H*)-one) only partially blocks AP-1 activation (as shown by a decrease in relaxin-3 potency). In contrast in HEK-RXFP3 cells, JNK inhibition completely blocks human relaxin-3-stimulated AP-1 reporter activation, whereas p38 MAPK or MEK inhibition partially blocked AP-1 activation (van der Westhuizen et al., 2010). This suggests that although all three MAPKs are involved in human relaxin-3-mediated AP-1 activation, the hierarchy of the different signaling pathways varies with the cell background. Several studies show that MAPK signaling is activated in forced swim tests in rats, where there are dramatic increases in pMEK1/2, pERK1/2, and pJNK1/2/3 (Shen et al., 2004). Although this is associated with an increase in relaxin-3 mRNA in the nucleus incertus (NI) (Tanaka et al., 2005), direct links between human relaxin-3, RXFP3, MAPK phosphorylation, and stress responses remain to be demonstrated in brain.

In another reporter gene assay, in CHO-K1 and HEK293 cells transiently expressing human RXFP3 and in SN56 cells endogenously expressing mouse RXFP3, activation of RXFP3 by human relaxin-3 increased NF $\kappa$ B reporter gene activation (van der Westhuizen et al., 2010). Activation of NF $\kappa$ B was blocked by PTX pretreatment (van der Westhuizen et al., 2010), suggesting that the response occurs downstream of  $G_{\alpha_{i/o}}$ . The physiologic significance of this pathway remains to be determined.

*2. Ligand-Directed Signaling Bias at Relaxin Family Peptide Receptor 3.* Ligand-directed signaling bias (as described in section II.C.2) has been described for relaxin-3, relaxin (van der Westhuizen et al., 2005), and the RXFP3 antagonist R3(B $\Delta$ 23–27)R/I5 (Kocan et al., 2014). The first indication of a functional relaxin-RXFP3 interaction came from examination of metabolic responses recorded by microphysiometry (van der Westhuizen et al., 2005). In this study, human relaxin caused a small change in the extracellular acidification rate in CHO-RXFP3 cells. In CHO-K1, HEK293, and SN56 cell backgrounds, subsequent studies demonstrated that human relaxin, porcine relaxin, and human INSL3 caused weak inhibition of forskolin-stimulated cAMP accumulation compared with the strong inhibition mediated by human relaxin-3 (van der Westhuizen et al., 2010) (Table 3). Interestingly, the ability of INSL3 to activate RXFP3 appears to be specific for the human but not the mouse receptor (van der Westhuizen et al., 2010). Previous studies reported no inhibition of cAMP accumulation by either porcine relaxin or human INSL3 (Liu et al., 2003b), but because sensitivity of inhibitory cAMP assays is highly dependent on both the degree of activation of AC by forskolin and the time of stimulation, the differences observed most likely result from distinct experimental paradigms.

Similar to relaxin-3, relaxin and porcine relaxin also caused AP-1 reporter gene activation (van der Westhuizen et al., 2010) in CHO-K1, HEK293, and



**Fig. 5.** Signal transduction pathways activated by RXFP3. The cognate ligand relaxin-3 activates RXFP3 that couples to  $G\alpha_{i/o}$  proteins to inhibit adenylyl cyclase and activate ERK1/2 and p38MAPK phosphorylation to promote AP1 transcription. Stimulation of RXFP3 by relaxin activates only ERK1/2 and AP1 transcription. There is an allosteric site on RXFP3 that when occupied by a positive allosteric modulator 135PAM1 sensitizes responses to relaxin-3 amide. In tissues where they coexist, activation of RXFP3 may cause transactivation of the EGFR.

SN56 cell backgrounds, with an order of potency relaxin > relaxin-3 > porcine relaxin. Again, some AP-1 activation appears to be independent of  $G\alpha_{i/o}$  coupling, because pretreatment with PTX failed to block porcine relaxin-stimulated AP-1 reporter gene activation in CHO-RXFP3 cells. Thus, as for relaxin-3, porcine relaxin can activate AP-1 reporter genes by a  $G\alpha_{i/o}$ -independent mechanism, suggesting ligand-directed signaling bias (van der Westhuizen et al., 2010). In contrast, all of the AP-1 reporter gene responses observed after stimulation of RXFP3 in SN56 cells were blocked by PTX, suggesting that different pathways were involved in mediating the response downstream of the mouse RXFP3 receptor (van der Westhuizen et al., 2010).

In the CHO-K1 cell background, the relaxin-stimulated AP-1 response is strongly inhibited by the p38 MAPK inhibitor (RWJ67657) or the JNK inhibitor (SP600125), whereas the MEK inhibitor (PD98059) only weakly

inhibited the AP-1 response, implicating p38 MAPK and JNK as the major MAPKs involved in the response to relaxin. In contrast in the HEK293 cell background, relaxin-stimulated AP-1 reporter gene activation was decreased after p38 MAPK or MEK inhibition but not by JNK inhibition, suggesting that p38 MAPK and ERK were the major MAPKs involved in mediating this response. In SN56 cells, endogenously expressing mouse RXFP3, p38 MAPK, JNK, or MEK inhibition was equally effective in blocking the response to relaxin, suggesting that all three kinases were equally important (van der Westhuizen et al., 2010). Clearly species and cell background are important determinants of the signaling pattern after RXFP3 activation. Direct measurement of pERK1/2, p38 MAPK, and pJNK after addition of relaxin family peptides has confirmed the findings of these inhibitor-based studies (Kocan et al., 2014) and demonstrates signaling bias; however, the  $G\alpha_{i/o}$ -independent pathway remains to be

identified for human relaxin-3 and porcine relaxin. Direct MAPK assays in CHO-RXFP3 cells show that human relaxin activates p38 MAPK and ERK1/2 with lower efficacy than human relaxin-3 but the two peptides have similar efficacy for JNK1/2/3 phosphorylation. Both human relaxin and human relaxin-3 activation of p38 MAPK, JNK1/2/3, or ERK1/2 involved PTX-sensitive G proteins (van der Westhuizen et al., 2007, 2010; Kocan et al., 2014).

Evidence for signaling bias at RXFP3 also comes from studies utilizing an RXFP3 antagonist (Kocan et al., 2014). The RXFP3 antagonist R3(B $\Delta$ 23–27)R/I5 blocked human relaxin-3 AP-1 reporter gene activation but not human relaxin AP-1 activation or human relaxin-3 NF $\kappa$ B activation. The antagonist itself R3(B $\Delta$ 23–27)R/I5 activated the SRE reporter but did not inhibit either human relaxin or human relaxin-3 SRE activation. Finally, although the antagonist R3(B $\Delta$ 23–27)R/I5 blocked human relaxin-3-stimulated p38MAPK and ERK1/2 phosphorylation, it was also a weak partial agonist for p38MAPK and ERK1/2 signaling (Table 3). Interestingly, in findings similar to previous studies, p38MAPK activation by R3(B $\Delta$ 23–27)R/I5 was found to be G protein independent. Direct examination of interactions between RXFP3 and G proteins using BRET showed that human relaxin-3-activated RXFP3 interacts with G $\alpha_{i2}$ , G $\alpha_{i3}$ , G $\alpha_{oA}$ , and G $\alpha_{oB}$ , whereas human relaxin or R3(B $\Delta$ 23–27)R/I5 can only induce interactions with G $\alpha_{i2}$  or G $\alpha_{oB}$ . Interestingly, only human relaxin-3 promoted RXFP3/ $\beta$ -arrestin interactions that were blocked by R3(B $\Delta$ 23–27)R/I5 (Kocan et al., 2014). This is compelling evidence for ligand-directed signaling bias at RXFP3.

**3. Allosteric Modulation of Relaxin Family Peptide Receptor 3.** The RXFP3 allosteric modulator 135PAM1 has been examined only in receptor binding studies and in recombinant systems expressing the chimeric G protein G $\alpha_{q15}$  or a CRE reporter gene (Alvarez-Jaimes et al., 2012). It is not known what pattern of signaling is observed in systems that naturally express RXFP3, and given that 135PAM1 has poor solubility and displays selectivity for the C-terminal amides that are not naturally occurring, it has limited use experimentally (Alvarez-Jaimes et al., 2012).

#### D. Relaxin Family Peptide Receptor 4 Signaling

**1. Canonical Signaling Pathways.** Stimulation of cells expressing human RXFP4 by INSL5 or human relaxin-3 increases GTP $\gamma$ S binding and inhibits forskolin-stimulated cAMP accumulation, suggesting that RXFP4 is G $\alpha_{i/o}$  coupled (Liu et al., 2003a, 2005b). Coexpression of RXFP4 with the promiscuous G protein G $\alpha_{16}$  in HEK293 cells produces a strong calcium signal in response to INSL5 and human relaxin-3 (Liu et al., 2003a, 2005b) that is not present in normal cells. Although relatively little information exists at present regarding RXFP4 signaling, it is expected that this will rapidly change given the identification of INSL5 as an incretin (Grosse et al., 2014).

Thus both RXFP3 and RXFP4 are G $\alpha_{i/o}$ -coupled receptors that show GTP $\gamma$ S binding and inhibition of cAMP accumulation. More extensive studies of RXFP3 signaling have also revealed coupling to ERK1/2, p38MAPK, and JNK as well as ligand-directed signaling bias and allosteric modulation.

## IV. Receptor-Protein Signaling and Regulatory Complexes

It is increasingly recognized that GPCRs are important components of protein complexes and that this close relationship with other proteins affects how the receptor signals and is regulated. Receptor complexes can be constitutive (see section III.A.3) or may form after activation by agonists. Few studies have examined relaxin family peptide receptors in this light, and substantial information exists for only RXFP1 and to a lesser extent RXFP3.

### A. Relaxin Family Peptide Receptor 1

**1. G Proteins That Couple to Relaxin Family Peptide Receptor 1.** Although it has long been assumed that stimulation of RXFP1 produces an increase in cAMP by G $\alpha_s$  activation of AC, a direct functional interaction between RXFP1 and G $\alpha_s$  has been demonstrated only recently. Application of a specific G $\alpha_s$  inhibitor (NF449; 4,4',4'',4'''-[carbonylbis(imino-5,1,3-benzenetriyl)-bis(carbonylimino)]*tetrakis*-1,3-benzenedisulfonic acid, octasodium salt) to HEK293 cells expressing RXFP1 significantly decreased receptor-stimulated cAMP accumulation, whereas a low concentration of cholera toxin (a G $\alpha_s$  activator) substantially enhanced the cAMP response to relaxin (Halls and Cooper, 2010). Other supporting evidence includes the increase in cAMP after PTX pretreatment in the early phase (<10 minutes) after stimulation of RXFP1. Furthermore, peptide fragments of ICL3 of RXFP1 (minimum length 615–629) added to rat tissues (striatum, cardiac, and skeletal muscle membranes) increased cAMP and "antagonized" the response to relaxin, whereas addition of peptide fragments of G $\alpha_s$  "antagonized" both relaxin stimulation and RXFP1-ICL3 peptide stimulation of rat tissues (Shpakov et al., 2007). Many of the responses linked to RXFP1 display sensitivity to PTX (Halls et al., 2006), suggesting coupling to G $\alpha_{i/o}$  proteins, and the specificity of the receptor for particular G $\alpha_{i/o}$  isoforms was identified using PTX-insensitive G proteins (Halls et al., 2006). In HEK293 cells stably expressing RXFP1, restoration of the expected cAMP signaling profiles (after PTX pretreatment) occurred only with the expression of G $\alpha_{oB}$  and G $\alpha_{i3}$ . Activation of G $\alpha_{oB}$  and G $\alpha_{i3}$  by RXFP1 was subsequently confirmed using [<sup>35</sup>S]GTP $\gamma$ S immunoprecipitation, and these studies also demonstrated that activation of G $\alpha_{i3}$  occurs within 3 minutes of relaxin stimulation, suggesting that the observed delay in activation of the G $\alpha_{i3}$ -cAMP pathway occurs downstream of receptor coupling to this

G protein (Halls et al., 2009b). Subsequent studies using a site-directed mutagenesis approach demonstrated that activation of the  $G\alpha_{i3}$ -cAMP pathway involves the final 10 amino acids of the RXFP1 C terminus and in particular R752 (Halls et al., 2009b). Taken together, this suggests that all G protein coupling occurs rapidly after receptor activation and that  $G\alpha_s$  (and probably  $G\alpha_{oB}$ ) couple to RXFP1 within the ICL3, whereas coupling to  $G\alpha_{i3}$  is dependent upon the final 10 amino acids of the receptor C terminus.

*2. Interactions between Relaxin Family Peptide Receptor 1 and  $\beta$ -Arrestins.*  $\beta$ -Arrestins were initially identified for their role in receptor desensitization and internalization. Prototypical GPCR signaling is terminated after activation by receptor phosphorylation,  $\beta$ -arrestin binding followed by uncoupling from the G protein, and internalization (Luttrell and Gesty-Palmer, 2010). However  $\beta$ -arrestins are increasingly recognized as scaffold proteins that recruit a variety of catalytically active proteins that influence a wide range of signaling pathways, protein translation, and gene transcription. Unlike many other GPCRs, when expressed in HEK293T or Cos-7 cells, RXFP1 activation does not result in significant receptor phosphorylation, desensitization, or internalization (Callander et al., 2009). Little phosphorylation and internalization could be measured by whole cell radioligand binding, and there was minimal cell surface localization of GFP-tagged  $\beta$ -arrestin visualized by confocal imaging (Callander et al., 2009). In human primary decidual cells or HEK293 cells stably expressing RXFP1, there is weak internalization as measured by cell surface enzyme-linked immunosorbant assay (ELISA) that is enhanced by overexpression of  $\beta$ -arrestin-2 (Kern and Bryant-Greenwood, 2009). Constitutive interaction between RXFP1 and  $\beta$ -arrestin-2 has been identified in the RXFP1 signalosome (Halls and Cooper, 2010), where  $\beta$ -arrestin-2 scaffolds PKA and PDE4D3 to regulate cAMP levels formed in response to subpicomolar concentrations of relaxin (see section III.A.3 for details). The signalosome dissociates in response to nanomolar concentrations of relaxin, and  $\beta$ -arrestin-2 has no role in modulating canonical RXFP1 signaling. Thus, there does appear to be a significant role for  $\beta$ -arrestins in RXFP1 signaling after receptor activation, because there is little  $\beta$ -arrestin recruitment and minimal receptor internalization.

