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REVIEW

Constitutive formation of an RXFP1-signalosome: a novel paradigm in GPCR function and regulation

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The classical second messenger cAMP is important in diverse physiological processes, where its spatial and temporal compartmentalization allows precise control over multiple cellular events. Within this context, G-protein-coupled receptors (GPCRs) govern specialized pools of cAMP, which are functionally specific for the unique cellular effects attributed to a particular system. The relaxin receptor, RXFP1, is a GPCR that exerts pleiotropic physiological effects including a potent anti-fibrotic response, increased cancer metastases, and has efficacy as a vasodilator in heart failure. On a cellular level, relaxin stimulation of RXFP1 results in the activation of multiple G-protein pathways affecting cAMP accumulation. Specificity and diversity in the cAMP signal generated by RXFP1 is controlled by differential G-protein coupling dependent upon the background of cellular expression, and cAMP compartmentalization. Further complexity in cAMP signalling results from the constitutive assembly of an RXFP1–signalosome, which specifically responds to low concentrations of relaxin, and activates a distinct cAMP pathway. The RXFP1–signalosome is a higher-order protein complex that facilitates receptor sensitivity to attomolar concentration of peptide, exhibits constitutive activity and dual coupling to G-proteins and β-arrestins and reveals a concentration-biased agonism mediated by relaxin. The specific and directed formation of GPCR-centered signalosomes allows an even greater spatial and temporal control of cAMP, thus rationalizing the considerable physiological scope of this ubiquitous second messenger.

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Abbreviations

AC, adenylyl cyclase; AKAP, A-kinase anchoring protein; CRE, cAMP response element; GRK, G-protein-coupled receptor kinase; INSL, insulin/relaxin-like peptide; LDLa, low-density lipoprotein class A; LGR, leucine-rich repeat-containing GPCR; LH, leuteinizing hormone; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C; RXFP, relaxin family peptide receptor; TSH, thyroid-stimulating hormone

The physiological relevance and consequence of cAMP generation is wide and varied, with this classical second messenger generating diverse, temporally and spatially specific actions. Increasingly, the enzymes responsible for cAMP generation themselves are implicated in multiple physiological roles, with knockout studies indicating an essential requirement of the adenylyl cyclases (ACs) in learning and memory, olfaction, cardiac contraction and alcohol addiction (reviewed in Sadana and Dessauer, 2009).

Compartmentalization of cAMP signalling generated by the formation of protein signalling complexes, or signalosomes, is a highly important emerging mechanism, whereby cAMP can affect numerous aspects of cell control in a specific and orchestrated manner (reviewed in Houslay, 2010; Pidoux and Taskén, 2010; Halls and Cooper, 2011). Despite the importance of this concept, the structural specificity of such signalosomes, and the relevance of the associated cAMP compartmentalization to downstream physiological responses, is poorly understood. Moreover, the evidence for the directed and specialized formation of these complexes in a cellular context is sparse.

The directed and specific compartmentalization of cAMP signalling, due to higher-order protein complexes, is a particularly important and relevant notion in the field of G-protein-coupled receptors (GPCRs; receptor nomenclature herein adheres to the recommendations published in the

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British Pharmacological Society's *Guide to Receptors and Channels*; Alexander *et al.*, 2011). When considered within the environment of GPCR signalling, signalosome-mediated focussing of the spatial and temporal properties of an important second messenger such as cAMP significantly extends the established paradigms of GPCR compartmentalization, ligand-directed signalling and cell context-specific responses. Thus, the co-expression of a variety of cAMP-organizing entities in the same cellular compartment – including GPCRs, ACs and A-kinase anchoring proteins (AKAPs) – provides enormous scope for the specific and selective formation of 'focused centres' for cAMP signalling. These localized focal points not only facilitate the production of this classical second messenger but also allow the organization and scaffolding of effectors that are regulated by cAMP and the associated co-ordination of multiple regulatory elements. In this context, the relaxin family peptide receptor 1, RXFP1, is a GPCR that increasingly exemplifies the complexity and importance of compartmentalization and specificity in cAMP signalling.

The relaxin family peptide receptor, RXFP1, activates cAMP pathways to exert pleiotropic physiological responses

The RXFPs: a unique receptor family

The receptor for relaxin, the relaxin family peptide receptor 1 (RXFP1), is a GPCR of growing complexity that principally signals via multiple cAMP effectors. Relaxin is implicated in numerous physiological processes; initially discovered for its role during parturition in lower species (Hisaw, 1926), in humans, relaxin is now credited with an essential involvement in implantation and the maintenance of pregnancy during the first trimester (Stewart *et al.*, 1990; Telgmann and Gellersen, 1998; Unemori *et al.*, 1999; Bond *et al.*, 2004; Hayes *et al.*, 2004; Shirota *et al.*, 2005). The effects of relaxin are far-reaching, with actions including vasodilation (Dschietzig *et al.*, 2001; 2009a; Fisher *et al.*, 2002; Novak *et al.*, 2006; Teerlink *et al.*, 2009; Teichman *et al.*, 2010; Xu *et al.*, 2010), direct effects on the heart (including increased inotropic and chronotropic responses; Kakouris *et al.*, 1992; Dschietzig *et al.*, 2009a; Teerlink *et al.*, 2009), potent anti-fibrotic effects (Unemori *et al.*, 1996; Garber *et al.*, 2001; Mookerjee *et al.*, 2006; Samuel *et al.*, 2009), wound healing and increased angiogenesis (Unemori *et al.*, 2000; Lewis *et al.*, 2001), and a role for relaxin in enhanced invasiveness and tumour metastases during cancer (Binder *et al.*, 2002; 2004; Hombach-Klonisch *et al.*, 2006; Kamat *et al.*, 2006; Thompson *et al.*, 2006; Vinall *et al.*, 2006; Feng *et al.*, 2007).

There are four recently deorphanized GPCRs for the relaxin family peptides. The four RXFPs can be categorized into two quite distinct groups: RXFP1 (formerly LGR7) and RXFP2 (formerly LGR8; G-protein-coupled receptor affecting testis descent, GREAT; GPR106) are leucine-rich repeat-containing GPCRs (LGRs), that respond to relaxin and insulin/like peptide 3 (INSL3), respectively; whereas RXFP3 (formerly GPCR135; somatostatin- and angiontensin-like peptide receptor, SALPR) and RXFP4 (formerly GPCR142; GPR100) are small-peptide like GPCRs (with similarity to the somatostatin and angiotensin II receptors), that respond to the neuropeptide relaxin-3 and INSL5 respectively (Hsu *et al.*, 2002; Kumagai *et al.*, 2002; Liu *et al.*, 2003; 2005). RXFP1 and RXFP2 are categorized as family C LGRs and resemble the glycoprotein hormone receptors – family A LGRs that include both the leuteinizing hormone, LH, and thyroid-stimulating hormone, TSH, receptors (Hsu *et al.*, 2000; 2002). The family C LGRs are distinguished from other related receptors due to their unique extracellular domain, which encompasses a low-density lipoprotein class A (LDLa) module at the extreme N-terminus, followed by 10 leucine-rich repeats, leading into the transmembrane and C-terminal regions. The LGR family can be evolutionarily traced back to nematodes and insects (Hsu *et al.*, 2000), and as such is thought to represent one of the earliest forms of GPCR signalling (Bathgate *et al.*, 2006). Despite their early divergence, the receptors are far from simple in terms of their activation and regulatory mechanisms, with evidence accumulating for multiple ligand binding sites (Sudo *et al.*, 2003; Halls *et al.*, 2005), receptor dimerization (Kern *et al.*, 2008), negative co-operativity (Svendsen *et al.*, 2008a,b) and a conspicuous lack of significant desensitization and internalization (Callander *et al.*, 2009; Kern and Bryant-Greenwood, 2009) that distinguishes them from the more prototypical GPCRs.