## B. Relaxin Family Peptide Receptor 2

*1. G Proteins That Couple to Relaxin Family Peptide Receptor 2.* By using the same approach as that used for RXFP1, the specificity of RXFP2 for particular  $G\alpha_{i/o}$  isoforms was identified using PTX-insensitive G protein mutants (Halls et al., 2006). After expression of three  $G\alpha_i$  and two  $G\alpha_o$  isoforms in HEK293 cells stably expressing RXFP2, the restoration of the expected cAMP signaling profile (after PTX pretreatment) occurred only with  $G\alpha_{oB}$  expression. This was subsequently

confirmed using [ $^{35}$ S]GTP $\gamma$ S immunoprecipitation (Halls et al., 2009b). Unlike RXFP1, RXFP2 does not couple to  $G\alpha_{i3}$ ; this is likely because receptor coupling to  $G\alpha_{i3}$  requires the final 10 amino acids of the RXFP1 C terminus (specifically R752) that are missing in RXFP2 (Halls et al., 2009b). It is highly likely that in a manner similar to RXFP1, RXFP2 also couples to  $G\alpha_s$ , because PTX pretreatment increases the cAMP generated after stimulation.

Both RXFP1 and RXFP2 couple to  $G\alpha_s$  and  $G\alpha_{oB}$ , but only RXFP1 also couples to  $G\alpha_{i3}$ . There is constitutive interaction between RXFP1 and  $\beta$ -arrestin-2 in the signalosome that dissociates with the advent of canonical signaling. There is only minimal internalization after activation of RXFP1, suggesting little interaction with  $\beta$ -arrestin-2 after signalosome dissociation. RXFP2 does not appear to interact with  $\beta$ -arrestins or internalize (Callander et al., 2009).

## C. Relaxin Family Peptide Receptor 3

*1. G Proteins That Couple to Relaxin Family Peptide Receptor 3 after Activation by Human Relaxin-3.* The early deorphanization studies suggested that RXFP3 was a  $G\alpha_{i/o}$ -coupled receptor that inhibited forskolin-stimulated cAMP accumulation (Liu et al., 2003b; van der Westhuizen et al., 2010). This was supported by studies in the cytosensor microphysiometer, where pretreatment of CHO-RXFP3 cells with PTX strongly inhibited the extracellular acidification rate, suggesting that the signaling pathways activated by RXFP3 were downstream of  $G\alpha_{i/o}$  (van der Westhuizen et al., 2005). As for RXFP1 and RXFP2, PTX-insensitive G proteins were used to determine the particular G proteins involved (van der Westhuizen, 2008). PTX pretreatment completely abrogated the ERK1/2 response in both CHO-RXFP3 and HEK-RXFP3 cells. In CHO-RXFP3 cells, the response was partially restored by transfection of  $G\alpha_{i2}$  or  $G\alpha_{oB}$  only. Similarly, in HEK-RXFP3 cells, signaling was partially restored by expression of mutant  $G\alpha_{i2}$  or  $G\alpha_{oB}$  but also by mutant  $G\alpha_{oA}$ . These differences may relate to different colocalization of receptors and G proteins in particular cell types. More recent studies used BRET to investigate ligand-induced interactions between RXFP3-RLuc8 and G proteins (G $\gamma$ 2-Venus) in live cells. Similar to previous studies, treatment of CHO-RXFP3-RLuc8 cells with human relaxin-3 caused activation of  $G\alpha_{i2}$ ,  $G\alpha_{oA}$ , and  $G\alpha_{oB}$ , but also revealed an interaction between RXFP3 and  $G\alpha_{i3}$  (Kocan et al., 2014). Thus RXFP3 has the potential to couple to  $G\alpha_{i2}$ ,  $G\alpha_{i3}$ ,  $G\alpha_{oA}$ , and  $G\alpha_{oB}$  after activation by human relaxin-3, but the receptor preferentially couples to discrete subsets of these G proteins dependent on cell type.

*2. G Proteins That Couple to Relaxin Family Peptide Receptor 3 after Activation by the Biased Ligands Human Relaxin and R3(B $\Delta$ 23–27)R/15.* The different signaling patterns observed with the biased ligands human relaxin and R3(B $\Delta$ 23–27)R/15 (see section III.C.2 for details) were also reflected in a distinct pattern of G protein coupling in

BRET studies (Kocan et al., 2014). The biased ligands coupled only with  $G\alpha_{i2}$  or  $G\alpha_{oB}$ , and the signal was much lower than that observed for human relaxin-3 (Kocan et al., 2014). To determine the potential biologic relevance of these findings, studies will be needed in cells that express RXFP3 at physiologic levels.

**3. Interactions between Relaxin Family Peptide Receptor 3 and  $\beta$ -Arrestins.** Interactions between RXFP3 and  $\beta$ -arrestins occur after administration of human relaxin-3 but not relaxin or the RXFP3 antagonist R3 (B $\Delta$ 23–27)R/I5 as measured by real-time kinetic BRET between RXFP3-Rluc8 and a  $\beta$ -arrestin fusion protein ( $\beta$ -arrestin-1-Venus or  $\beta$ -arrestin-2-Venus). Preincubation with R3(B $\Delta$ 2327)R/I5 completely inhibits the human relaxin-3 stimulated recruitment of  $\beta$ -arrestin-1 and  $\beta$ -arrestin-2 to RXFP3 (Kocan et al., 2014). Furthermore, PTX pretreatment partially blocked  $\beta$ -arrestin recruitment, suggesting that RXFP3 recruits  $\beta$ -arrestins via  $G\alpha_{i6}$ -dependent and -independent pathways. The involvement of  $\beta$ -arrestin in ERK1/2 activation downstream of RXFP3 was investigated by comparing cells expressing dominant negative (V53D) versus wild-type (WT)  $\beta$ -arrestin-1. There was no inhibitory effect of the dominant negative  $\beta$ -arrestin-1 on ERK1/2 activation, suggesting that, unlike several other GPCRs (Cottrell et al., 2009; Luttrell and Gesty-Palmer, 2010), the RXFP3/ $\beta$ -arrestin-1 interaction contributes little to ERK1/2 signaling (Kocan et al., 2014).

Previous studies demonstrated internalization of RXFP3 after 10 minutes of stimulation with human relaxin-3 but not human relaxin, porcine relaxin, or INSL3 as assessed by radioligand internalization assays (70–90% of receptors internalized) and confocal microscopy (van der Westhuizen et al., 2010). Taken together, these studies suggest that treatment with only the cognate ligand human relaxin-3 causes RXFP3 to undergo classic  $\beta$ -arrestin-dependent internalization. The detailed mechanisms involved in RXFP3 internalization, phosphorylation, and recycling/degradation all remain to be determined.

#### D. Relaxin Family Peptide Receptor 4

**1. G Proteins That Couple to Relaxin Family Peptide Receptor 4.** There is currently a paucity of information regarding signaling pathways activated by RXFP4. The receptor is G protein-coupled because activation by INSL5 or human relaxin-3 increases GTP $\gamma$ S binding. cAMP accumulation in response to forskolin is inhibited, suggesting that RXFP4 couples to  $G\alpha_{i6}$  proteins (Liu et al., 2003a, 2005b). Calcium signaling is activated only in cells coexpressing RXFP4 and the promiscuous G protein  $G\alpha_{16}$ . Direct evidence for RXFP4-G protein coupling is currently lacking.

In contrast to RXFP1, the information available on proteins interacting with RXFP3 and RXFP4 is largely confined to G proteins and  $\beta$ -arrestins. RXFP3 couples to  $G\alpha_{oA}$ ,  $G\alpha_{oB}$ ,  $G\alpha_{i2}$ , and  $G\alpha_{i3}$ , although the pattern observed varies with cell type and ligand. There is also

good evidence for an interaction of RXFP3 with  $\beta$ -arrestins that modulates receptor internalization. At present, RXFP4 is known to couple only to proteins of the  $G\alpha_{i6}$  family.

## V. Physiologic Roles of Relaxin Family Peptide Receptors and Their Cognate Ligands

The physiologic roles of relaxin family peptides and their receptors have been extensively reviewed (Ivell and Anand-Ivell, 2009; Smith et al., 2011; Bathgate et al., 2013a). In particular, the role of relaxin in female and male reproductive physiology have been well studied (Sherwood, 1994, 2004; Bathgate et al., 2013a, 2006c). For this reason, only brief overviews will be given here.

### A. Relaxin and Relaxin Family Peptide Receptor 1

**1. Reproduction.** The discovery of relaxin in 1926 (Hisaw, 1926) and the demonstration of its effects on the pubic ligament led to the peptide being regarded as a hormone of reproduction for many years. Subsequent studies showed that relaxin produced by the corpus luteum and/or placenta has important roles in pregnancy and parturition and is a major circulating hormone during pregnancy in all mammalian species acting on the pubic symphysis, cervix, uterus, vagina, and mammary glands. It also is responsible for many of the cardiovascular changes that occur during pregnancy (Debrah et al., 2006; Conrad, 2011).

Relaxin causes increased flexibility and elasticity of the interpubic ligament during pregnancy in several species (O'Connor et al., 1966; Steinetz et al., 1983), and in the relaxin knockout mouse the interpubic ligament fails to develop (Zhao et al., 1999). The peptide also causes softening and hypertrophy of the cervix during the second half of pregnancy in mammals (Bathgate et al., 2006c), and these effects are impaired in both relaxin-deficient rats (Burger and Sherwood, 1998) and relaxin knockout mice (Zhao et al., 1999; Bathgate et al., 2006c). In humans, although relaxin levels increase during cervical ripening, this still occurs after embryo transfer where circulating relaxin levels are undetectable (Eddie et al., 1990a). In clinical trials, although direct application of porcine relaxin to the cervix appeared to assist in ripening (Evans et al., 1983; MacLennan et al., 1986b), later studies with recombinant human relaxin failed to confirm this finding (Bell et al., 1993; Weiss et al., 2009).

Relaxin reduces uterine contractility (Krantz et al., 1950) and increases uterine growth (Steinetz et al., 1957) in rat, mouse, guinea pig, hamster, and pig (Bathgate et al., 2006c) but in humans, has little effect on uterine tone (MacLennan et al., 1986a, 1995). In humans, relaxin is probably important for implantation rather than the later stages of pregnancy. Relaxin promotes growth of the vagina during pregnancy in mice (Hall, 1960; Schink and Struck, 1968) and rats (Burger and Sherwood, 1995; Zhao et al., 1996, 2001), and consistent with these effects, the growth of the vagina was profoundly decreased in



relaxin knockout mice (Zhao et al., 2000). These effects are essential for normal delivery in some species but not humans.

Relaxin has trophic effects on the mammary gland and/or nipple in several species. Nipple development is completely blocked in relaxin knockout mice with limited effects on the mammary gland (Zhao et al., 1999). Pups born to relaxin-knockout mice die within 24 hours unless crossfostered to wild-type mothers, an effect entirely due to poor nipple development, because female knockout mice produce milk normally (Zhao et al., 1999). The same phenotype is displayed by RXFP1 knockout mice (Krajnc-Franken et al., 2004) and is not rescued by transgenic overexpression of INSL3 (Kamat et al., 2004). Relaxin binding sites are present in the mammary glands of pigs, rats, and humans; in humans, RXFP1 receptors are localized to the nipple, epithelial cells (Kohsaka et al., 1998), and stromal tissue (Ivell et al., 2003).

Although relaxin has many well defined roles in reproduction in many species, in humans these effects are often absent or ill defined. Relaxin is produced in the human ovary during the luteal phase of the menstrual cycle and secreted into the circulation (Eddie et al., 1990b; Stewart et al., 1990; Chen et al., 2003). In the clinical trial of relaxin for the treatment of scleroderma (Erikson and Unemori, 2001), women received 24-week subcutaneous infusion of relaxin and reported heavy, irregular, or prolonged menstrual bleeding (Unemori et al., 1999). In humans and other primates, the peak of relaxin secretion is in the first trimester of pregnancy. However relaxin is clearly not mandatory during the induction or maintenance of pregnancy, because women without ovaries can become pregnant by ovum donation even though they have undetectable levels of circulating relaxin (Johnson et al., 1991). Relaxin secretion also correlates with increased expression of RXFP1 mRNA and relaxin binding in the human endometrium in the secretory phase of the menstrual cycle (Bond et al., 2004; Campitiello et al., 2011).

Perhaps somewhat surprisingly given its history as a hormone acting on the female reproductive tract, relaxin is also found in the male reproductive tract in most mammals. Relaxin from human seminal plasma is identical to luteal relaxin (Winslow et al., 1992) and appears to be produced in the prostate (Yki-Järvinen et al., 1983; Sokol et al., 1989). RXFP1 is expressed in sperm in both mice (Krajnc-Franken et al., 2004) and humans (Carrell et al., 1995; Ferlin et al., 2012) and increases sperm motility and facilitates penetration into oocytes (Weiss, 1989). Another potential role for seminal relaxin may be to act on the female reproductive tract to prepare the endometrium for implantation (Telgmann and Gellersen, 1998; Unemori et al., 1999; Anand-Ivell and Ivell, 2014).

**2. Central Nervous System.** In mammals, there are high concentrations of RXFP1 in the SFO and OVLTLT that are activated by relaxin to cause a reduction in plasma osmolality (Sunn et al., 2002). This effect in rats

is associated with increased serum relaxin levels during the second half of pregnancy (Sherwood et al., 1980; Lindheimer et al., 1989) and is absent in pregnant rats that have undergone ovariectomy or treatment with relaxin antibodies (Novak et al., 2001). Similarly, the decrease in plasma osmolality with late pregnancy is not observed in relaxin knockout mice (Zhao et al., 1999). In humans, there is a decrease in plasma osmolality with pregnancy, but this may not be completely attributable to relaxin because women who become pregnant after ovum donation (and hence lack circulating relaxin) display attenuated (Smith et al., 2006b) or no decrease in plasma osmolality (Johnson et al., 1991, 1996). However, the apparent lack of relaxin in the absence of a corpus luteum may have to be reassessed using more sensitive methods of detection since the discovery of responses to femtomolar concentrations of relaxin (Halls and Cooper, 2010), well below the level of detection using current ELISA methods; relaxin is also produced locally in many tissues including the uterus. Relaxin administered intracerebroventricularly or intravenously is dipsogenic in nonpregnant rats (Summerlee et al., 1998a), and rats given relaxin monoclonal antibodies in the second half of pregnancy show a reduction in water consumption. Administration of porcine or human relaxin intravenously causes activation (increased *c-fos* expression) of neurons in the peripheral and dorsal segments of the SFO and in the dorsal cap region of the OVLTLT as well as in the supraoptic and paraventricular nuclei of the hypothalamus (McKinley et al., 1997, 1998; Sunn et al., 2001, 2002), all sites of localization of RXFP1 and accessible to circulating relaxin (Weisinger et al., 1993).

Relaxin acting on RXFP1 in the circumventricular organs and hypothalamic nuclei may have a role in the timing of parturition, because this is disrupted by central administration of a relaxin monoclonal antibody (Summerlee et al., 1998b). RXFP1 is also highly expressed in the basolateral amygdala, and administration of relaxin to this region impairs fear-related memory consolidation in rats (Ma et al., 2005). However, no specific agonist or antagonist studies have been carried out to determine whether these effects are mediated by RXFP1 or to determine the source of endogenous relaxin that activates these receptors (Callander and Bathgate, 2010). Although RXFP1 is also highly expressed in other regions associated with memory formation, such as the neocortex, thalamic nuclei, hippocampus, and supramammillary nucleus, there are no studies to date that examine effects on memory. RXFP1 is also present in the oxytocin-containing cells of the paraventricular and supraoptic hypothalamic nuclei (Burazin et al., 2005), and intravenous relaxin administration increases oxytocin neuron activity and oxytocin release (Way and Leng, 1992).

**3. Blood Vessels.** Relaxin plays an important role in many of the adaptive cardiovascular changes that occur in pregnancy (Conrad, 2011). These include increases in cardiac output, heart rate, and a decrease in vascular



resistance (Conrad et al., 2004; Conrad and Novak, 2004; Debrah et al., 2005, 2006). Increases in plasma volume and decreased blood pressure are not consistently observed (Ahokas et al., 1989; Conrad, 2004, 2010, 2011). Ovariectomy or passive immunization with monoclonal antibodies for rat relaxin prevent these adaptive changes (Novak et al., 2001). In both female and male rats, chronic relaxin administration (osmotic minipump) increases renal plasma flow and glomerular filtration rate (Danielson et al., 1999). In humans, a few studies suggest similar effects of relaxin on the cardiovascular system (Conrad and Shroff, 2011). In the clinical trial for scleroderma, long-term (6 months) infusion of relaxin increased creatinine clearance and modestly decreased blood pressure (Erikson and Unemori, 2001; Teichman et al., 2009). In the pilot trial in patients suffering from stable chronic heart failure, a short (24 hours) infusion of relaxin was associated with decreased systemic and pulmonary vascular resistance and pulmonary wedge pressure and elevated cardiac index without significantly affecting systolic or mean blood pressure (Dschietzig et al., 2009c; Teichman et al., 2009).

Vasodilation in arterioles, capillaries, and venules is a common response to relaxin in reproductive tissues (Vasilenko et al., 1986; Bani et al., 1988; Lee et al., 1992), heart (Bani-Sacchi et al., 1995; Masini et al., 1997; Bani et al., 1998b), liver (Bani et al., 2001), and cecum (Bigazzi et al., 1986). Relaxin is a potent vasodilator in arteries (Conrad, 2010; McGuane et al., 2011b), although the effect is vessel specific (McGuane et al., 2011b). Relaxin is a physiologic antagonist of vasoconstrictors in mesenteric arteries (St-Louis and Massicotte, 1985; Massicotte et al., 1989), primary bovine aortic smooth muscle cells (Bani et al., 1998a), and uterine artery (Longo et al., 2003). The vasodilator effects in guinea pig and rat coronary arteries and in bovine cultured smooth muscle cells are associated with increased NO synthesis (Bani-Sacchi et al., 1995; Bani et al., 1998a). The rise in intracellular calcium produced by  $\alpha$ -thrombin or angiotensin II is also reduced by relaxin (Bani et al., 1998a; Failli et al., 2002). In humans, there are vasodilator effects in gluteal resistance or subcutaneous arteries but little or no effect in pulmonary, myometrial, or placental vessels (Petersen et al., 1991; Fisher et al., 2002; McGuane et al., 2011b). In gluteal arteries, the vasodilator responses likely involve NO and interestingly were influenced by the medication being administered to patients. Arteries obtained from patients on angiotensin converting enzyme inhibitors showed marked attenuation of the vasodilator response to relaxin, effects that appeared to be further enhanced by inhibition of cyclooxygenase (Fisher et al., 2002). However, in patients not receiving angiotensin converting enzyme inhibitors, inhibition of cyclooxygenase had little effect on the vasodilator response to relaxin (Fisher et al., 2002). The vasodilator mechanisms suggested for relaxin in both humans and in animal models involve activation of NOS (Nistri and Bani, 2003; Conrad and Novak, 2004), VEGF,

placental growth factor (PGF), matrix metalloproteinases, ET<sub>B</sub> receptors (Novak et al., 2002; Dschietzig et al., 2003), and modification of the extracellular matrix of the vessel walls (Jeyabalan et al., 2003; Leggabe et al., 2005; Xu et al., 2010; McGuane et al., 2011b). These responses have a distinct temporal hierarchy. The acute (within minutes) responses are endothelium dependent and blocked by NOS inhibitors, the PI3K inhibitors Wortmannin and LY294002, and by PTX pretreatment but not by the VEGF receptor antagonist SU5416 [(3Z)-3-[(3,5-dimethyl-1H-pyrrol-2-yl)methylidene]-1,3-dihydro-2H-indol-2-one] (McGuane et al., 2011b). The effects are consistent with G $\beta$  $\gamma$  activation of PI3K, Akt phosphorylation, and eNOS. Treatment with NOS inhibitors or removal of the endothelium enhances contractions of vascular smooth muscle to agonists in both pregnant rats (Danielson and Conrad, 1995; Gandley et al., 2001) and in rats treated chronically with relaxin (Novak et al., 2002). This does not appear to be attributable to changes in NOS expression, because alterations in renal hemodynamics in pregnancy are not accompanied by large changes in NOS expression. In pregnant rats, eNOS expression in the renal artery fell by 39%, whereas iNOS and nNOS expression increased by 31 and 25%, respectively (Alexander et al., 1999).

Longer-term (within hours) vasodilator responses to relaxin also involve NOS, because blockade with NG-monomethyl-L-arginine prevents the renal hemodynamic and hyperfiltration responses (Danielson et al., 1999), and these effects can be recapitulated in vitro (Novak et al., 2002). The longer-term response involves endothelial ET<sub>B</sub> receptors that release NO (Dschietzig et al., 2003; Jeyabalan et al., 2003). Two mechanisms involving the relaxin-ET<sub>B</sub> receptor pathway have been found. In rodent and human vessels of ~100–300  $\mu$ m diameter, relaxin activates MMP-9 and later also MMP-2 to generate ET-1(1–32), rather than ET-1(1–21), from big ET-1 (Jeyabalan et al., 2003). The effect of MMPs on big ET-1 has been described independently of relaxin (Fernandez-Patron et al., 1999). ET-1(1–32) then preferentially binds to the endothelial ET<sub>B</sub> receptor causing NO release. In blood vessels from pregnant or relaxin-treated nonpregnant rats, pro-MMP-2 and MMP-2 activity and pro-MMP-2 protein and mRNA are increased (Jeyabalan et al., 2003, 2006). MMP-9 activity also appears to be increased somewhat, although more recent studies suggest that MMP-9 is more important in the relatively short-term responses to relaxin (4–6 hours) with reversal of the effects being produced by MMP-9 rather than MMP-2-neutralizing antibodies (Jeyabalan et al., 2007). This is supported by studies with the selective MMP-2 inhibitor cyclic CTTHWGF<sub>2</sub>TLC, the MMP inhibitor GM6001 [(2R)-N'-hydroxy-N-[(2S)-3-(5H-indol-3-yl)-1-methylamino-1-oxopropan-2-yl]-2-(2-methylpropyl)butanediamide], or a TIMP-2 or MMP-2 neutralizing antibody, all of which inhibit the vasodilator actions of relaxin in renal arteries (Jeyabalan et al., 2003),

whereas inhibition of the formation of ET(1–21) by phosphoramidon has no effect. These studies clearly implicate MMPs in the vasodilator actions of relaxin, although MMPs also cleave calcitonin gene-related peptide to promote vasoconstriction (Fernandez-Patron et al., 2000). These findings correspond well with those obtained with the ET<sub>B</sub> antagonist RES-701-1 [cyclic (Gly1-Asp9) (Gly-Asn-Trp-His-Gly-Thr-Ala-Pro-Asp-Trp-Phe-Phe-Asn-Tyr-Tyr-Trp)] that blocks renal hemodynamic changes produced by relaxin (Danielson et al., 2000) in rats and antagonizes inhibition of renal artery smooth muscle produced by relaxin or in pregnancy (Novak et al., 2002). Similar effects were produced in vitro by the ET receptor antagonist SB209670 [(+)-(1*S*,2*R*,3*S*)-3-(2-carboxymethoxy-4-methoxyphenyl)-1-(3,4-methylenedioxyphenyl)-5-(prop-1-yl-oxo)indane-2-carboxylic acid] but not by the ET<sub>A</sub> selective BQ123 [2-[(3*R*,6*R*,9*S*,12*R*,15*S*)-6-(1*H*-indol-3-ylmethyl)-9-(2-methylpropyl)-2,5,8,11,14-pentaoxo-12-propan-2-yl-1,4,7,10,13-pentazabicyclo[13.3.0]octadecan-3-yl]acetic acid (Gandley et al., 2001).

An alternative mechanism that occurs mainly in larger vessels and in the pulmonary circulation (Dschietzig et al., 2001a,b, 2003) involves relaxin-induced upregulation of endothelial ET<sub>B</sub> receptor requiring Raf-1, MEK-1, ERK-1/2, and NF $\kappa$ B (Dschietzig et al., 2003). This mechanism spares the smooth muscle ET<sub>B</sub> receptor, a contributor to ET-1–related vasoconstriction. Thus, relaxin acts as a functional ET-1 antagonist, because the endothelial ET<sub>B</sub> receptor mediates NO production and, equally importantly, ET-1 clearance (Dschietzig et al., 2001b, 2003). Although another study does not support this mechanism (Kerchner et al., 2005), this can be explained by the use of small rather than large arteries, no pulmonary endothelial cells, and significantly lower relaxin concentrations.

Evidence also exists for a role of VEGF in the longer-term vasodilator actions of relaxin. In human endometrial cells, relaxin increases cAMP levels and VEGF expression, and the effects are prevented by AC inhibition and mimicked by forskolin or a PDE inhibitor (Unemori et al., 1999), suggesting that relaxin-stimulated cAMP production mediates increased VEGF transcription and, thus, angiogenesis. However, although treatment of rat or mouse renal arteries or human subcutaneous arteries with the VEGF receptor antagonist SU5416 blocks the vasorelaxant effects of relaxin (McGuane et al., 2011a), the specificity of SU5416 has been questioned (Arora and Scholar, 2005; Loges et al., 2006), and in rat renal arteries, SU5416 potentiates the acute vasodilator effects of relaxin (McGuane et al., 2011b). Furthermore, although VEGF-neutralizing antibodies block the effects of relaxin, this can also be mimicked by placental growth factor antibodies (McGuane et al., 2011a), again making interpretation of the results difficult. It is possible that the antibodies, by blocking the vasodilator effects of VEGF and PGF, may produce physiologic antagonism of the response to relaxin without necessarily being directly related to the

primary mechanism of action. Alternatively, these results may reflect MMP-2- or MMP-9-mediated release of extracellular matrix-bound VEGF (and PGF) (Bergers et al., 2000; van Hinsbergh et al., 2006) that could underpin the MMP-ET<sub>B</sub> pathway by stimulating expression of prepro-ET-1.

Finally, relaxin promotes angiogenesis (Unemori et al., 1999; Unemori et al., 2000) by inducing VEGF and basic fibroblast growth factor synthesis. These effects are likely important in promotion of wound healing (Bitto et al., 2013) and also in the heavy, irregular, or prolonged menstrual bleeding that accompanies relaxin administration (Unemori et al., 1999). Relaxin also increases arterial compliance by its effects on geometric remodeling (increases in unstressed wall area and wall-to-lumen area ratio) and compositional remodeling (decrease in collagen-to-total protein ratio) in certain arteries (Chan and Cipolla, 2011; Debrah et al., 2011; Gooi et al., 2013). These properties expedite tissue and organ perfusion in the long term.