Activation of the relaxin receptor, RXFP1, occurs in a complex fashion, involving binding of ligand to both high- and low-affinity binding sites (Sudo *et al.*, 2003; Halls *et al.*, 2005; Svendsen *et al.*, 2008a,b) and an as yet undefined interaction of the LDLa domain to facilitate signalling (Scott *et al.*, 2006; Hopkins *et al.*, 2007; Kern *et al.*, 2007). Relaxin activation of RXFP1 results in stimulation of multiple signal transduction pathways including cAMP (influenced by a variety of Gα isoforms, discussed below), extracellular signal-regulated kinases (ERK) (Zhang *et al.*, 2002; Dschietzig *et al.*, 2003; 2009b; Mookerjee *et al.*, 2009), tyrosine kinases (Palejwala *et al.*, 1998; Kuznetsova *et al.*, 1999; Bartsch *et al.*, 2001; Anand-Ivell *et al.*, 2007; Heng *et al.*, 2008) and nitric oxide signalling (reviewed in Nistri and Bani, 2003; Conrad and Novak, 2004), in addition to the activation of signalling pathways associated with connective tissue metabolism (including inhibition of transforming growth factor-β signalling and activation of matrix metalloproteinase production; Unemori and Amento, 1990; Unemori *et al.*, 1996; Bennett *et al.*, 2003; Masterson *et al.*, 2004; Samuel *et al.*, 2004; Heeg *et al.*, 2005; Ho *et al.*, 2007) and direct stimulation of the glucocorticoid receptor (Dschietzig *et al.*, 2009b,c).

RXFP1 exerts physiological effects via cAMP

Despite evidence for relaxin-activation of multiple second messengers, the major signalling pathway activated by relaxin (and the most studied to date) is associated with the stimulation of cAMP accumulation. In studies that pre-dated receptor identification, relaxin was found to increase cAMP accumulation in a number of cells and tissues, including THP-1 cells (Parsell *et al.*, 1996), MCF-7 cells (Bigazzi *et al.*, 1992), uterine tissue from rodents (Sanborn *et al.*, 1980; Osa *et al.*, 1991), cultures of human endometrial cells (Chen *et al.*, 1988; Fei *et al.*, 1990) and rat anterior pituitary cells (Cronin *et al.*, 1987). Furthermore, following the discovery of a receptor for relaxin, mutants of RXFP1 were generated based on the conserved behaviour of constitutively active LH and TSH

receptors (Hsu *et al.*, 2000); mutant LH and TSH receptors identified in patients with male-limited precocious puberty and non-immune hyperthyroidism, respectively, contain point mutations in transmembrane domain 6 that are associated with constitutive activity (Parma *et al.*, 1993; 1997; Shenker *et al.*, 1993; Laue *et al.*, 1995; 1996; Kosugi *et al.*, 1996; 1998). Within this region, a 'Phe-Thr-Asp' motif is completely conserved between the glycoprotein hormone receptors and RXFP1; subsequent mutation of the last residue in this motif engendered constitutive activity in RXFP1 ($Asp^{637}Tyr$) that was associated with increased cAMP accumulation (Hsu *et al.*, 2000). However, not all cells yield robust increases in cAMP in response to relaxin. Stimulation of rat ventricular fibroblasts or rat renal myofibroblasts causes only weak and transient cAMP production (Samuel *et al.*, 2004; Mookerjee *et al.*, 2009), and there is no increase in cAMP following peptide stimulation of human lower uterine segment fibroblasts (Palejwala *et al.*, 1998; 2001).

Despite these subtle inconsistencies, the physiological relevance of a cAMP response to relaxin is well demonstrated. In human endometrial stromal cells, basal and relaxin-stimulated cAMP levels are enhanced by inhibition of phosphodiesterase (PDE) 4, and relaxin stimulation and PDE4 inhibition act synergistically to induce decidualization (Bartsch *et al.*, 2004), an important process required to support implantation of the developing embryo. Such increases in cAMP in endometrial cell cultures in response to relaxin (Huang *et al.*, 1987; Tabanelli *et al.*, 1992) mimic the addition of a cell permeable cAMP analogue (Tang *et al.*, 1993). Indeed, only relaxin and cAMP itself are able to stimulate the decidualization of endometrial stromal cells *in vitro* in the absence of progesterone (Callander *et al.*, 2009). Increases in cAMP mediated by relaxin are also linked to the physiological effects of the peptide upon angiogenesis; treatment of a murine model with human relaxin increased the degree of angiogenesis at wound sites, which was associated with an increased expression of vascular endothelial growth factor (VEGF), an important pro-angiogenic protein (Unemori *et al.*, 2000). Interestingly, in cultures of normal human endometrial cells (NHE cells), human relaxin also increased the expression of VEGF, and these effects of relaxin were prevented by AC inhibition, and mimicked by either the AC activator forskolin or a PDE inhibitor (Unemori *et al.*, 1999). This suggests that relaxin-stimulated cAMP production mediates increased VEGF transcription and, consequently, angiogenesis. The positive inotropic effects of relaxin on the atrial myocardium (Kakouris *et al.*, 1992; Ward *et al.*, 1992) are also linked to activation of cAMP pathways; the increased inotropy induced by relaxin was completely abolished by a PKA inhibitor (Piedras-Rentería *et al.*, 1997a,b; Dschietzig *et al.*, 2011), or an inhibitor of the rapidly inactivating component of the transient K^+ outward current (I_{to}, carried by the $K_v4.3$ channel; Piedras-Rentería *et al.*, 1997a,b; Dschietzig *et al.*, 2011), and partially inhibited by a phosphatidylinositol 3-kinase (PI3K; Dschietzig *et al.*, 2011) or $G\alpha_{i/o}$ inhibitor (Kompa *et al.*, 2002; Dschietzig *et al.*, 2011). This suggests that the cAMP generated via the $G\alpha_{i/o}$–PI3K pathway (see below) facilitates PKA-phosphorylation of $K_v4.3$, leading to increased Ca^{2+} influx and thus increased inotropy. To this end, relaxin is currently in clinical trials for its efficacy in acute heart failure. Clearly, cAMP signalling is a very important and central mechanism, whereby relaxin exerts multiple physiological outcomes.

Multiplicity in relaxin-stimulated cAMP signalling generates great physiological potential, controlled by differential G-protein coupling, compartmentalization of cellular responses and concentration-biased agonism

The molecular identity of the proteins involved in generating cAMP downstream of RXFP1 activation has been the focus of many recent studies. Although this research has revealed the complexity of the cAMP pathways activated by RXFP1, principally due to the promiscuous coupling of the receptor to different Gα isoforms (RXFP1 couples to $G\alpha_s$, $G\alpha_{i3}$ and $G\alpha_{oB}$, which together can both stimulate and inhibit AC activity via different mechanisms; generally, these G-proteins can also affect Ca^{2+} channel, K^+ channel, phospholipase C and phospholipase A_2 activity), it has also suggested great scope for the pleiotropic physiological effects mediated by relaxin.