Compared with arteries, remarkably little is known of the effects of relaxin on veins. It was reported that the vasodilator potency of relaxin is lower in veins than arteries (Li et al., 2005). Rat mesenteric veins, in contrast to arteries, are not remodeled by relaxin (Jelinic et al., 2014). It is important to recognize that venous tone is significantly more dependent on sympathoadrenergic innervation and pacemaker cells (Cajal cells) than arterial tone (Gelman, 2008). Given the interest in the utility of vasodilators in the treatment of cardiac failure (Bhushan et al., 2014), this promises to be an important future area of research.

Relaxin protects against endothelial dysfunction that denotes an inflammatory and oxidative stress-related vascular pathology characterized by compromised vasodilator capacity on the basis of reduced NO bioavailability. In a rat in vitro model (Dschietzig et al., 2012) in which TNF- $\alpha$  impairs endothelial function, relaxin, by a mechanism involving PI3K and GR signaling, attenuates the upregulation of arginase-II that deprives NOS of its substrate L-arginine; antagonizes the increased ET-1 expression that promotes vasoconstriction and inflammation; upregulates SOD-1 expression to combat oxidative/nitrosative stress; and finally, causes partial reversal of dephosphorylation of eNOS, which impairs NO generation. Others have confirmed the SOD-1 finding and also reported elevated SOD-2 expression (Collino et al., 2013). This vascular protection is further complemented by NO-mediated inhibition of platelet and neutrophil activation (Bani et al., 1995a,b; Masini et al., 2004). Relaxin also attenuates VCAM-1 and MCP-1 stimulation that mediate the initial monocyte-endothelium contact and are both key events in early vascular inflammation (Brecht et al., 2011).

**4. Heart.** Relaxin acts directly on the heart. The presence of RXFP1 in the heart was first suggested by the demonstration of high-affinity binding sites for

relaxin in rat atria (Osheroﬀ et al., 1992; Osheroﬀ and Ho, 1993; Tan et al., 1999). Subsequently, relaxin was shown to be a powerful inotropic and chronotropic agent (Kakouris et al., 1992). Positive chronotropic eﬀects of relaxin occur in perfused intact hearts (Thomas and Vandlen, 1993; Bani-Sacchi et al., 1995; Coulson et al., 1996; Toth et al., 1996) and isolated right atria (Kakouris et al., 1992; Tan et al., 1998; Ward et al., 1992; Wade et al., 1994; Mathieu et al., 2001), and the positive inotropic eﬀects occur in left atria (Kakouris et al., 1992; Ward et al., 1992; Wade et al., 1994; Tan et al., 1998; Mathieu et al., 2001). Chronotropic eﬀects of relaxin are accompanied by the secretion of atrial natriuretic peptide in isolated perfused rat hearts (Toth et al., 1996). In rabbit sinoatrial node cells, relaxin increases the rate of spontaneous action potentials and the L-type calcium current (Han et al., 1994) by a PKA-dependent mechanism. In rat atrial myocytes, relaxin inhibits outward potassium currents, increases action potential duration, and enhances calcium entry; this is the mechanism underlying the positive inotropic eﬀect (Piedras-Renteria et al., 1997a,b). Regarding inotropic eﬀects in the ventricle, there is one report in mice (Shaw et al., 2009) indicating that relaxin increases myoﬁlament calcium sensitivity by promoting phosphorylation of myosin-binding protein C, troponin I, and troponin C. This results in higher force generation at normal calcium levels and is dependent on myoﬁlament-associated PKC $\delta$ . This is the only report of inotropic responses to relaxin in ventricles. Although the cardiac actions of relaxin are largely conﬁned to rodents, a recent study (Dschietszig et al., 2011) demonstrates inotropic eﬀects in human atria (but not in ventricular myocardium), which are preserved in failing hearts and involve PKA, outward potassium currents, and PI3K. The eﬀects of relaxin on the cardiovascular system are not gender speciﬁc.

Relaxin also protects against myocardial injury caused by ischemia and reperfusion (Bani et al., 1998b). Pre-treatment of rats with relaxin 30 minutes before cardiac ischemia produced by ligating the left anterior descending coronary artery markedly reduced the size of the penumbra and reduced cardiac arrhythmias, mortality, myeloperoxidase activity, malonyldialdehyde production, and calcium content as well as causing an improved morphology (Bani et al., 1998b). These ﬁndings were conﬁrmed in an *in vivo* pig model of myocardial infarction in which relaxin, administered during reperfusion of the left anterior descending coronary artery, attenuated leukocyte recruitment and oxidative damage and improved contractile recovery (Perna et al., 2005). In a mouse infarction model, relaxin improves postinfarction remodeling by suppressing reactive ﬁbrosis in vital myocardium, without aﬀecting reparative ﬁbrosis (scarring) within the infarcted area (Samuel et al., 2011). This postinfarction ﬁbrosis-modulating eﬀect could also assist stem cell-based reparative therapies that appear to be promoted by the peptide. Mouse C2C12 skeletal myoblasts engineered to

overexpress human relaxin demonstrate growth and functional maturation of neonatal immature cardiomyocytes assessed by the expression of myocardium-speciﬁc structural genes (connexin 43, troponin T, and HCN4 ion channel) (Formigli et al., 2009). Coronary infusion of C2C12 skeletal myoblasts overexpressing relaxin in conjunction with administration of exogenous relaxin starting at day 30 after infarction in rats improves myocardial viability in the infarcted area compared with myoblast therapy alone (Bonacchi et al., 2009).

Relaxin also attenuates hypertrophy in rat neonatal cardiomyocytes by inhibiting myoﬁbroblast activation and the subsequent paracrine release of growth factors (Moore et al., 2007), conﬁrming an earlier study in spontaneously hypertensive rats and suggesting that endogenous relaxin has antihypertrophic actions (Dschietszig et al., 2005).

Atrial ﬁbrillation, a common arrhythmia that develops during ageing-related ﬁbrosis, hypertension, or heart failure, is suppressed by relaxin in spontaneously hypertensive rats, an established model of end-organ damage in response to hypertension (Parikh et al., 2013). The aforementioned eﬀects of relaxin on atrial hypertrophy and ﬁbrosis resulted in restoration of normal conduction velocity, a reduced action potential duration, and hence decreased atrial ﬁbrillation.

Relaxin may be a naturally occurring cardioprotective agent, because in chronic heart failure, the expression of human relaxin-1 and human relaxin is increased in both atria and ventricles, and the level of expression correlates with the degree of failure (Dschietszig et al., 2001b; Fisher et al., 2003) but is not a predictor of clinical outcomes (Fisher et al., 2003). Increased circulating relaxin levels in human heart failure were not conﬁrmed in two subsequent clinical studies (Kupari et al., 2005; Heringlake et al., 2009), possibly due to a methodological issue related to the utilization of a new batch of antiserum (Kupari et al., 2005; Heringlake et al., 2009) for the ELISA (Dschietszig et al., 2001b; Fisher et al., 2002). Increased relaxin gene expression in chronic heart failure was conﬁrmed by two independent studies using the rat infarction model (Kompa et al., 2002; Zhang et al., 2005).

After the demonstration of protective actions in the cardiovascular system, relaxin was tested in human heart failure. A hemodynamic pilot study in patients with stable chronic heart failure (Dschietszig et al., 2009c) demonstrated that a 24-hour intravenous infusion of recombinant human relaxin markedly elevated cardiac index without aﬀecting heart rate and decreased pulmonary wedge pressure, systemic, and pulmonary vascular resistance without aﬀecting systolic or mean arterial blood pressure and central venous pressure. Hemodynamic changes occurred within 45 to 60 minutes, and relaxin infusion improved creatinine and blood urea nitrogen clearance and was free of relevant side eﬀects.

The use of relaxin for acute heart failure (defined as new onset or, more frequently, worsening of known heart failure) is promising. In the Pre-RELAX (phase II) and RELAX (phase III) trials (Teerlink et al., 2009, 2013), relaxin moderately improved dyspnea (the primary endpoint), was exceptionally safe, improved renal function, and lowered all-cause as well as cardiovascular mortality at day 180. Because both trials were not powered statistically to assess mortality, another adequately powered mortality trial in acute heart failure is in progress. If successful, relaxin would become the first evidence-based remedy for this highly challenging clinical syndrome.

**5. Kidney.** The pronounced effects of relaxin on renal arteries imply a pivotal role in the regulation of renal function. Indeed, relaxin is *the* major renal vasodilator responsible for the increases in renal plasma flow and glomerular filtration rate during rat pregnancy (Novak et al., 2001). The increase in renal plasma flow and glomerular filtration rate with relaxin also occurs in nonpregnant female rats and male rats (Danielson et al., 1999; Danielson and Conrad, 2003) and is accompanied by natriuresis (Bogzil et al., 2005).

In humans, circumstantial evidence for a role for relaxin comes from a clinical study in which women who were pregnant through egg donation, and therefore relaxin deficient, exhibited a decreased glomerular filtration rate compared with that observed in normal pregnancies (Smith et al., 2006b). In a small study in human volunteers (Smith et al., 2006a), relaxin administered for 5 hours increased renal plasma flow by ~75% and fractional sodium excretion by ~25% but did not affect glomerular filtration rate (Smith et al., 2006a). The authors suggest that the volunteers may not have been volume replete, which may offset the effects of relaxin at the afferent arteriole. Alternatively, humans, in contrast to rats, are not typically in glomerular filtration equilibrium, thereby allowing dissociation of the effects of relaxin on renal plasma flow and glomerular filtration rate (Smith et al., 2006a), or the effect may take longer to develop via alteration of the filtration coefficient. For example, during chronic relaxin infusion in the scleroderma trials, the estimated glomerular filtration rate rose significantly in the treated group (Seibold et al., 2000; Khanna et al., 2009). However, the vasodilator effects of relaxin may also influence creatinine handling in the bowels, a process that is usually upregulated in states of renal insufficiency such as scleroderma or congestive heart failure. The renal effects of relaxin in humans therefore remain to be confirmed.

**6. Fibrosis.** The antifibrotic effects of relaxin were the first biologic effects to be recorded (Hisaw, 1926), and a number of studies have examined this property as a possible treatment of the connective tissue disease scleroderma. Relaxin was safe and well tolerated in clinical trials and effective in some patients in a phase II trial (Seibold et al., 2000) but failed to show clinical efficacy in a larger scale phase III trial (Erikson and

Unemori, 2001). Despite these disappointing findings there are many studies in animals that show a role for relaxin in controlling collagen turnover. In relaxin knockout mice, there is a progressive increase in tissue fibrosis with age in male mice that is prevented (Samuel et al., 2007) or reversed in lung (Samuel et al., 2003b), kidney (Samuel et al., 2004b), and heart (Samuel et al., 2004a) by the administration of relaxin.

In the lung, treatment with relaxin reduces expression of collagen types I and III, increases levels of MMPs, and reduces fibrosis (Unemori et al., 1996). In kidney-derived fibroblasts, relaxin inhibits profibrotic changes induced by TGF- $\beta$  by a mechanism involving the NO/guanylyl cyclase pathway with associated decreases in Smad2 phosphorylation and nuclear localization (Mookerjee et al., 2009). Relaxin has antifibrotic effects in rat renal fibrosis models, including fibrosis produced by bromoethylamine treatment (Garber et al., 2001), in an antiglomerular basement membrane model (McDonald et al., 2003) and in spontaneously hypertensive rats (Lekgabe et al., 2005). In cardiac fibroblasts, relaxin reduces collagen type I and III expression and increases MMPs (Samuel et al., 2004a). In cardiac fibrosis after chronic stimulation of  $\beta$ -adrenoceptors by isoprenaline (Zhang et al., 2005) or by cardiac-specific transgenic overexpression of  $\beta_2$ -adrenoceptors (Bathgate et al., 2008), relaxin treatment markedly reduced cardiac fibrosis. In streptozotocin-treated mRen-2 rats (a model for diabetic cardiomyopathy), relaxin reduced left ventricular collagen, myocardial stiffness, and diastolic dysfunction (Samuel et al., 2008), associated with a significant decrease in TIMP-1 expression and an increase in extracellular matrix-degrading MMP-13 (Samuel et al., 2008). Relaxin also has antifibrotic actions in a number of models of liver fibrosis (Bennett, 2009). However in a chronic pressure overload model in mice, relaxin was ineffective (Xu et al., 2008), possibly because cardiac RXFP1 may be downregulated in this model or the serum concentrations of relaxin are insufficient to offset the extensive fibrosis.

Relaxin acting via RXFP1 in primary fibrochondrocytes increases the expression of mRNA for MMP-9 and MMP-13 (Ahmad et al., 2012) associated with activation of PI3K, Akt, PKC $\zeta$ , and ERK1/2; inhibitors of these proteins blocked MMP-9 induction and the antifibrotic effects of relaxin. Relaxin-mediated increases in MMP-9 expression were also blocked by transfection of a dominant negative form of Akt or by small interfering RNA knockdown of ERK1/2, PKC $\zeta$ , Elk-1, c-fos, and, to a lesser extent, NF $\kappa$ B (Ahmad et al., 2012). This important study connects many of the known pathways of relaxin/RXFP1 signaling to a well recognized physiologic response to relaxin and provides insights that could be useful in translating the antifibrotic effects of relaxin into a clinical setting.

The apparent discrepancy between the highly successful preclinical antifibrosis studies and the failure of

relaxin in the phase III human scleroderma trial can be explained by an incomplete understanding of the pathophysiology of scleroderma and by the enrolment of patients in the terminal stages of the disease. It is of interest to note that the dermal fibrosis of the relaxin knockout mouse is rescued by exogenous relaxin at 6 months but not rescued by administration at 12 months of age (Samuel et al., 2005).