Differential G-protein coupling is directed by the cellular context of RXFP1 expression

Upon receptor activation, RXFP1 couples to $G\alpha_s$, which stimulates AC activity and results in increased cAMP production (Hsu *et al.*, 2000; 2002; Halls *et al.*, 2006). Recent studies suggest that the interaction between RXFP1 and $G\alpha_s$ occurs within the third intracellular loop. A peptide derived from this loop (residues 615–629; Figure 2) increased AC activity independently of RXFP1 stimulation, and functionally antagonized receptor activation (Shpakov *et al.*, 2007). This observation is also consistent with the gain-of-function receptor mutants (described above) that constitutively increase cAMP following a point mutation in the adjacent transmembrane 6 (Hsu *et al.*, 2000; Figure 2). In addition to $G\alpha_s$ activation, RXFP1 also couples to $G\alpha_{oB}$, which inhibits AC activity (Halls *et al.*, 2006; 2009a; Mookerjee *et al.*, 2009). Additional complexity in cAMP accumulation is engendered by the simultaneous coupling of RXFP1 to $G\alpha_{i3}$, which activates a further surge of cAMP accumulation via a Gβγ-PI3K-protein kinase C (PKC) ζ pathway to specifically activate AC5 (Nguyen *et al.*, 2003; Nguyen and Dessauer, 2005a,b; Halls *et al.*, 2006; 2009a). Activation of this $G\alpha_{i3}$ pathway is dependent upon the final 10 amino acids of the RXFP1 C-terminal tail (requiring Arg^{752}; Figure 2) and localization of the receptor within lipid-rich membrane domains (Halls *et al.*, 2009a).

While RXFP1 simultaneously couples to all three pathways when expressed in HEK293 cells, endogenous expression of RXFP1 appears to allow more selective Gα isoform coupling in a manner dependent upon cell type. Endogenous expression of RXFP1 in primary cultures of rat cardiac fibroblasts and THP-1 cell lines allows receptor coupling to all three G-protein pathways linked to cAMP that were originally described in HEK293 cells: $G\alpha_s$, $G\alpha_{oB}$ and $G\alpha_{i3}$ (Halls *et al.*, 2009b; Halls and Cooper, 2010). In distinct cellular back-

grounds – when RXFP1 is endogenously expressed in primary cultures of rat renal myofibroblasts or Colo16 cell lines – the receptor couples only to the $G\alpha_s$ and $G\alpha_{oB}$ pathways and is thus unable to activate the additional and sustained increase in cAMP mediated by $G\alpha_{i3}$ signalling (Halls *et al.*, 2009b; Mookerjee *et al.*, 2009). Even greater functional limitation occurs in a T-47D cell line, where endogenous RXFP1 is only able to couple to $G\alpha_s$ to affect cAMP accumulation (Halls *et al.*, 2009b).

Clearly, the cellular context of receptor expression dictates the degree of potential pleiotropy in G-protein coupling, thereby either greatly limiting or extending the functionality of a particular receptor system. This cell-directed variation in receptor functionality has far-reaching consequences in the associated activation of downstream cellular responses. Recently, this has been clearly demonstrated in human atrial myocardium from failing hearts (Dschietzig *et al.*, 2011). In non-failing atrial myocardium, the positive inotropic effects of relaxin were partially inhibited by $G\alpha_{i/o}$ and PI3K inhibitors; however, in failing myocardium, the response was completely abolished (Dschietzig *et al.*, 2011). This coincided with a significant (approximately 200%) increase in $G\alpha_{i3}$ expression levels (Dschietzig *et al.*, 2011) despite a slight decrease in RXFP1 expression (Kompa *et al.*, 2002); in fact, the failing heart is classically characterized by increased expression of $G\alpha_i$ proteins and unchanged expression of $G\alpha_s$ (Eschenhagen *et al.*, 1992; Böhm *et al.*, 1994), which is deemed responsible for compromised β_2-adrenoceptor–cAMP signalling (Rau *et al.*, 2003). Thus, differential expression levels of $G\alpha_{i3}$ in the same tissue manifested by disease pathogenesis can shift the principal mediators of the RXFP1–cAMP response, from $G\alpha_s$ to $G\alpha_{i3}$-focussed cAMP pathways.

Compartmentalization of cellular responses by a single second messenger

The coupling of RXFP1 to combinations of $G\alpha_s$, $G\alpha_{oB}$ or $G\alpha_{i3}$ dependent upon the cellular context allows scope for differential degrees of cAMP pathway activation and thus diverse cellular responses. Further scope for diversity in the cellular response to relaxin activation of RXFP1 derives from compartmentalization of the cAMP pool downstream of receptor coupling to G-proteins. Thus, $G\alpha_s$ and $G\alpha_{oB}$ pathways affect cAMP-response element (CRE)-controlled gene transcription, whereas the $G\alpha_{i3}$ pathway does not; in addition, only the $G\alpha_{i3}$ pathway can affect nuclear factor of κB (NFκB)-mediated gene transcription (Halls *et al.*, 2007). The physiological consequence of this selective and specific gene transcription initiated by the different G-protein pathways influencing cAMP accumulation is yet to be demonstrated. However, taken together within the context of differential G-protein–cAMP pathway activation dependent upon a particular cellular background, this observation provides further rationalization for the pleiotropy of relaxin's physiological actions.

Additionally, the opposing activity of $G\alpha_s$ and $G\alpha_{oB}$ upon a single second messenger, and associated downstream transcriptional regulation, suggests that the levels of cAMP stimulated by relaxin–mediated activation of RXFP1 are tightly regulated and maintained within a defined range depending on the cellular background. Thus in HEK293 cells overexpressing RXFP1, or indeed in THP-1 cells, the $G\alpha_s$ response tends to overwhelm the inhibition exerted by $G\alpha_{oB}$ (Halls *et al.*, 2006; 2009a,b). Conversely, in HeLa cells or primary cultures of rat cardiac fibroblasts, the inhibition of AC activity exerted by pertussis toxin-sensitive $G\alpha_{i/o}$ proteins overwhelms the $G\alpha_s$-mediated AC stimulation, and concentration-dependent increases in cAMP in response to relaxin are only revealed following pertussis toxin treatment (Halls and Cooper, 2010). Thus, even though cells may not increase cAMP in response to relaxin, a small degree of AC stimulation could be functionally masked due to the overwhelming effect of $G\alpha_{i/o}$ inhibition. This raises the interesting possibility that this signalling pathway could be highly susceptible to hijacking by other GPCRs or viral systems (Nijmeijer *et al.*, 2010; Tschische *et al.*, 2010), reversing the relaxin-mediated cellular effects to a more $G\alpha_s$-dominant signalling system and thus affecting physiological endpoints.

Concentration-biased agonism: a role for circulating relaxin?

The coupling of RXFP1 to the $G\alpha_s$, $G\alpha_{oB}$ and $G\alpha_{i3}$ cAMP signalling pathways, and indeed the physiological effects attributed to this ligand-receptor pair, occurs in response to nanomolar concentrations of relaxin, with EC_{50} values typically reported in the 0.1–0.5 nM range (Sudo *et al.*, 2003; Halls *et al.*, 2005; 2006; Summers *et al.*, 2010). Consequently, relaxin is thought to mediate its physiological actions in an autocrine or paracrine manner, in order to achieve the concentrations required for receptor activation. In addition to this localized relaxin production and release, there is also evidence for the secretion of relaxin into the circulation; however, in this capacity, the ligand is only present at concentrations much lower than those currently established to generate effective cellular responses (Sherwood, 2004). In fact, the measurement of relaxin in the circulation is hampered by the sensitivity limits of ELISA assays, and thus, the peptide may actually be present in much smaller amounts than presently thought (Sherwood, 2004). Although there is as yet no specific physiological role attributed to circulating relaxin, vanishingly low concentrations of the peptide can generate a unique cellular response; this occurs via an RXFP1–signalosome that specifically responds to sub-picomolar concentrations of relaxin (Halls and Cooper, 2010). As such, this unique role for circulating compared to locally synthesized relaxin demonstrates a new paradigm of concentration-biased agonism in this receptor system (Figure 1).

GPCR–signalosomes: high-resolution signalling systems

An RXFP1-signalosome facilitates attomolar ligand responses, constitutive activity and concentration-biased agonism

Upon expression of RXFP1, the receptor induces the formation of a constitutively active and tightly regulated signalosome, which constitutes a highly sensitive mechanism whereby attomolar concentrations of relaxin can induce cAMP accumulation. RXFP1 is scaffolded to AC2 via AKAP79, allowing efficient activation of the AC by $G\alpha_s$ and $G\beta\gamma$ subunits. The cAMP produced is tightly regulated by the activity

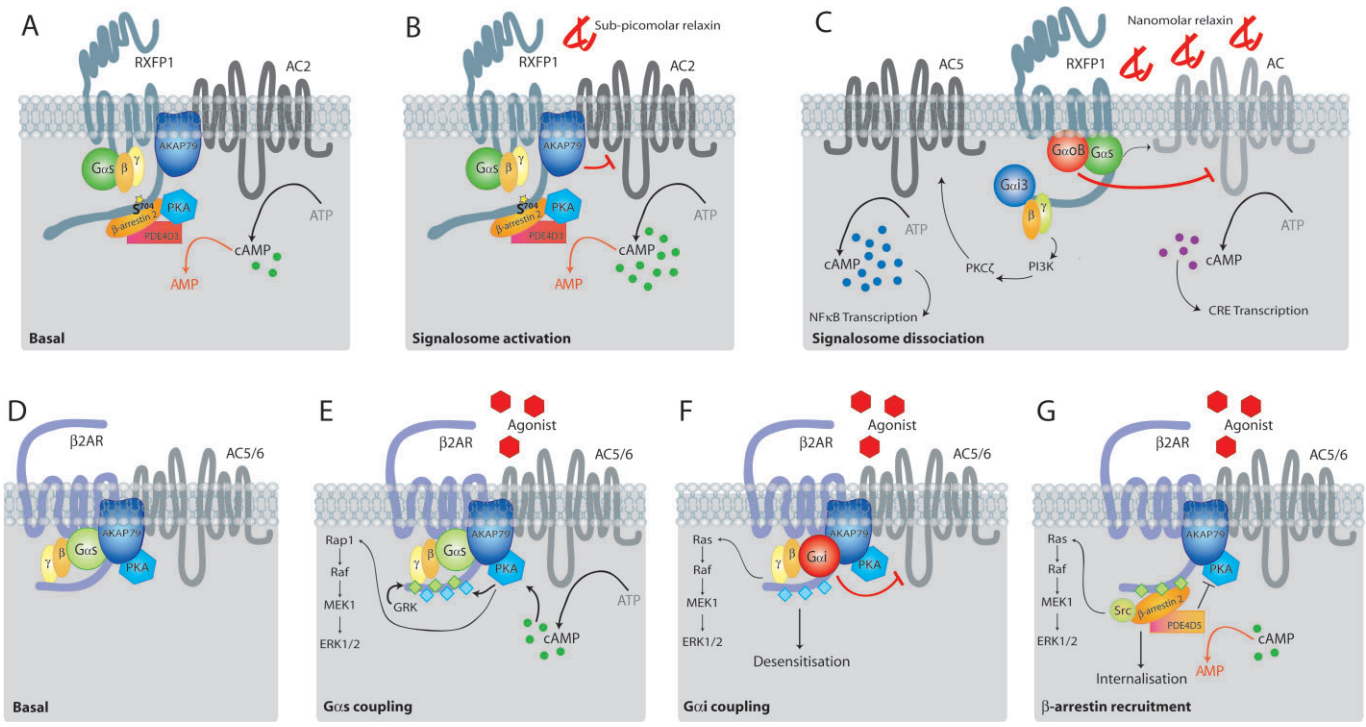

Figure 1

Concentration-biased signalling at the relaxin receptor, RXFP1, compared with prototypical β_2-adrenoceptor signalling. The relaxin receptor RXFP1 demonstrates differential activation of intracellular signalling pathways, leading to increased cAMP accumulation in response to increasing concentrations of ligand; this is in contrast to the prototypical activation, desensitization and internalization paradigm demonstrated by the β_2-adrenoceptor. A. Under basal conditions, expression of RXFP1 induces the formation of an active signalosome. AKAP79 interacts with helix 8 of the RXFP1 C-terminal tail and thereby scaffolds AC2 to the vicinity of the receptor; this allows efficient activation of AC2 by both $G\alpha_s$ and $G\beta\gamma$-subunits. The cAMP generated by the stimulation of AC2 is tightly controlled by the activity of PDE4D3. This phosphodiesterase is activated by PKA (in a negative feedback loop) and additionally interacts with β-arrestin 2, which binds to Ser^{704} of the RXFP1 C-terminus, thereby anchoring the regulatory proteins to the signalosome. B. Sub-picomolar concentrations of relaxin (down to attomolar levels) further activate the pre-assembled, receptor-driven signalosome. The cAMP generated following signalosome activation is maintained within a tightly defined range by the activity of PDE4D3, and this is probably complemented by direct AKAP79-mediated inhibition of AC2 activity. C. Increasing concentrations of relaxin appear to induce dissociation of the RXFP1-signalosome (Halls and Cooper, 2010). At nanomolar concentrations, relaxin stimulation of RXFP1 activates the classical cAMP signalling pathways. RXFP1 can couple to $G\alpha_s$ to activate AC, and $G\alpha_{oB}$, which inhibits AC activity. The cAMP generated by the combined influence of these two G-protein pathways, ultimately affects CRE-controlled gene transcription. RXFP1 can additionally couple to $G\alpha_{i3}$, which activates a $G\beta\gamma$-PI3K-PKCζ pathway, resulting in increased AC5 activity. The additional and sustained increases in cAMP generated by this pathway will only affect NFκB-mediated gene transcription. D. Under basal conditions, the β_2-adrenoceptor is also associated with AKAP79, which scaffolds AC5/6 and PKA to the vicinity of the receptor. There is no evidence for cAMP turnover within this complex. E. Following receptor stimulation, the liberation of $G\alpha_s$ activates AC5/6 to increase cAMP. This results in PKA activation, which phosphorylates the receptor, and can also activate ERK1/2 signalling. The activated receptor can be phosphorylated by GRKs. F. PKA phosphorylation of the receptor C-terminus, results in an uncoupling or signal switching of the β_2-adrenoceptor from $G\alpha_s$ to $G\alpha_i$, leading to receptor desensitization. The $G\beta\gamma$ subunits liberated from $G\alpha_i$ can also activate ERK1/2 signalling. G. GRK phosphorylation of the receptor C-terminus leads to β-arrestin recruitment and receptor internalization. Recruitment of β-arrestin also allows the scaffolding of Src and PDE4D5 proteins: Src can activate ERK1/2 signalling; whereas PDE4D5 hydrolyses cAMP and inhibits the activity of PKA, preventing signal switching to $G\alpha_i$ and facilitating desensitization.