One of the interesting aspects that have emerged is that the antifibrotic properties of relaxin are clearly seen in disease conditions associated with excessive collagen deposition. Explanations for this unusual and potentially useful property of relaxin are now starting to emerge. In rat kidney myofibroblasts, it was discovered that the antifibrotic actions of relaxin are completely abolished by the AT<sub>2</sub>R antagonist PD123319 (Chow et al., 2014). In an in vivo fibrosis model in mice, produced by unilateral ureteric obstruction, the protective effects of relaxin are lost when the AT<sub>2</sub>R is either absent (in AT<sub>2</sub>R<sup>-ly</sup> mice) or blocked by PD123319, confirming that the AT<sub>2</sub>R is obligatory for the antifibrotic actions of relaxin. These effects are not produced by relaxin interacting directly with the AT<sub>2</sub>R, because the peptide does not bind to this receptor. However, it was found that RXFP1 can form constitutive heterodimers with AT<sub>2</sub>R in cell-based models, and the RXFP1-AT<sub>2</sub>R heterodimers may mediate the downstream signaling pathways originally attributed to relaxin (Heeg et al., 2005; Mookerjee et al., 2009; Chow et al., 2012), including inhibition of the TGF- $\beta$ <sub>1</sub>/pSmad2 axis and reduced TGF- $\beta$ <sub>1</sub>-induced collagen deposition. Because AT<sub>2</sub>Rs are expressed at low levels in tissues (Matsubara, 1998; Carey, 2005; Jones et al., 2008) and fibroblasts under physiologically quiescent states but are dramatically increased in number and activity under pathologic conditions, their increased availability in injured/diseased tissues increases their functional importance (Siragy and Carey, 1997; Savoia et al., 2006). Hence, pathologic conditions create an environment in which RXFP1-AT<sub>2</sub>R heteromerization is more likely to take place and may help explain why relaxin displays its antifibrotic effects to a pronounced extent under pathologic conditions.

Relaxin also has beneficial effects in wound healing (Casten and Boucek, 1958) that may involve the vasodilator effects mentioned above but, in addition, may involve the synthesis of new blood vessels by enhancing the local production of VEGF (Unemori et al., 2000).

**7. Organ Protection.** Relaxin not only protects the myocardium from ischemia-reperfusion (IR) injury (see above) but also protects other tissues. Thus, there is protection from IR injury in rat liver (Boehnert et al., 2005, 2008, 2009) by attenuating oxidative stress, leukocyte activation, and improving oxygen supply. In kidney IR in rats, relaxin reduces renal apoptosis and TNF- $\alpha$  levels as well as creatinine and urea levels (Yoshida et al., 2013). In a similar model, relaxin decreases inflammatory cytokines, counteracts oxidative damage by increasing SOD-1 and

SOD-2 expression, and ameliorates neutrophil-related injury as assessed by myeloperoxidase levels (Collino et al., 2013). Finally, the beneficial effects of relaxin are also observed in IR experiments in isolated rat lungs (Alexiou et al., 2010, 2013) where there is inhibition of leukocytes and oxidative surge, as well as prevention of ET-1 stimulation, resulting in less edema formation and lower pulmonary vascular pressure.

NO appears to be critically involved in organ protection (Masini et al., 1997; Alexiou et al., 2010, 2013; Collino et al., 2013). In rat lung, relaxin evokes an early and moderate increase in iNOS dependent on ERK1/2 activation that is counterbalanced by PI3K recruitment (Alexiou et al., 2013). In rat kidney (Collino et al., 2013), the peptide upregulates iNOS and activates eNOS via PI3K. It remains to be investigated whether over the long term, additional modes of action, such as the actions of relaxin on the GR pathway, come into play.

**8. Formation and Spread of Tumors.** Relaxin is expressed in endometrial (Kamat et al., 2006), mammary (Tashima et al., 1994), thyroid (Hombach-Klonisch et al., 2006), and prostate tumors (Thompson et al., 2006; Feng et al., 2007). The peptide is associated with breast cancer (Bani, 1997; Silvertown et al., 2003), and relaxin treatment of breast cancer cells implanted into nude mice increases their invasive potential (Binder et al., 2002). In contrast, longer-term application of relaxin (up to 8 days) reduces mammary xenograft growth in mice (Radestock et al., 2008). The elevated serum relaxin levels that have been reported in breast cancer patients are associated with metastatic disease (Binder et al., 2004). Relaxin is also associated with prostate cancer progression in the mouse xenograft model (Silvertown et al., 2006), and blocking the actions of relaxin or its receptor in this model decreases cancer growth (Feng et al., 2010). In patients with prostate cancer, elevated relaxin levels are linked to cancer progression, metastasis, and androgen independence (Thompson et al., 2006). Likewise, relaxin heightens the collagenolytic potency of thyroid cancer cells by upregulating MMP-2 that facilitates greater in vitro invasiveness (Bialek et al., 2011). In human osteosarcoma cells, relaxin promotes faster in vitro growth, invasion, and angiogenesis via the Akt and VEGF pathways, and small interfering RNA-mediated knockdown of relaxin greatly mitigates these effects (Ma et al., 2013a,b). The peptide hastens the proliferation of human leiomyoma cells (Suzuki et al., 2012). A clinical study in 108 patients with hepatocellular carcinoma identified expression of relaxin in tumor tissue as an independent predictor of poor prognosis (Pan et al., 2013). Similarly, high plasma relaxin levels were associated with a worse prognosis in 146 patients with esophageal squamous cell carcinoma (Ren et al., 2013). A recent study also suggests that CTRP8 interacts with RXFP1 to facilitate cell migration in brain cancer, dependent on activation of PI3K and PKC (Glogowska et al., 2013). In summary, relaxin does

not appear to initiate cancer, but, like other growth and angiogenic hormones, it may promote cancer growth and/or spread by its actions on enhanced matrix degradation and angiogenesis. To date, though lacking definitive proof, this might be clinically relevant for mammary, thyroid, and prostate cancer.

9. *Diabetes*. Although relaxin shares many structural similarities with insulin (Schwabe and McDonald, 1977), the peptides interact with different receptors and activate quite different signal transduction mechanisms. RXFP1 is a GPCR, whereas insulin receptors are tyrosine kinases and relaxin and insulin do not display cross-reactivity. Although this would appear to argue against any connection between the hormones, there are studies that suggest that relaxin can influence carbohydrate metabolism. For instance, many insulin-dependent diabetic women have hypoglycemic episodes during the first trimester of pregnancy, when circulating levels of relaxin are higher, whereas increased insulin may be required during the third trimester, when relaxin concentration are lowest (O'Byrne et al., 1978). Additionally, intraperitoneal dosing of human relaxin inhibits ad libitum feeding in male rats when given during the early dark phase of the circadian clock (McGowan et al., 2010).

Relaxin concentrations in the serum of diabetic women are higher than those observed in the serum of non-diabetic women during each trimester of pregnancy (Steinetz et al., 1992; Whittaker et al., 2003). One possible explanation is that the increased relaxin is a physiologic response to the insulin-resistance that occurs during pregnancy. More circumstantial evidence comes from studies showing that circulating relaxin levels correlate positively with insulin sensitivity and inversely with  $\beta$ -cell function in women with type-2 diabetes mellitus (T2DM) (Szepietowska et al., 2008) and studies showing an increase in plasma relaxin after successful antidiabetic therapy in men with T2DM (Schondorf et al., 2007). The cellular mechanism is not clear, but it has been reported that relaxin increases the binding of insulin to its receptors on adipocytes (Olefsky et al., 1982; Jarrett et al., 1984) an effect that would enhance glucose uptake. In rat cardiac fibroblasts, high-glucose conditions increase the expression of endogenous relaxin (Wang et al., 2009). In human amniotic epithelial cells, relaxin enhances expression of insulin-like growth factor-2 (Millar et al., 2003). In pre-clinical studies in lean mice that are insulin-resistant after a high-fat diet, relaxin increases glucose uptake into skeletal muscle by improving hemodynamics and angiogenesis that is known to be an important factor in determining the response to insulin (Bonner et al., 2013). More recently, relaxin applied over 12 days in a genetic mouse model of T2DM lowered blood glucose levels, from an average of 660 mg/dl in vehicle-treated animals to 490 mg/dl (Bitto et al., 2013). In addition, relaxin has also been shown to activate peroxisome proliferator-activated

receptor- $\gamma$  and enhance the actions of the peroxisome proliferator-activated receptor- $\gamma$  activator rosiglitazone in cells expressing RXFP1 (Singh and Bennett, 2010). In this way relaxin treatment would be expected to influence insulin sensitivity.

Originally identified for its effects on the female reproductive tract, relaxin has now been recognized to have actions in the central nervous system (CNS), cardiovascular system, and kidney, on glucose metabolism and in cancer metastasis in both males and females. Although it has important and well defined roles in pregnancy and reproduction in many species, it is not mandatory for successful pregnancy in humans. The vasodilatory and antifibrotic actions of relaxin on blood vessels are the subject of intense interest, and relaxin was recently shown to produce inotropic responses in human atria. Studies of the antifibrotic actions of relaxin indicate that the effects are dependent on expression of the AT<sub>2</sub>R, raising the question whether heterodimer formation between RXFP1 and other GPCRs underpins other physiologic responses to the hormone. Connections have also been established between relaxin and the formation and spread of tumors, although the mechanisms involved are less clear. Relaxin also appears to improve insulin sensitivity and improves glucose tolerance in animal models of T2DM.

### *B. Insulin-Like Peptide 3 and Relaxin Family Peptide Receptor 2*

1. *Reproduction*. INSL3 is primarily a reproductive hormone and was first cloned from testicular cDNA libraries (Adham et al., 1993; Pusch et al., 1996). The peptide is secreted by testicular Leydig cells (Adham et al., 1993; Pusch et al., 1996) but is also found in human cyclic corpora lutea (Tashima et al., 1995); ruminant ovary, uterus, and placenta (Bathgate et al., 1996; Roche et al., 1996); mouse (Zimmermann et al., 1997); and marmoset ovary (Zarreh-Hoshiyari-Khah et al., 1999). Importantly INSL3 is a circulating hormone in both males (Foresta et al., 2004) and females (Anand-Ivell et al., 2013), although the levels are much higher in males.

The best characterized physiologic role for INSL3 is in the male where it controls testis descent. Male INSL3 knockout mice show normal development but are infertile and bilaterally cryptorchid, with the testis located high in the abdominal cavity adjacent to the kidney (Nef and Parada, 1999; Zimmermann et al., 1999). A similar phenotype is displayed in RXFP2 knockout mice, confirming the cryptorchid phenotype (Overbeek et al., 2001; Gorlov et al., 2002). Thus, the INSL3/RXFP2 system appears to be essential for the development of the gubernaculum during embryogenesis and for normal transabdominal testicular descent.

Cryptorchidism is the most common birth defect of the male genitalia; affecting 1–4% of live male births, with a greater incidence in premature infants (Ivell and

Hartung, 2003). Although the studies in *INSL3* or *RXFP2* knockout mice suggested that mutations in these genes could account for cryptorchidism, this is unlikely to be the case in all human infants, because few of these mutations result in a functional change in either the peptide or the receptor (Tomboc et al., 2000; Gorlov et al., 2002). Furthermore, because all mutations of *INSL3* and *RXFP2* identified in cryptorchid patients have been found only as heterozygotes and the expression of *INSL3* in testicular Leydig cells is high, it is unlikely that a mutation in a single allele would be sufficient to induce bilateral cryptorchidism.

*INSL3* is highly expressed in the testicular Leydig cells in adult mammals and is considered an important marker of Leydig cell function (Ivell and Anand-Ivell, 2009; Ivell et al., 2014). Additionally, *INSL3* circulates at nanogram per milliliter levels in males and therefore has potential endocrine functions such as that on bone function. *RXFP2* receptors are expressed on Leydig cells (Anand-Ivell et al., 2006) and on germ cells in the seminiferous tubules (Kawamura et al., 2004; Anand-Ivell et al., 2006; Filonzi et al., 2007). A recent study suggested that *INSL3* influences steroid production by Leydig cells (Pathirana et al., 2012), and studies in male rats suggest that *INSL3* is a paracrine factor involved in male germ cell survival (Kawamura et al., 2004). However, it should be noted that androgen production is normal in *INSL3* and *RXFP2* knockout mice (Nef and Parada, 1999; Zimmermann et al., 1999; Overbeek et al., 2001; Gorlov et al., 2002). Additionally, conditional deletion of *RXFP2* in male germ cells resulted in normal fertility (Huang et al., 2012).

In females, *INSL3* is produced in the thecal cells of the ovary and in the corpus luteum (Ivell and Bathgate, 2002). In mouse ovaries, *Insl3* expression is higher in the follicular than the luteal phase (Zimmermann et al., 1997), and female *INSL3* knockout mice demonstrate impaired fertility associated with longer estrous cycle length, smaller litter sizes, accelerated follicular atresia and luteolysis, and premature loss of corpora lutea (Nef and Parada, 1999; Spaniel-Borowski et al., 2001). Further evidence that *INSL3* is a circulating hormone of ovarian origin in females comes from the observation that postmenopausal women have undetectable plasma *INSL3* levels (Ivell and Anand-Ivell, 2009). Studies in cohorts of adult women suggest that *INSL3* plasma levels are indicative of the number of ovarian antral follicles recruited and growing within a follicular wave (Anand-Ivell et al., 2013). As *INSL3* is a product of the thecal cells of ovarian follicles, plasma *INSL3* levels have been investigated and shown to be elevated in polycystic ovary syndrome, a common female endocrine disorder characterized by excessive production of androgens from ovarian follicles (Gambineri et al., 2007; Szydlarska et al., 2012; Anand-Ivell et al., 2013). Importantly, a recent study in cows demonstrated that *INSL3* has a positive autoregulatory role in maintaining thecal androgen production that

is essential for normal ovarian follicle development (Glister et al., 2013).

2. *Brain*. *RXFP2* expression has been found in human brain by RT-PCR (Hsu et al., 2002) and in the rat parafascicular nucleus, dorsolateral, ventrolateral, and posterior thalamic nuclei and medial habenula by in situ hybridization histochemistry (Shen et al., 2005; Sedaghat et al., 2008). These sites correspond to the distribution of *RXFP2* determined by [<sup>125</sup>I]*INSL3* binding (Sedaghat et al., 2008). *Insl3* expression has been demonstrated in the rat brain (Gundlach, personal communication) and also in the bovine hypothalamus by RT-PCR (Bathgate et al., 1996) and Northern blotting (Bathgate et al., 1999). The effects of *INSL3* administration to rat brain suggest that *RXFP2* influences sensorimotor function (Sedaghat et al., 2008).

3. *Bone and Other Tissues*. Physiologic functions of *INSL3* in other tissues have been postulated based on *INSL3* and *RXFP2* expression patterns. In young adults with cryptorchid hypogonadism associated with the T222P mutation of *RXFP2*, 64% had reduced bone density but normal plasma testosterone levels (Ferlin et al., 2008). *RXFP2* mRNA and protein are present in human osteoblasts, and an osteoblast cell line responded to *INSL3* with a concentration-dependent increase in cAMP. Mouse osteoblasts also express *RXFP2*, but not *Insl3*, and *RXFP2* knockout mice are osteopenic and have functional osteoblast impairment (Ferlin et al., 2008). *INSL3* regulates expression of genes required for proliferation and differentiation, matrix deposition, and osteoclastogenesis in cultured human osteoblasts (Pepe et al., 2009). Deficits in *INSL3/RXFP2* signaling are correlated with reduced bone mass (Ferlin et al., 2008, 2009). Thus *INSL3* may have a role in bone physiology, and *RXFP2* mutations may be linked with osteoporosis in men (Ferlin et al., 2013). Interestingly, recent studies suggest that *RXFP2* is associated with horn growth in sheep (Johnston et al., 2013) and cows (Wiedemar et al., 2014), suggesting actions of *INSL3* on bone is a common feature in male mammals.

In tumor biology, there is altered expression of *INSL3* and a splice variant in human hyperplastic thyroid adenoma and thyroid cancer (Hombach-Klonisch et al., 2003). *INSL3* is also present in human prostate carcinomas and increases motility in the human androgen-insensitive prostate carcinoma cell line PC-3 (Klonisch et al., 2005). All human thyroid adenomas, three types of human thyroid carcinomas, and mouse noncancerous follicular epithelial cells of the thyroid express *RXFP2* mRNA (Hombach-Klonisch et al., 2010). Thyroid cancer cells expressing *INSL3* demonstrate enhanced motility and increased colony formation in vitro and enhanced tumor growth in vivo. Finally, *INSL3* increases levels of the calcium binding protein S100A4, which increases cancer cell mobility and enhances tumor tissue vascularization, suggesting that S100A4 is a downstream target of *RXFP2* signaling in thyroid cells (Hombach-Klonisch



et al., 2010). Thus INSL3 and RXFP2 appear to play a role in cancer metastasis.

*RXFP2* mRNA expression is highest in rat kidney at late stage gestation and decreases dramatically at birth with lowest levels in adulthood (Fu et al., 2006). *RXFP2* is expressed in mesangial cells in mature glomeruli and inhibits proliferation of cultured primary glomerular cells, suggesting that INSL3 and *RXFP2* influence the genesis or maturation of renal glomeruli and regulate mesangial cell density (Fu et al., 2006). *Pod1* is one of the main transcription factors involved in glomerulogenesis (Quaggin et al., 1999), and an E-box consensus sequence capable of binding *Pod1* in conjunction with other helix loop helix transcription factors is located upstream of the gene for *RXFP2* (Funato et al., 2003). Therefore, *Pod1* may regulate expression of *RXFP2* in the kidney during development to facilitate glomerular cell proliferation (Familarì et al., 2009). *RXFP2* expression levels in the embryonic kidney were significantly greater in *Pod1* knockout mice than in heterozygous or wild-type controls, indicating that *RXFP2* is downstream of *Pod1* and *Pod1* negatively regulates expression of *RXFP2* in the glomeruli (Familarì et al., 2009).

Although the INSL3 and *RXFP2* system has primarily been studied for its specialized roles in both male and female reproductive physiology, there is emerging evidence to suggest that this system may have other roles in the CNS, in cancer metastasis, in bone physiology and in the kidney.

### C. Relaxin-3 and Relaxin Family Peptide Receptor 3

Relaxin-3 and *RXFP3* expression is highest in the brain, and most studies examine roles in the CNS. The evidence for the receptor-ligand pair includes their coevolution (Wilkinson et al., 2005a), their coexpression in brain (Bathgate et al., 2002; Burazin et al., 2002; Liu et al., 2003b; Tanaka et al., 2005; Ma et al., 2009b,c; Smith et al., 2010, 2011), and the high affinity of relaxin-3 for *RXFP3* (Liu et al., 2003b; van der Westhuizen et al., 2007, 2010). Although relaxin-3 may interact with *RXFP1* (Sudo et al., 2003) and *RXFP4* (Liu et al., 2003a) and possibly *RXFP2* in some species (Scott et al., 2005a), these interactions are probably not important physiologically because the expression pattern of relaxin-3 suggests that it would be unlikely to interact with the other *RXFP* receptors.

**1. Stress.** Relaxin-3 and *RXFP3* are present in hypothalamic and extrahypothalamic regions involved in the hypothalamic-pituitary-adrenal axis (Sutton et al., 2004; Liu et al., 2005a; Ma et al., 2007a; Smith et al., 2010). Corticotropin releasing factor (CRF) is synthesized and released from the paraventricular nucleus of the hypothalamus (PVN) in response to stress. *RXFP3* is highly expressed in the PVN, as well as other regions associated with stress and anxiety, such as the bed nucleus of the stria terminalis, lateral septum, periaqueductal gray, and dorsal raphe (Liu et al., 2005a). Neural responses to CRF are

mediated by the CRF<sub>1</sub> receptor that is expressed in most relaxin-3-containing neurons in the NI (Sutton et al., 1982; Tanaka et al., 2005). Administration of CRF intracerebroventricularly to rats caused activation of relaxin-3-containing neurons, and neurogenic stressors increased relaxin-3 mRNA in the NI (Tanaka et al., 2005), where relaxin-3 is found in presynaptic vesicles. Relaxin-3 mRNA in the rat NI is increased after a forced swim stress paradigm (Banerjee et al., 2010), and these effects are partly blocked by pretreatment with the CRF<sub>1</sub> antagonist antalarmin. Although the precise CRF neuronal projection(s) to the NI relaxin-3 cells have not yet been described, hypothalamic and preoptic areas containing CRF neurons have been shown to project to the NI (Goto et al., 2001). CRF levels in rat, monkey, or human CSF are in the picomolar range even in pathologic states (Garrick et al., 1987; Arborelius et al., 1999). Altered relaxin-3 expression in the NI due to stress is therefore unlikely to result from CRF in the spinal fluid and most likely comes from CRF neuronal projection(s). Ascending projections from the NI (Olucha-Bordonau et al., 2003) are consistent with *RXFP3* receptor autoradiography (Sutton et al., 2004) and with involvement of relaxin-3/*RXFP3* in neuropsychiatric disease (Smith et al., 2010).

**2. Feeding and Metabolism.** In rodents, *RXFP3* is expressed in the PVN and supraoptic nucleus (Smith et al., 2010, 2011), suggesting a role in metabolic control. Injection of relaxin-3 intracerebroventricularly to rats either in the early light or early dark phase, transiently increased food intake (McGowan et al., 2005). This stimulatory effect did not result from increased spontaneous activity or arousal and is likely mediated by *RXFP3* and not *RXFP1*, because human relaxin administration had no effect when given during the early light phase (McGowan et al., 2005) or decreased feeding when given during the early dark phase (McGowan et al., 2010) in ad libitum-fed rats. The inhibitory feeding response to human relaxin when administered in the dark as opposed to the light phase suggests a circadian influence involving *RXFP1* that is also expressed in hypothalamic centers associated with feeding (Ma et al., 2006).

Both acute and chronic relaxin-3 infusions into the PVN increased food intake (McGowan et al., 2006). Injection into the supraoptic nucleus, arcuate nucleus, and anterior preoptic area also transiently increased food intake. However, it is unclear how relaxin-3 exerts its effects in the arcuate nucleus or anterior preoptic area, because *RXFP3* is not present in these nuclei. Finally, intracerebroventricular infusion of the selective *RXFP3* agonist R3/I5 also increased food intake, an effect blocked by preadministration of the antagonist R3 (BA23–27)/I5 (Kuei et al., 2007). Administration of R3 (BA23–27)/I5 intracerebroventricularly alone has no effect on food intake (Sutton et al., 2009), suggesting that under resting conditions there is minimal tone in the relaxin-3/*RXFP3* system.

Although relaxin-3 influences food intake, effects on body weight are equivocal. In two studies, chronic intracerebroventricular relaxin-3 increased body weight (Hida et al., 2006; Sutton et al., 2009), but others show no weight change after intracerebroventricular or intrahypothalamic injection of relaxin-3 (McGowan et al., 2005, 2006). In rats, chronic intracerebroventricular administration of the antagonist R3(B $\Delta$ 23–27)R/I5 did not affect body weight (Sutton et al., 2009). There is also little evidence to suggest that body weight is influenced by endogenous relaxin-3. Although relaxin-3 knockout mice were initially reported to have lower body weights, the first study (Sutton et al., 2009) was performed on a mixed 129:B6 mouse strain before the mice were backcrossed, and the differential phenotype could not be reproduced on a C57/B16N background. This dependence of phenotype on strain suggests there may be compensation for a chronic absence of relaxin-3 by other systems.

Several studies investigating the effects of relaxin-3 infusion on feeding also measured blood hormone levels. In rats, chronic infusion of RXFP3 agonist (relaxin-3 or R3/I5) intracerebroventricularly increased plasma leptin, insulin (Hida et al., 2006), adiponectin, testosterone, angiotensinogen, and decreased growth hormone levels (Sutton et al., 2009). Administration into the PVN caused increases in leptin in ad libitum-fed animals and in thyroid-stimulating hormone in ad libitum- and pair-fed animals (McGowan et al., 2006). Intra-PVN agonist dosing increased feeding but failed to significantly change other behaviors such as drinking, grooming, burrowing, rearing, general locomotion, apparent sleep, head down, or tremor (McGowan et al., 2005). Similarly, chronic R3(B $\Delta$ 23–27)R/I5 dosing seemed only to decrease plasma levels of growth hormone (Sutton et al., 2009) while not altering the other hormones measured. These effects occurred in the absence of changes in energy expenditure.

Studies of the metabolic role of relaxin-3 have provided only limited supporting mechanisms, although considerable evidence supports the actions of the peptide on feeding behavior. Although there is RXFP3 expression in the pancreas and in vitro relaxin-3 suppression of insulin secretion has been reported in isolated tissue (Yamamoto et al., 2009), human plasma relaxin-3 levels are in the picograms per milliliter (low picomolar) range and do not vary in the diabetic state (Zhang et al., 2013). Considering relaxin-3/RXFP3 expression occurs in CNS areas associated with neuroendocrine and behavioral control (Sutton et al., 2004; Ma et al., 2009a; Ganella et al., 2013b), metabolic effects of an RXFP3 agonist may be largely secondary to increased feeding behavior. As noted above, central injection of an RXFP3 agonist in rats increased feeding both acutely (McGowan et al., 2005) and chronically (Hida et al., 2006; Sutton et al., 2009), suggesting a lack of tolerance. In a second rat model, chronic viral expression of an RXFP3 agonist achieved similar results (Ganella et al., 2013a,b). Acute or chronic dosing of an antagonist alone to rats was ineffective (Sutton et al., 2009), suggesting that relaxin-3

tone is low under resting physiologic conditions. Conversely in mice, RXFP3 agonist dosing does not increase feeding under resting conditions (Ganella et al., 2013b). A recent study showed that an RXFP3 antagonist, although ineffective in altering feeding in baseline/sated mice, blocked motivated feeding behavior (Smith et al., 2014). In this study, R3(B1–22)R/I5 blocked food anticipatory activity after a 4-hour food restriction when administered intracerebroventricularly to C57/B16J control mice but not to congenic relaxin-3 knockout mice. Antagonist treatment also significantly reduced feeding in mice trained to expect palatable food at a given time. Although relaxin-3 and RXFP3 neuroanatomy is consistent with effects on feeding and body weight in monkeys (Ma et al., 2009b,c), more studies are needed to predict translation of these effects to humans. In addition, detailed studies are also needed to tease out potential confounding effects on reward, motivation and arousal (see below) and to determine whether there is a true feeding response to relaxin-3 stimulation.

**3. Behavioral Activation and Arousal.** Evidence for a role for the relaxin-3/RXFP3 system in behavioral activation and arousal is derived from studies that examined colocalization of projections of relaxin-3 neurons from the NI to septal GABAergic and cholinergic neurons that project to the hippocampus (Ma et al., 2009a). The rodent septohippocampal pathway is heavily innervated by relaxin-3 positive projections from the NI. This pathway generates the hippocampal theta rhythm, with oscillations at 4–12 Hz that is controlled by pacemaker neurons of the medial septum (MS). The hippocampal theta rhythm is involved in behaviors such as vigilance, exploration, orientation, navigation, locomotor control, and working memory. Electrical stimulation of the NI causes theta rhythm in the hippocampus and lesions of the NI disrupt theta rhythm initiated by stimulation of the reticularis pontine oralis (Nunez et al., 2006). In both anesthetized and conscious rats, RXFP3 modulates neuronal activity in the hippocampus and MS to promote hippocampal theta rhythm (Ma et al., 2009a) and blockade of RXFP3 in the MS with the antagonist R3(B $\Delta$ 23–27)R/I5 dose dependently impairs performance in a paradigm investigating theta rhythm-dependent spatial working memory, effects that are reversed by coadministration of the RXFP3 selective agonist R3/I5 (Ma et al., 2009a).