of protein kinase A (PKA)-activated PDE4D3, scaffolded to the receptor C-terminus (specifically requiring Ser^{704}) by β-arrestin 2 (Halls and Cooper, 2010). The stimulatory (AKAP79 and AC2) and regulatory (β-arrestin 2, PKA and PDE4D3) components of the signalosome appear to be both spatially and functionally distinct (Figure 2): knockdown of AKAP79 does not affect the association between the regulatory components and the receptor, whereas knockdown of β-arrestin 2 does not prevent the interactions between RXFP1 and the stimulatory components. Furthermore, the protein constituents of the complex exhibit isoform selectivity, determined using targeted protein knockdown or over-expression of dominant negative mutants (Halls and Cooper, 2010): thus, of the PKA-activated PDE4D isoforms expressed in HEK293 cells (reviewed in Houslay, 2010), the complex is specific for PDE4D3; assembly of the regulatory components is dependent upon a constitutive association between the receptor and β-arrestin 2, but not β-arrestin 1 (reviewed in DeFea, 2011); and the cAMP activity of the RXFP1-signalosome is dependent upon the constitutive association between helix 8 of the receptor and AKAP79, but not gravin (AKAP250) or AKAP149 (reviewed in Baillie *et al.*, 2005; Des-

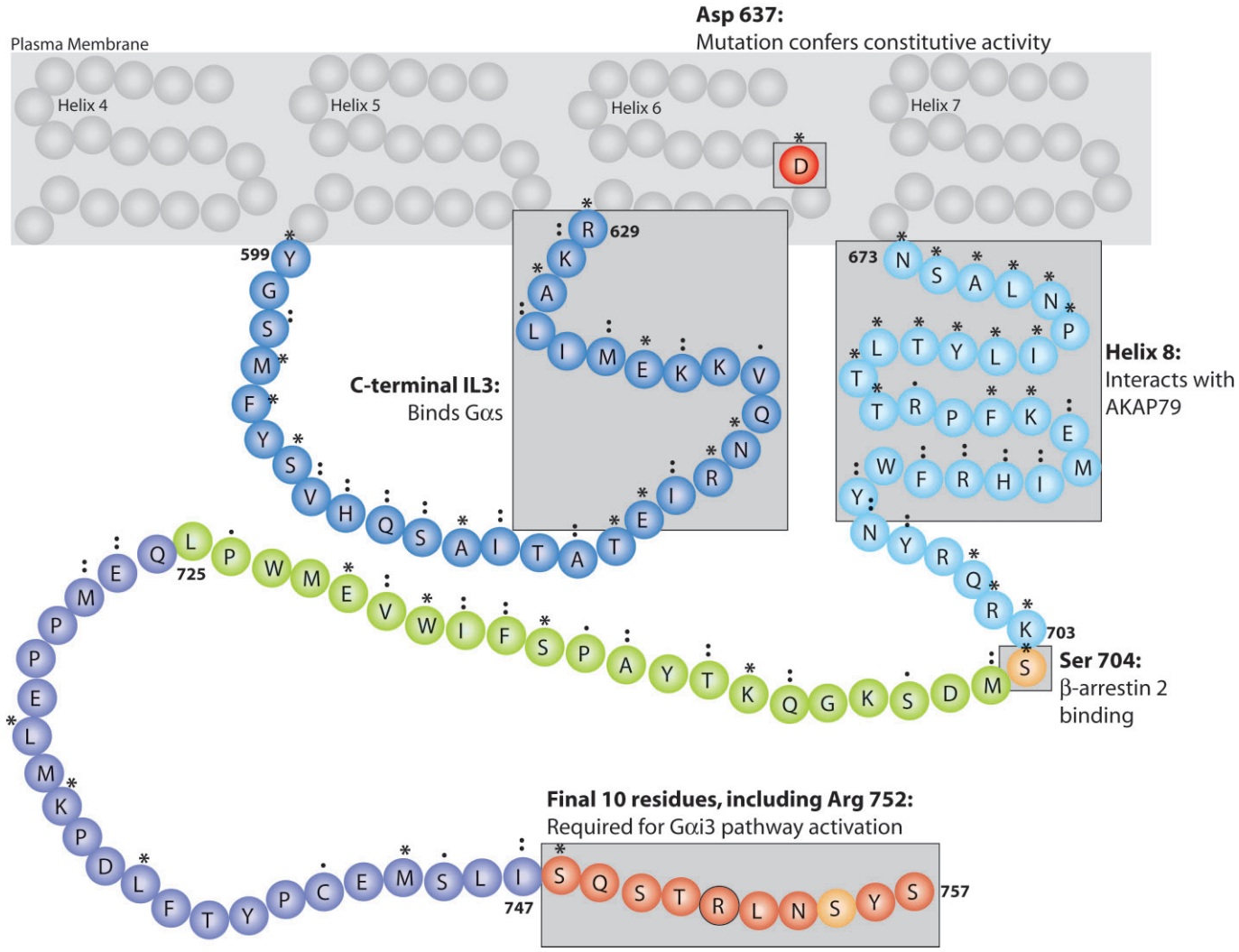

Figure 2

The importance of the third intracellular loop and C-terminus of RXFP1 in cAMP signalling. Many important regions for activation of cAMP signalling pathways within the intracellular loops and C-terminal tail of RXFP1 have now been identified. The third intracellular loop and transmembrane domain 6 are important for the activation of cAMP signalling pathways: the C-terminal portion of the third intracellular loop (residues 615–629) is suggested to couple to $G\alpha_s$, and mutation of Asp^{637} induces a constitutive activation of cAMP signalling. The first section of the C-terminal tail regulates the formation of the RXFP1-signalosome: putative helix 8 (residues 673–703) is required for the interaction between RXFP1 and AKAP79 (which scaffolds AC2), and Ser^{704} is absolutely required for the interaction between the C-terminus and β-arrestin 2 (anchoring the regulatory sub-complex). The final 10 residues of the C-terminus (748–757), and specifically Arg^{752} are absolutely required for activation of the $G\alpha_{i3}$–Gβγ–PI3K–PKCζ–AC5 cAMP signalling pathway. The RXFP1 C-terminal tail is not palmitoylated. Symbols above the amino acid residues indicate the relative conservation between RXFP1 and RXFP2: * identical residue, conserved residue and semi-conserved residue.

sauer, 2009; Skroblin *et al.*, 2010). Importantly, and uniquely, this signalling mechanism is absolutely distinct from the cAMP signalling pathways activated by higher concentrations of relaxin; indeed, the complex appears to dissociate following activation of RXFP1 with nanomolar concentrations of peptide. Supra-picomolar concentrations of relaxin instead increase cAMP as previously described, via $G\alpha_s$ stimulation of AC, negatively modulated by the inhibitory activity of $G\alpha_{oB}$, with a sustained, further rise in cAMP generated via a $G\alpha_{i3}$–Gβγ–PI3K–PKCζ–AC5 pathway (Hsu *et al.*, 2002; Nguyen *et al.*, 2003; Nguyen and Dessauer, 2005a,b; Halls *et al.*, 2006; 2009a).

Relaxin is therefore a concentration-biased agonist at RXFP1; activation of RXFP1 by relaxin proceeds by a biphasic concentration–response mechanism (Figure 1) with increases in cAMP detected following stimulation with as little as 10 aM of peptide. This degree of sensitivity has been demonstrated in only a few other physiological systems, including the suppression of pro-inflammatory cytokine production by interleukin-15 (Alleva *et al.*, 1997), proliferation of a helper T-cell line by interleukin-1 (Orencole and Dinarello, 1989), the effects of neuropeptides and neurosteroids in nociception (Sánchez-Blázquez and Garzón, 1995; Ueda *et al.*, 2001) and the long-term effects of transforming growth factor-β on

basal FSH levels (Ying *et al.*, 1986). Activation of a GPCR however does not typically occur in response to such small concentrations of ligand. Thus, the specific assembly of a GPCR–signalosome facilitates a substantially increased sensitivity of the receptor system to ligand, and these protein complexes thereby constitute high-resolution signalling systems.

A particularly interesting feature of the RXFP1–signalosome is the paradigm of concentration-biased signalling exhibited by the pre-assembled proteins. Concentration-biased signalling can be considered analogous to the well-established concept of ligand-biased signalling, whereby a ligand preferentially activates a specific signalling pathway; similarly, in this case a particular concentration of ligand activates a specific signalling pathway. Thus, at concentrations below the nanomolar level, relaxin appears only to stimulate a small increase in cAMP via the signalosome, whereas at supra-nanomolar concentrations, relaxin preferentially activates the classical relaxin signalling pathways affecting cAMP accumulation, defined by $G\alpha_s$, $G\alpha_{oB}$ and $G\alpha_{i3}$. Importantly, there is no effect of inhibitors of classical pathway-specific proteins (including $G\alpha_{i/o}$, PI3K and PKC) on cAMP generated in response to sub-picomolar concentrations of relaxin. The reciprocal also holds – there is no effect of inhibition of signalosome-specific proteins (including AC2, AKAP79 and β-arrestin 2) upon classical relaxin cAMP signalling. Furthermore, higher concentrations of relaxin appear to dissociate the signalosome, thereby allowing activation of the classical signalling pathways. Thus, the signalosome and classical relaxin signalling pathways appear to be quite distinct in composition. It is also interesting to consider the potential contrast of these concentration-specific signalling pathways in terms of lipid raft dependence. It is established that AC2 is preferentially excluded from lipid-rich domains (reviewed in Willoughby and Cooper, 2007), whereas activation of the $G\alpha_{i3}$ pathway following supra-nanomolar relaxin stimulation of RXFP1 depends upon lipid-rich domains in HEK293 cells (Halls *et al.*, 2009a).

The physiological application of this highly sensitive, concentration-dependent signalosome is not yet apparent. However, it is intriguing to consider this complex within the context of the role of relaxin during embryo implantation. In a number of tissues, relaxin exerts its effects over an extended period of time, exemplified by endometrial decidualization (the differentiation of endometrial stromal cells), which is crucial for embryo implantation and the maintenance of pregnancy. Both circulating and locally produced relaxin have been implicated in this process (Einspanier *et al.*, 1999; Unemori *et al.*, 1999; Palejwala *et al.*, 2002; Hayes *et al.*, 2004), which requires the continued elevation of cAMP (reviewed in Telgmann and Gellersen, 1998; Gellersen and Brosens, 2003). Thus, the sensitivity of RXFP1 to sub-picomolar concentrations of relaxin may suggest a mechanism that extends a physiological, perhaps homeostatic, role for the concentrations of peptide present within the circulation and thus may be linked to some of the long-term physiological effects of this hormone. Future research, perhaps utilizing relaxin knockout models or single cell models of physiologically relevant targets (i.e. atrial cardiomyocytes, endometrial cells, fibroblasts or neurons), will be required to fully elucidate the downstream consequences and physiological significance of this ultra-sensitive cAMP signalling pathway.

Physiological relevance of attomolar-agonism: an essential link to AC2?

Increased cAMP production in response to sub-picomolar concentrations of relaxin was found to be specific for, and dependent upon, the expression of AC2. AC2 belongs to the group II ACs (including AC2, AC4 and AC7) that are defined by their conditional stimulation by Gβγ-subunits (dependent upon co-activation by $G\alpha_s$) and activation by PKC phosphorylation (reviewed in Halls and Cooper, 2011). In addition to AC2, HEK293 cells also endogenously express AC7 from this same sub-group of ACs. However, over-expression of AC7 abolished the sub-picomolar relaxin response, which directly opposed the enhancement of sub-picomolar cAMP signalling observed following AC2 over-expression. Thus, the RXFP1-controlled signalosome is specific for the expression of AC2 and consequently may not exist in all cell types.

AC2 is principally expressed in brain, lung, skeletal muscle, heart and uterus (myometrium) (reviewed in Defer *et al.*, 2000; Willoughby and Cooper, 2007; Sadana and Dessauer, 2009), as well as the spinal cord (Ehnert *et al.*, 2004). Within the spinal chord, ACs are thought to have a role in the pain response (Sadana and Dessauer, 2009), a system that is sensitive to attomolar concentrations of stimuli (Sadana and Dessauer, 2009). There is also evidence for the expression and function of AC2 in the olfactory system, another system that is designed to be highly sensitive to physiological stimuli. Within the olfactory system, AC2 expression predominates in the vomeronasal system, which is responsible for pheromone detection, rather than the odorant-detecting main olfactory epithelium, where the expression of AC3 dominates (Sadana and Dessauer, 2009). Both the pain response within the spinal chord and the detection of pheromones within the vomeronasal system are dependent upon the cAMP produced by AC2 and respond to very low concentrations of stimuli. Based on these observations it is tantalizing to speculate that a signalosome specific for AC2 could be present and be required for these sensitive physiological responses. In terms of the RXFP1–signalosome, there is potential for the expression of AC2 and RXFP1 to overlap in the brain, lung, heart and uterus, and this may suggest a high likelihood for a physiological role of this specific signalosome in these tissues.

The importance of AKAPs in cAMP–signalosomes: scaffolds and negative regulators of signalling

The high-sensitivity activation of AC2 within the RXFP1-signalosome is dependent upon the scaffolding of the AC to RXFP1 by AKAP79. The AKAPs constitute a large and growing family of highly divergent proteins, with the exception of a conserved PKA interaction motif (reviewed in Wong and Scott, 2004). Due to the very limited homology between AKAPs, a general AC–AKAP interaction motif has not been identified. In fact, it appears that different AKAPs use disparate mechanisms to interact with the same AC, and further, that different ACs bind to disparate regions of the same AKAP (reviewed in Dessauer, 2009). Nonetheless, AKAPs have an emerging importance in directing compartmentalization and

control of the sub-cellular localization and molecular specificity for targets of cAMP signalling pathways. This is achieved by their action as scaffolds, not only for ACs but also by tethering cAMP effectors, downstream targets and regulatory proteins.

AKAP79 is a scaffold for calcineurin, PKC, GPCRs (including the β_1- and β_2-adrenoceptors, AMPA ionotropic glutamate receptors and the relaxin receptor RXFP1) and a variety of channels [including the voltage-activated Ca^{2+} channel $Ca_v1.4$ (L-type channel), voltage-activated K^+ channel family K_v7 (M-type/KCNQ channels) and the transient receptor potential cation channel TRPV1] (reviewed in Dessauer, 2009; Halls and Cooper, 2010). More recent studies have also demonstrated the ability of AKAP79 to anchor a number of AC isoforms, including AC2, AC3, AC5, AC6, AC8 and AC9 (Bauman *et al.*, 2006; Efendiev *et al.*, 2010; Willoughby *et al.*, 2010). By this multivalent activity, AKAP79 can therefore potentially direct the differential regulation of distinct protein complexes merely by its scaffolding function.