The relaxin-3/RXFP3 system also influences locomotor activity in rodents. Although chronic intracerebroventricular relaxin-3 had no effect on locomotor activity in male Wistar rats (Hida et al., 2006), acute injection of the selective RXFP3 agonist R3/I5 increased locomotor activity, whereas the antagonist R3(B $\Delta$ 23–27)R/I5 given alone had no effect (Sutton et al., 2009). Effects of the RXFP3 agonist on general locomotor activity, although statistically significant in one study (Sutton et al., 2009), seem to be small in magnitude and were not reproduced after intra-PVN injections (McGowan et al., 2005). Interestingly,

female relaxin-3 knockout mice were hypoactive relative to wild-type littermates in several paradigms including the locomotor cell, large open field, Y-maze, and novel object tests (Smith et al., 2009; Hosken et al., 2014). A similar phenotype is displayed in mice receiving the RXFP3 antagonist R3(B1–22)RI5 or R3(B1–22)R (Smith et al., 2014).

Because serotonin (5-hydroxytryptamine [5-HT]) has well established roles in cognitive, emotional, and behavioral control (reviewed in Cools et al., 2008) and the NI is located close to the dorsal raphe, a region enriched in 5-HT neurons, studies were carried out on the effects of 5-HT on relaxin-3 expression (Miyamoto et al., 2008); most relaxin-3-containing neurons of the NI were found to coexpress 5-HT<sub>1A</sub> receptors. Inhibition of 5-HT synthesis for 3 days increased relaxin-3 mRNA in the NI. The correlation between 5-HT/5-HT<sub>1A</sub> inactivity (because of 5-HT depletion) and altered relaxin-3 expression in the NI suggests a role for these systems in anxiety and/or depression. Recent studies show anxiolytic and antidepressant effects of intracerebroventricular RXFP3 agonist dosing in behavioral rat models (Ryan et al., 2013a). These studies emphasize the need to determine the relationship between 5-HT and relaxin-3/RXFP3 systems.

Recent studies in rats link RXFP3 circuits to ethanol self-administration and reinstatement behaviors (Ryan et al., 2013b). The R3(BΔ23–27)RI5 or R3(B1–22)R RXFP3 antagonists administered intracerebroventricularly reduced alcohol self-administration without concomitant alterations in feeding, locomotor behaviors, or memory. The effects on alcohol self-administration were specific, because the antagonists had no effect on sucrose self-administration. In addition, RXFP3 antagonism blocked both cue and stress-induced reinstatement of alcohol seeking. The reduction of alcohol self-administration/reinstatement was linked to the stress-responsive bed nucleus of the stria terminalis, because direct injection of the RXFP3 agonists into the bed nucleus of the stria terminalis also reduced alcohol self-administration.

Extensive brain mapping and behavioral studies have established that the relaxin-3 and RXFP3 system is involved in stress, metabolic control, and behavioral activation and arousal. Recent studies suggest that modulation of the system could be useful in the treatment of forms of addiction.

#### *D. Insulin-Like Peptide 5 and Relaxin Family Peptide Receptor 4*

Although RXFP4 was originally identified as a receptor for relaxin-3 (Liu et al., 2003a), the dramatically different expression patterns of ligand and receptor suggested that relaxin-3 was not the preferred cognate ligand. *RXFP4* mRNA is present in a variety of human tissues including brain, kidney, testis, thymus, placenta, prostate, salivary gland, thyroid, and colon (Liu et al., 2005b; Burnicka-Turek et al., 2012; Mashima et al.,

2013; Thanasupawat et al., 2013). Many of these tissues also express *INSL5* (Burnicka-Turek et al., 2012; Mashima et al., 2013; Thanasupawat et al., 2013), and both *RXFP4* and *Insl5* are pseudogenes in rats and dogs (Chen et al., 2005; Wilkinson et al., 2005a). *INSL5* was shown to be a high-affinity ligand for RXFP4 (Liu et al., 2005b), and thus the receptor-ligand coevolution, pharmacology, and similarities in expression profiles strongly indicate that RXFP4 is the endogenous receptor for *INSL5*.

*1. Metabolism.* There is considerable interest in GPCRs that are located in pancreatic islet  $\beta$ -cells and represent potential targets for the treatment of T2DM (Ahren, 2009). One of the most studied systems is the incretin hormone system: glucagon-like peptide 1 (GLP-1) and its receptor GLP-1R. GLP-1 is secreted from enteroendocrine-L cells that are located in the gastrointestinal tract and concentrated in the colon and rectum. The peptide acts on GLP-1R located in a number of tissues including the GI tract, heart, kidney, lung, pancreatic  $\beta$ -cells, and brain. GLP-1 increases insulin release from the pancreas and improves glucose tolerance (Ahren, 2009). The *INSL5* and *RXFP4* system displays similarities with the incretins. Like GLP-1, *INSL5* is concentrated and released from enteroendocrine-L cells (Grosse et al., 2014) into the circulation to act on RXFP4 located in a number of other tissues. In the mouse, RXFP4 expression has been detected by Western blotting in hypothalamus, pituitary, testis, epididymis, ovary, uterus, pancreas, and liver, with immunohistochemical localization to pancreatic islets, anterior pituitary, and Leydig cells in the testis (Burnicka-Turek et al., 2012). In humans, using a similar approach, RXFP4 was detected in heart, placenta, skeletal muscle, and pancreas (Mashima et al., 2013). Most interestingly, the development of the *Insl5* knockout mouse showed that these animals have impaired glucose homeostasis. Glucose levels increase with age, and in *Insl5*<sup>-/-</sup> mice older than 6 months, glucose levels are significantly greater than age-matched control littermates. Glucose tolerance tests revealed impaired glucose tolerance in *Insl5*<sup>-/-</sup> mice that does not appear to be associated with changes in insulin sensitivity. The knockouts also had reduced pancreatic islet area compared with controls, which was associated with a reduced number of  $\beta$ -cells and lower circulating insulin levels. However there was no change in circulating GLP-1 levels (Burnicka-Turek et al., 2012). *Insl5*<sup>-/-</sup> mice also displayed impaired fertility due to a reduction in sperm motility and alterations in the estrus cycle (Burnicka-Turek et al., 2012). Interestingly, recent association studies in humans suggest that polymorphisms of RXFP4 are associated with a high body mass index and show a trend to association with obesity (Munro et al., 2012). Studies in mice show an increase in food intake in wild-type mice after intraperitoneal injection of *INSL5* that is not seen in RXFP4 knockout mice (Grosse et al., 2014). Plasma *INSL5* levels increase with fasting or calorie restriction but are lowered by feeding adding weight to the suggestion that

INSL5 is an orixigenic hormone released from the enteroendocrine-L cells in the gut (Grosse et al., 2014). At present, little is known of the potential mechanisms used by INSL5 and RXFP4 to produce metabolic effects, and a number of apparent anomalies will have to be addressed. For instance, to date all  $\beta$ -islet cell GPCRs that increase insulin secretion are either  $G\alpha_s$  or  $G\alpha_q$  coupled, and  $G\alpha_i$ -coupled receptors actually reduce insulin secretion (Ahren, 2009). This appears to conflict with current evidence suggesting that RXFP4 is  $G\alpha_i$ -coupled (Liu et al., 2003a, 2005a), although very little work has focused on RXFP4 and no studies have directly examined receptor function in pancreatic islets.

The emergence of recent evidence that INSL5 is an incretin secreted from the enteroendocrine-L cells and that both INSL5 and RXFP4 knockout mice display impaired glucose tolerance has awakened considerable interest in this ligand/receptor pair. However, more detailed biologic studies are required to firmly establish its role in metabolic control processes.

## VI. Unresolved Pharmacological and Therapeutic Issues and Future Directions

Although there have been significant advances in our understanding of the physiologic roles of relaxin family peptides and their receptor systems in recent years, there are still significant knowledge gaps. There is increasing recognition of the complexity of relaxin signaling, with recent studies suggesting that some of the pleiotropic actions of relaxin may be explained by the formation of heterodimer complexes with other GPCRs. Although this may unveil new opportunities for therapeutic intervention it may also explain why relaxin has failed in several clinical trials despite a promising preclinical profile. Clearly if heterodimer complexes are formed and are functional in pathologic conditions and blockade of the partner receptor can prevent the actions of relaxin, then a thorough understanding of the condition and treatments being received by the patient will be necessary to obtain the required therapeutic outcome. Significant new tools have emerged for the study of RXFP receptors, including small molecule allosteric agonists at RXFP1 and refined peptide agonists and antagonists at all RXFP receptors, together with a better understanding of the structure-activity relationships of the peptides and their receptors. However much remains to be done to identify the signaling patterns associated with the new molecules and how these properties can be used therapeutically. Likewise, although we now know a great deal more about the functional domains of the RXFP1 LDLa module, it is not yet apparent how it mediates signaling in response to relaxin. It is possible that it constitutes a tethered ligand, but definitive evidence has yet to be obtained.

A long-standing unresolved issue is whether there are physiologically relevant levels of relaxin circulating in nonpregnant women or in men. As discussed previously

(see section V.A.1), relaxin is produced in the human ovary during the luteal phase of the menstrual cycle and can be measured in the circulation at this time (Eddie et al., 1990b; Stewart et al., 1990; Chen et al., 2003). Levels at other stages of the cycle are very low, and there is no evidence of circulating relaxin in postmenopausal women. It is still contentious as to whether there are physiologically relevant levels of circulating relaxin in men. Various studies have examined plasma relaxin levels in both male and female patients with cardiovascular disease with varied results (see section V.A.4). One problem with these studies is the use of numerous different immunoassays, most of which are incompletely characterized and the fact that relaxin levels in female patients have never been correlated with reproductive status (Du et al., 2010). However it should also be noted that relaxin is produced locally in many tissues, and it is possible that many actions of relaxin are associated with paracrine production rather than circulating relaxin in nonpregnant women and men. It is also feasible that the normally low circulating relaxin concentrations that are difficult to measure by current methods do have a physiologic function. The recent evidence for relaxin signaling at highly sensitive RXFP1 signalosomes that respond to subpicomolar concentrations of relaxin provides a basis for responses to circulating peptide although their physiologic function is still unclear.

### A. Signaling Paradigms

1. *Heterodimerization of Relaxin Family Peptide Receptor 1: A Possible Explanation for the Selective Actions of Relaxin in Diseased or Pathologic States?* Although there is evidence that RXFP1 and RXFP2 form homo- and heterodimers in cell lines (Kern et al., 2008; Svendsen et al., 2008a,b), a recent study demonstrated that the antifibrotic effects of relaxin in kidney are dependent on heterodimerization between RXFP1 and the angiotensin  $AT_2R$  (see section III.A.4; Chow et al., 2014). The inhibitory effects of relaxin on excess collagen deposition were completely lost in mice treated with the  $AT_2R$  antagonist PD123319 or in  $AT_2R$  knockout mice, and by using BRET in cell studies, the two receptors were found to constitutively associate. These findings are particularly interesting, because they provide an explanation for the established observation that the antifibrotic effects of relaxin are seen only in pathologic conditions, with no effect on healthy tissue. Under normal conditions,  $AT_2R$  are typically expressed at very low levels, but their expression dramatically increases after injury or disease (Matsubara, 1998; Carey, 2005). RXFP1 is also expressed at extremely low levels at the cell surface, with much higher levels intracellularly within the ER (Kern et al., 2007). Because many GPCRs require dimerization for trafficking from the ER to the cell surface (Milligan, 2009), the injury-induced increase in  $AT_2R$  expression may result in an associated enhanced trafficking of RXFP1 (as an

AT<sub>2</sub>R-RXFP1 heterodimer) to the cell surface, thus explaining why functional effects of relaxin treatment only occur under specific pathologic conditions.

The relevance of RXFP1 heterodimerization—with the AT<sub>2</sub>R and perhaps other GPCRs—in many of the other physiologic roles of relaxin remains to be determined. This may be a general requirement for relaxin/RXFP1 function, given the wide and pleiotropic effects of relaxin compared with the relatively limited and variable phenotype of relaxin and RXFP1 knockout and transgenic mice (Zhao et al., 1999, 2000; Samuel et al., 2003a, b, 2004b; Kamat et al., 2004; Krajnc-Franken et al., 2004; Feng et al., 2006; Ganesan et al., 2009). IUPHAR requires two of the following three conditions to be met to definitively determine the existence of heterodimers in native tissue: 1) evidence for physical association in native tissues or primary cells; 2) a specific functional property for the heterodimeric receptor in native tissue; and 3) use of knockout animals or RNAi to demonstrate heterodimers in vivo (Pin et al., 2007). Although conditions 2 and 3 were demonstrated for the RXFP1-AT<sub>2</sub>R interaction, questions still remain regarding the interaction between RXFP1 and AT<sub>2</sub>R, including a direct demonstration of receptor interaction in endogenous expression systems, whether RXFP1 usually exists as a monomer and only dimerizes with the AT<sub>2</sub>R under pathologic conditions and whether this new knowledge can be exploited to specifically target RXFP1 in disease states.