However, the influence of AKAP79 (on ACs at least) extends further than its ability to act as a protein scaffold. In fact, AKAP79 has been demonstrated to interact with and affect the activity of most AC isoforms. The association of AKAP79 with both AC5 and AC6 results in the facilitation of PKA-mediated AC phosphorylation and a subsequent inhibition of AC activity (Bauman *et al.*, 2006). Similarly, the anchoring of AC8 by AKAP79 in both over-expression and endogenous neuronal systems limits the sensitivity of the AC to activation by Ca^{2+} (Willoughby *et al.*, 2010). In a similar manner, the activity of AC2 is also inhibited by the presence of AKAP79 (Efendiev *et al.*, 2010). However, AKAP79 merely acts as a neutral scaffold for AC3 and AC9 (Efendiev *et al.*, 2010).

The role of AKAP79 in scaffolding AC2 to the RXFP1–signalosome appears to be of a dual nature. While AKAP79 may have a small degree of inhibitory influence on the activity of AC2, its principal role in this case appears to be the scaffolding of RXFP1 and AC2 within the complex. Thus, removal of AKAP79 from the system results in a loss of sub-picomolar cAMP signalling, rather than relieving any substantial negative influence upon AC activity.

A new GPCR regulatory paradigm demonstrated by signalosome formation: lack of desensitization and internalization, a constitutive association with β-arrestins and dual coupling to G-proteins

The regulatory sub-complex of the RXFP1–signalosome is dependent upon the association of β-arrestin 2 with Ser^{704} of the receptor C-terminal tail (Figure 2), which exclusively occurs following the expression of RXFP1 and is independent of ligand stimulation (Halls and Cooper, 2010). This contrasts the accepted paradigm for GPCR/β-arrestin interactions, which typically occur following receptor activation and phosphorylation (Figure 1). Indeed, although β-arrestin binding was traditionally thought to only occur following GRK or second messenger (i.e. PKA or PKC) phosphorylation of an activated receptor, it is now apparent that associations between GPCRs and β-arrestins can occur in the absence of both receptor activation and phosphorylation (reviewed in DeFea, 2011). Furthermore, the regulation of RXFP1 activity following stimulation with supra-nanomolar concentrations of relaxin does not appear to follow the prototypical desensitization and internalization paradigm described by studies of the β_2-adrenoceptor (Callander *et al.*, 2009; Kern and Bryant-Greenwood, 2009). This classical GPCR paradigm predicts that ligand stimulation of the receptor facilitates activation of G-proteins, phosphorylation of the receptor C-terminus by G-protein receptor kinases (GRKs), recruitment of β-arrestins (which uncouple the receptor from its G-protein partners) and receptor internalization, which corresponds with the associated activation of β-arrestin-mediated signalling pathways (Figure 1; reviewed in Kelly *et al.*, 2008; Tobin *et al.*, 2008; Rajagopal *et al.*, 2010).

Well-established observations made prior to the deorphanization of RXFP1 suggested that the receptor was not desensitized; activation of the relaxin receptor in numerous target tissues was associated with prolonged physiological effects that persisted for longer than 6 h with constant washing (Summers *et al.*, 1995; Tan *et al.*, 1998). This observation is still consistently replicated in cell population and single cell signalling assays (albeit over smaller time scales; Halls ML, unpubl. obs.). Furthermore, recent evidence specifically looking for the prototypical GPCR regulatory features, suggested an absence of significant receptor internalization following ligand binding (Callander *et al.*, 2009). Finally, the basal interaction between RXFP1 and β-arrestin 2, when considered in conjunction with the constitutive activity of the signalosome, suggests dual coupling of both $G\alpha_s$ and β-arrestin 2 to the intracellular regions of RXFP1. Again, this conflicts with the general paradigm of GPCR signalling, which suggests mutually exclusive coupling of a GPCR to either G-protein- or β-arrestin-mediated signalling pathways.

Interestingly, this alternate paradigm of constitutive β-arrestin association, dual G-protein coupling and an absence of appreciable receptor desensitization and internalization has also been demonstrated by another GPCR. The dopamine D_4 receptor does not exhibit significant desensitization or internalization following stimulation with dopamine. Instead, this receptor constitutively co-immunoprecipitates with β-arrestin 2, and this association does not decrease following receptor stimulation (Rondou *et al.*, 2010; Spooren *et al.*, 2010), which classically activates $G\alpha_{i/o}$ proteins. Further examples of dual G-protein coupling are evident in other GPCR systems, which do exhibit typical internalization characteristics. The calcium-sensing (CaS) receptor is constitutively associated with β-arrestin 1, and this association does not change following agonist stimulation (Bouschet *et al.*, 2007), despite the receptor coupling to $G\alpha_{q/11}$, $G\alpha_{12/13}$ and $G\alpha_{i/o}$ proteins (Brown *et al.*, 2010). Furthermore, two mutants of the V_2 vasopressin receptor (responsible for nephrogenis syndrome of inappropriate antidiuresis, NSIAD) exhibit constitutive cAMP accumulation, and BRET studies show a constitutive association between the receptors and β-arrestin 1 (Tenenbaum *et al.*, 2009).

Taken together, these observations of the behaviour of RXFP1, the D_4, CaS and V_2 receptors, which conflict with the classical GPCR regulatory paradigm, may suggest a situation whereby constitutively active receptors that are basally associated with β-arrestins are also able to tolerate and coordinate dual coupling to G-proteins. Alternatively, the phosphorylation state of the target receptor could induce differential conformational states of β-arrestins, which subsequently dictates their function (Shenoy *et al.*, 2006). A mutant of the β_2-adrenoceptor, which lacks G-protein coupling but retains β-arrestin binding, exhibited weak receptor phosphorylation, moderate β-arrestin recruitment, but robust ERK1/2 phosphorylation in contrast to the wild-type receptor (Shenoy *et al.*, 2006). The authors suggest that the β-arrestin 2 recruited to a GRK2-phosphorylated receptor may not be optimally suited for engaging efficient ERK1/2 activation (Shenoy *et al.*, 2006). This raises the possibility of distinct β-arrestin 2 conformations, dictated by the receptor to which they are bound, that may also permit dual coupling of the receptor to G-proteins.

Interestingly, the RXFP1, D_4 dopamine and CaS receptors all behave unexpectedly in terms of their constitutive associations with β-arrestins, when examined in imaging studies. In each case, confocal studies using GFP-tagged β-arrestins showed only cytosolic expression of the tagged protein, which did not change following receptor expression (which was localized to the plasma membrane) or ligand stimulation (Bouschet *et al.*, 2007; Callander *et al.*, 2009; Rondou *et al.*, 2010; Spooren *et al.*, 2010). This may suggest that a relatively small population of endogenously expressed β-arrestin is responsible for constitutive GPCR-interactions, or perhaps that prior recruitment of endogenous β-arrestins to the receptors subsequently prevents an exchange with the GFP-tagged variant following expression. Alternatively, the addition of the GFP-tag to the β-arrestin itself may interfere with the constitutive association between β-arrestins and these more unusual GPCRs. One might also add the caveat that in instances of over-expression, it may be difficult to see subtle recruitment of a cytosolic GFP-tagged protein to the plasma membrane unless expression levels of this protein are kept very low. Nonetheless, these receptors clearly display relatively unique interactions with β-arrestins.