*2. The Development of ML290, a Small-Molecular-Weight Allosteric Agonist at Relaxin Family Peptide Receptor 1.* Relaxin binding to and activation of its receptor RXFP1 involves a number of complex interactions and requires many essential receptor regions from the N-terminal LDLa module to the extracellular LRR and TM loops to the full-length C-terminal tail (see section I.B.1). As such, the development of novel molecules that mimic the actions of relaxin at RXFP1 has been a difficult challenge over a number of decades. However, the recent description of a small molecule agonist for RXFP1, ML290 (Xiao et al., 2013), has reignited the search for small molecule RXFP1 ligands.

Based on the size discrepancy between relaxin and ML290 (6000 versus 500 kDa) and some preliminary studies, it was considered unlikely that ML290 bound to the same regions of RXFP1 as the cognate ligand. ML290 also displayed species-specific actions, and studies of chimeras of mouse and human RXFP1 showed that ML290 binds to a region in ECL3 (Xiao et al., 2013) (see section II.A.3). Binding to a topographically distinct region of the receptor suggests a number of exciting and unique attributes for small molecule activators of RXFP1. First, although there was no evidence for signal bias by native relaxin peptides or peptide-based derivatives at RXFP1 (M. Kocan, C. Siwek, et al., personal communication), small molecule ligands binding to a distinct receptor region are likely to engender unique receptor conformations distinct from those induced by relaxin. A common

corollary of this is that the signaling profile of ML290 is likely to be biased relative to relaxin. Second, binding of a small molecule to a site topographically distinct from the orthosteric (relaxin) binding sites, suggests an allosteric mode of action. Allosteric modulation of endogenous ligands has been widely documented for many other GPCRs (reviewed in Christopoulos, 2002, and May et al., 2007). Allosteric ligands are considered highly attractive as therapeutics, because of their ability to fine tune the spatial and temporal subtleties of endogenous ligand-receptor interactions (as both allosteric and orthosteric ligands can be simultaneously bound) and their potential for increased receptor selectivity (as these sites have not typically evolved to accommodate a common endogenous ligand). The potential for fine tuning of the relaxin response by a positive allosteric modulator would be highly beneficial in pathologic fibrosis, whereas a negative allosteric modulator could be beneficial for the prevention of cancer metastases. Finally, although the synthesis of peptide derivatives of relaxin has not yet produced a neutral antagonist, the discovery of a series of small agonist molecules suggests that the development of antagonists for RXFP1 may be possible in the future (because of the complicated activation mechanism of RXFP1, agonists are much more difficult to design than simple blocking/antagonist compounds). Not only are these exciting possibilities likely to result in useful experimental tools to accelerate our understanding of RXFP1 function, they will also provide novel avenues for the future design of disease-specific RXFP1 therapeutics.

*3. Novel Activators of RXFP Receptors.* Over the last few years, a number of novel RXFP ligands have been described, ranging from small molecule or novel peptide activators of RXFP1 to a synthetic biased antagonist and positive allosteric modulator for RXFP3.

A recent study identified a novel protein in brain tumors that can interact with RXFP1 and promote cell invasion in the absence of relaxin (Glogowska et al., 2013). By using primary glioblastomas isolated from human patients, tumor cell migration and invasion in response to CTRP8 was found to be dependent on RXFP1 expression and involved activation of PI3K and PKC. Subsequent experiments showed an interaction between CTRP8 and RXFP1 after coimmunoprecipitation of tagged proteins coexpressed in HEK293 cells. Molecular modeling suggested that CTRP8 may interact with LRR7 and LRR8 of the RXFP1 extracellular domain, although this was not validated experimentally. This study raises some intriguing possibilities that yet again expand the repertoire of this pleiotropic receptor. First, the ability of additional peptides to bind to and activate RXFP1 may provide a further explanation (in addition to RXFP1-signalosomes responding to very low concentrations of relaxin, see section III.A.3) for the surprisingly widespread effects of RXFP1 activation relative to the limited sites of relaxin production. If nonrelaxin peptides can

activate the receptor in different cells, tissues, or pathologies, this also suggests a potential endogenous signaling bias for RXFP1 and may illuminate the pleiotropic effects of receptor activation. Second, and in contrast to the cognate ligand relaxin that requires binding to both the extracellular and TM domains of RXFP1, CTRP8 and ML290 together may provide examples of alternate mechanisms for RXFP1 activation by interacting with a single receptor domain. Although these observations are inherently intriguing, they must still be considered with caution. In both cases, there has been no direct demonstration of receptor binding using traditional radioligand binding paradigms or demonstration of interactions in isolated protein systems, and in the case of CTRP8, no RXFP1 mutagenesis to determine precise sites of receptor binding. Until these basic parameters of ligand-receptor interactions are demonstrated, it is impossible to exclude that the RXFP1-dependent functional effects might occur via interactions with a third protein or even a higher order protein complex.

A number of peptides with unique properties at RXFP3 and RXFP4 have been synthesized and represent powerful scientific tools for the study of this receptor subgroup. A positive allosteric modulator of RXFP3, 135PAM1, was recently described that showed a probe dependence for the C-terminal amidation state of the peptide agonist (Alvarez-Jaimes et al., 2012). Thus, although 135PAM1 positively modulated the cAMP inhibition stimulated by relaxin-3<sub>NH2</sub> and the selective agonist R3I5<sub>NH2</sub> (as determined using a CRE reporter gene), there was no effect of the modulator in combination with nonamidated peptides. A previously described antagonist for RXFP3, R3(BΔ23–27)R/I5 was recently shown to have agonist properties at some signaling pathways (Kocan et al., 2014). Although the peptide antagonized some pathways downstream of relaxin or relaxin-3 activated RXFP3, it did not block others and activated SRE-mediated gene transcription itself. Such biased efficacy of so-called "antagonists" is an increasingly well-documented phenomenon for many GPCRs (Evans et al., 2010). Finally, a minimized INSL5 variant was recently synthesized (Belgi et al., 2013) that contains only the two interchain disulfide bonds that are characteristic of the relaxin family of peptides, with the intra-A-chain disulfide bond and eight residues from the N terminus of the A-chain removed. The minimized INSL5 analogs exhibited higher affinity and efficacy than native INSL5 and are considerably easier to synthesize. This represents an important progression for the INSL5/RXFP4 pairing and should precipitate more studies on this hitherto neglected RXFP receptor.

There have been significant advances in recent years in both our understanding and in the development of tools for the study of signaling pathways activated by relaxin family peptides. The pleiotropic actions of relaxin are now beginning to be understood thanks to the recognition that RXFP1 is able to promote exquisitely

sensitive signaling platforms or signalosomes and that it can form both homo- and heterodimers that have distinct signaling properties. The development of RXFP1 antagonists and small molecular weight allosteric agonists provides novel tools for elucidating the roles of this receptor in physiologic and pathologic conditions. RXFP2 and its cognate ligand INSL3, although structurally closely resembling RXFP1 and relaxin, appear to have relatively few and highly specialized functions. The role of relaxin-3 and RXFP3 as a neuropeptide-receptor pairing is now quite well understood, and there are examples appearing of potent and selective antagonists that promise to be useful tools. The identification of ligand bias at RXFP3 is likely of little physiologic importance owing to the distinct localization of relaxin peptides and receptors in vivo but may be a factor to be borne in mind when potential therapeutics are developed. The recognition of INSL5 as an incretin and the INSL5/RXFP4 system as players in metabolic control has stimulated increased interest in the biology of this, until now, relatively little studied receptor-ligand pair.

## B. Therapeutic Applications

1. *Is There Therapeutic Potential in Small-Molecule Targeting of Relaxin Family Peptide Receptor 1 Heterodimers?* The recent developments showing the importance of RXFP1 heterodimerization and the identification of the small molecule agonist ML290 (see above) may provide some exciting and unique opportunities to therapeutically target RXFP1 with minimal side effects. The therapeutic potential of many pharmaceutical drugs is often limited by the development of toxic effects that can result from drug binding to nonspecific sites or drug-target binding in nontherapeutically relevant tissues. If heterodimerization of RXFP1 with the AT<sub>2</sub>R (and perhaps other GPCRs) occurs exclusively at sites of fibrotic injury, small molecule compounds that specifically target the RXFP1-AT<sub>2</sub>R would be expected to exhibit exquisite selectivity, resulting in activation of the therapeutically relevant receptor to produce an antifibrotic effect only at the site of injury. Furthermore, this scenario has the capacity to incorporate endogenous temporal control of therapeutic action—thus reversal of the injury would correspondingly decrease the amount of RXFP1-AT<sub>2</sub>R heterodimer and thus the available sites of action for the small molecule. In this way, high-resolution knowledge of a system has the potential to translate into a precise spatial and temporal control of drug action at a site of injury. Whether other GPCRs heterodimerize with RXFP1 under particular injury or disease conditions and at specific locations and whether these dimers can be specifically targeted using small molecules remain to be seen.

2. *Relaxin Family Peptide Receptor 3 as a Drug Target.* Studies in rodents suggest that RXFP3 modulators have potential for the treatment of emotional disorders (depression and/or anxiety), social behavior deficits (autism), and inappropriate food/alcohol seeking (Smith et al.,



2011). In vivo studies show a link between relaxin-3/RXFP3 signaling and anxiety/depression (Banerjee et al., 2010; Ryan et al., 2013a); alcohol seeking/reinstatement (Ryan et al., 2013b) (the reinstatement portion of which could be partially related to stress; Weiss et al., 2001); and memory and hippocampal theta emissions (Ma et al., 2009a). The neuroanatomy of relaxin-3/RXFP3 expression is consistent with a role in these disease states and is conserved from the mouse (Smith et al., 2010) to the macaque (Ma et al., 2009b), suggesting at least some of the effects demonstrated in lower species disease models may translate to humans.

In vivo studies suggest that relaxin-3 neurons, which largely also express GABA (Ma et al., 2007a), provide predominantly inhibitory inputs. However, the RXFP3 agonist R3/I5 shows diverse effects in cells of the intergeniculate leaflet, with both depolarization and hyperpolarization recorded in different cells from the same preparation (Blasiak et al., 2013). Thus the phenotype of the relaxin-3 recipient neuron and its connections could be crucial to the mode of RXFP3 signaling. Additional work is needed to understand the pharmacology of RXFP3 and its potential therapeutic use.

The in vivo pharmacological studies performed so far have administered peptide modulators or viral constructs intracerebroventricularly or directly into CNS regions. Because this involves a labor-intensive surgical approach, RXFP3 agonists/antagonists with CNS bioavailability would greatly facilitate the extension of these studies. Unfortunately these are currently unavailable, and the positive allosteric modulator (135PAM1) that has been described displays probe selectivity precluding its use in native mammalian systems (Alvarez-Jaimes et al., 2012). Perhaps additional screening will identify useful pharmacological tools, allowing peripheral or enteric dosing of RXFP3 agonists and antagonists.

**3. Relaxin Family Peptide Receptors and Metabolism.** Recent evidence supports roles for relaxin family peptides in controlling metabolism. Relaxin infusion improves skeletal muscle perfusion and glucose uptake in lean mice. The effect is absent in mice fed a high-fat diet for 13 weeks (Bonner et al., 2013) but can be rescued by chronic infusion of relaxin for 3 weeks. The effect does not result from a direct action on myocytes but involves improvements in blood flow and antifibrotic actions of relaxin (Bonner et al., 2013). This may represent a novel approach to the treatment of insulin resistance.

Increased serum relaxin-3 levels have been described in women with metabolic syndrome (Ghattas et al., 2013), and it has been suggested that this may be related to the known effects of relaxin-3 on food intake (McGowan et al., 2005) and body weight (Hida et al., 2006). However, it is possible that relaxin-3 could also activate RXFP4, and it is interesting to note that there is also an increased body mass index in individuals with a polymorphism of RXFP4 (rs11264422; Munro et al., 2012). There is now strong evidence emerging that INSL5 is an incretin that acts on RXFP4 to have an orexigenic effect (Grosse et al., 2014)

(see section V.D.1). INSL5 mice have impaired glucose tolerance (Burnicka-Turek et al., 2012), and there is a report that RXFP4 knockout mice have similar phenotype (Takeda patent PCT/GB2008/003023). RXFP4 may be a promising target for the treatment of obesity and T2DM.

In conclusion, there have been a number of attempts to establish relaxin as a therapeutic for the promotion of cervical ripening, facilitation of implantation, prevention of preeclampsia, and for the treatment of scleroderma. Only recently has this been successful, and relaxin is now in extended phase III clinical trials with redefined endpoints for the treatment of acute heart failure. The relative lack of success of relaxin in previous trials may be illuminated by the recent discovery that the relaxin receptor RXFP1 may in some situations act as a heterodimer partner receptor. As is the case with the RXFP1/AT<sub>2</sub>R, the expression pattern and actions of the heterodimer may be highly localized and also susceptible to blockade by antagonists of the partner receptor. This work needs to be validated in models of human disease in human tissues but could explain the variability of response observed throughout the patient cohort in previous trials. Relaxin also consistently reduces blood pressure, and antagonists may have some potential in reducing the invasiveness of cancers. The relaxin-3 and RXFP3 system has been examined as a potential target for the treatment of metabolic diseases such as anorexia, cachexia, and obesity. However, inconsistent evidence from animal studies suggests that the complex regulation of energy homeostasis by the CNS, in addition to the redundancy of these pathways, is difficult to override by manipulation of a single system. Recent studies also imply that RXFP3 may be a target for the treatment of addiction. Although still at an early stage, the studies of the biologic role of the INSL5-RXFP4 system suggest that it could have some potential for the treatment of diabetes. Small molecule agonists and antagonists of both RXFP3 and RXFP4 would facilitate more in-depth studies of the biologic roles and therapeutic potential of these two receptors.

#### Authorship Contributions

*Wrote or contributed to the writing of the manuscript:* Halls, Bathgate, Sutton, Dschietzig, Summers.

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