The role of β-arrestins as independent and influential signalling scaffolds

The concept of β-arrestins acting as protein scaffolds in their own right is an emerging area that extends the role of these proteins, initially defined by their involvement in the prototypical desensitization and internalization of classical GPCRs (reviewed in DeWire *et al.*, 2007; Ma and Pei, 2007; DeFea, 2008; 2011). Indeed, β-arrestins are often considered multifunctional adaptor proteins, with evidence accumulating for associations with numerous signalling mediators including those with roles in signal transduction (i.e. protein kinases, phosphatases, trafficking proteins, small G-proteins), metabolic enzymes, proteins implicated in cellular organization (i.e. cytoskeletal proteins, motor proteins), chaperone and stress response proteins, ion channels and nucleic acid binding proteins (i.e. transcription factor, RNA processing, DNA binding and ribosomal proteins) (Xiao *et al.*, 2007).

In the context of the RXFP1–signalosome, it appears that β-arrestin 2 may be purely acting as a scaffolding protein, as there is still no evidence for β-arrestin 2-mediated activation of downstream signalling pathways in response to relaxin. Interestingly, this is in contrast to the role of β-arrestin 2 in the β_2-adrenergic receptor complex; in this case, β-arrestin 2 recruitment to the GRK-phosphorylated C-terminus allows the associated recruitment of PDE4D5, which reverses the AKAP79/PKA-mediated switching of the receptor from $G\alpha_s$-cAMP-PKA to $G\alpha_{i/o}$-ERK (Houslay and Baillie, 2005) and facilitates subsequent β-arrestin 2 stimulation of ERK pathways. Thus, the principal function of β-arrestin may be controlled by the receptor to which it is bound. In this context, the type of proteins scaffolded by β-arrestin also appear to be influenced by the receptor in question; thus, the RXFP1/AKAP79/β-arrestin 2 complex has a preference for PDE4D3 (Halls and Cooper, 2010) compared with the β_2-adrenoceptor/AKAP79/β-arrestin 2 complex that is specific for the PDE4D5 isoform (Bolger *et al.*, 2003; Lynch *et al.*, 2005; Willoughby *et al.*, 2007). It is interesting to speculate that this specificity in recruitment of a particular isoform of PDE4D may be dependent upon the conformation of β-arrestin 2 induced by the interaction with a particular receptor C-terminus. Indeed, previous studies have shown that β-arrestin preferentially interacts with PDE4D5 over other PDE4 isoforms, even in cell types where PDE4D5 is not the predominant species (Houslay and Baillie, 2005), due to an additional interaction site for β-arrestins within the N-terminal region, separate from the site within the catalytic unit (Bolger *et al.*, 2003). As such, the preference in the RXFP1-signalosome for PDE4D3 over PDE4D5 may be induced by the conformation of β-arrestin 2 adopted following binding to the RXFP1 C-terminus.

The concept of differential conformations of β-arrestins defined by their binding partners is not new; there is supportive evidence to suggest that β-arrestins may adopt different conformations with specific preferences for G-protein occupied versus unoccupied receptors, and other studies have demonstrated changes in β-arrestin binding partners following receptor activation (DeWire *et al.*, 2007). These specific conformational (and thus functional) states of β-arrestins are also demonstrated in studies examining the effect of mutation of potential phosphorylation sites within the C-terminal tail of various GPCRs; removal of phosphorylation sites either ablates β-arrestin recruitment and associated ERK activation (i.e. β_2-adrenoceptor), has no effect on the ability of the receptor to recruit β-arrestins and activate ERK phosphorylation (i.e. angiotensin II AT_{1A} receptor) or can result in the receptor retaining the ability to recruit β-arrestins but losing any associated activation of ERK signalling pathways (i.e. orexin OX_1 receptor) (DeWire *et al.*, 2007).

Thus, in the context of GPCR-signalosomes, β-arrestins do function as truly flexible adaptor proteins, whereby the relevant conformation of the β-arrestin is initially defined by receptor binding, and subsequently dictates the additional scaffolding of specific proteins to the signalosome.

Physiological implications of signalling complexity: the RXFP1–signalosome

Many, if not all, GPCRs can intrinsically exhibit a degree of constitutive, or ligand-independent, activity (reviewed in

Smit *et al*., 2007), and this has direct implications for the targeting of these receptors as therapeutics. The degree of constitutive activity exhibited by a particular receptor can be influenced by a specific ligand, or by point mutations, thus manipulating the balance between active and inactive receptor states. Correspondingly, variations in the constitutive activity of a receptor dependent upon the expression of the GPCR within different cell types, and associated variations in downstream signalling constituents, add an additional area of complexity; thus, a truncated CB_1 cannabinoid receptor that exhibits enhanced constitutive activity results in an increase in inverse agonist efficacy (SR141716A) but a decrease in agonist efficacy (WIN 55,212–2) (Nie and Lewis, 2001). The relative constitutive activity of a receptor will also affect the outcome of the interaction between the GPCR and a particular ligand. Furthermore, variation in the constitutive activity of a GPCR raises the concept of protean agonism (reviewed in Kenakin, 2001), whereby ligands can exhibit positive agonism in a system with low constitutive activity, but inverse agonism in a system with a high degree of constitutive activity. Indeed, many important therapeutic agents, initially considered competitive antagonists, are now recognized to be inverse agonists with negative intrinsic activities, including the α_1-adrenoceptor target prazosin and the dopamine D_2 receptor ligand haloperidol (Smit *et al*., 2007).

Many wild-type receptors demonstrate constitutive activity in the absence of any disturbance in equilibrium caused by either ligand or mutation, and some such as the histamine H_3 receptor actually require the presence of inverse agonists in order to maintain homeostasis (Morisset *et al*., 2000; Wieland *et al*., 2001). Similarly, many of the constitutively active wild-type GPCRs are receptors for neurotransmitters, which suggests a requirement of constitutive activity for the maintenance of neuronal tone (Seifert and Wenzel-Seifert, 2002). Thus, it is interesting to speculate such a homeostatic or neurotransmitter-like relevance for the RXFP1-signalosome, particularly in its physiological roles in decidualization and neuronal function. Furthermore, the identification of RXFP1 as a receptor with intrinsic constitutive activity, in the absence of any influence of a ligand or mutational manipulations, has interesting consequences for the interpretation of its known physiological roles and for the potential development of therapeutics targeting this system. Additional complexity arises when natural (due to expression of RXFP1 within different cell types) or induced (hijaacking of this signalling system by other receptors) variations in the expression of signalling constituents are considered, and this may perhaps reveal a cellular mechanism for the pleitropy of relaxin's physiological effects.

However, signalling complexity is not only derived from the constitutive activity exhibited by the RXFP1–signalosome but can also be attributed to the cAMP signalling bias that is directed by increasing concentrations of ligand. On a cellular level, the potential physiological consequences of specific cAMP pools generated in response to different concentrations of agonist, and by temporally distinct pathways, are vast. This not only implies a fine degree of spatial and temporal control over the cellular signal but also greatly increases the range of cellular responses that may be activated by a single ligand/receptor pair. This, in addition to other amplifying factors including ligand-directed signalling, and differences in protein composition between varying cell types, makes transparent the pleiotropic physiological effects of this interesting hormone. The physiological relevance of a highly sensitive GPCR–signalosome, and whether this protein complex functions purely in a homeostatic realm, remains to be determined. Nevertheless, the directed assembly of a finely tuned GPCR–signalosome that facilitates concentration-biased agonism would dramatically increase the physiological potential of any GPCR system. Future research is essential to discover how widespread the phenotype displayed by the RXFP1–signalosome is.

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Conflict of interest

The author states no conflict of interest.

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