Sub-picomolar relaxin signalling by a pre-assembled RXFP1, AKAP79, AC2, β-arrestin 2, PDE4D3 complex

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Biochemical studies suggest that G-protein-coupled receptors (GPCRs) achieve exquisite signalling specificity by forming selective complexes, termed signalosomes. Here, using cAMP biosensors in single cells, we uncover a pre-assembled, constitutively active GPCR signalosome, that couples the relaxin receptor, relaxin family peptide receptor 1 (RXFP1), to cAMP following receptor stimulation with sub-picomolar concentrations of peptide. The physiological effects of relaxin, a pleiotropic hormone with therapeutic potential in cancer metastasis and heart failure, are generally attributed to local production of the peptide, that occur in response to sub-micromolar concentrations. The highly sensitive signalosome identified here provides a regulatory mechanism for the extremely low levels of relaxin that circulate. The signalosome includes requisite $G\alpha_s$, $G\beta\gamma$ and adenylyl cyclase 2 (AC2); AC2 is functionally coupled to RXFP1 through AKAP79 binding to helix 8 of the receptor; activation of AC2 is tonically opposed by protein kinase A (PKA)-activated PDE4D3, scaffolded through a β-arrestin 2 interaction with Ser^{704} of the receptor C-terminus. This elaborate, pre-assembled, ligand-independent GPCR signalosome represents a new paradigm in GPCR signalling and provides a mechanism for the distal actions of low circulating levels of relaxin.

Introduction

cAMP is the prototypical second messenger for signal transduction by G-protein-coupled receptors (GPCRs), which impacts on virtually every aspect of cellular homeostasis and physiology. The diverse effects of cAMP require sophisticated targeting and regulation of intracellular cAMP pools, which is facilitated by organized entities of adenylyl cyclases (ACs), phosphodiesterases (PDE), protein kinase A (PKA) and

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A-kinase-anchoring proteins (AKAPs; Malbon *et al*, 2004; Wong and Scott, 2004; Willoughby and Cooper, 2007; Patel *et al*, 2008; Dessauer, 2009). In the case of GPCRs, this regulation can be further enhanced by the formation of GPCR signalosomes: macromolecular receptor-linked protein complexes that facilitate the preferential activation of downstream targets. The cAMP output of any such organized complex, in terms of spatiotemporal complexity or dynamics, cannot be addressed or observed by traditional cAMP assays, which examine heterogeneous populations of cells at very low resolution. Genetically encoded fluorescent-based cAMP sensors are a means for finely resolving and identifying such subcellular cAMP dynamics in single cells (Nikolaev *et al*, 2004).

Relaxin is a pleiotropic hormone with wide and varied physiological effects (Sherwood, 2004; van der Westhuizen *et al*, 2008). The peptide is involved in fibrosis, inflammation, wound healing, allergic responses, cancer metastasis and pregnancy. Consequently, there is considerable interest in therapeutic applications of relaxin; in particular, its potent anti-fibrotic effects, and the ability of relaxin to promote the growth, differentiation and invasiveness of tumour cells (Klonisch *et al*, 2007; Du *et al*, 2010). Furthermore, recent clinical trials for acute heart failure have shown therapeutic efficacy for relaxin as a vasodilator (Dschietzig *et al*, 2009; Teerlink *et al*, 2009). The physiological effects of relaxin are commonly attributed to local production of the hormone in a wide variety of human tissues; however some major targets—for example the normal heart, do not appear to synthesize relaxin (Bathgate *et al*, 2006; Samuel *et al*, 2006); so a function for circulating relaxin is envisaged. The relaxin that is found in the circulation could be speculated to exert such effects, but the concentrations are lower than are effective at any known signalling mechanism for the peptide (Sherwood, 2004; Gedikli *et al*, 2009).

The receptors for relaxin were recently de-orphanized (Hsu *et al*, 2002); the relaxin family peptide receptor 1 (RXFP1) is a GPCR, with two binding sites for relaxin: a high-affinity site within the extracellular-domain leucine-rich repeats, and a low-affinity site within the transmembrane extracellular loops (Sudo *et al*, 2003; Büllesbach and Schwabe, 2005; Halls *et al*, 2005). A second, highly homologous receptor, RXFP2, binds insulin/relaxin-like peptide 3 (INSL3) in addition to relaxin (Kumagai *et al*, 2002). The cAMP signalling pathways activated by the stimulation of RXFP1 with high concentrations of relaxin are well characterized. The receptor couples to $G\alpha_s$, and negative regulation of cAMP is exerted by $G\alpha_{oB}$ (Halls *et al*, 2006); further cAMP accumulation occurs through $G\alpha_{i3}$, activating a $G\beta\gamma$-phosphoinositide 3-kinase (PI3K)-protein kinase C (PKC) ζ-AC5 pathway (Nguyen *et al*, 2003; Nguyen and Dessauer, 2005a, b; Halls *et al*, 2006, 2009).

In this study, we have tackled the sub-cellular dynamics of relaxin-mediated cAMP signalling in single cells by using targeted FRET-based cAMP biosensors: Epac2-camps (glEpac2), containing the cAMP-binding domain of Epac2 (Nikolaev *et al*, 2004) and a modified version of this biosenor that targets it to the plasma membrane (pmEpac2; Wachten *et al*, 2010). This approach revealed a hitherto unsuspected, constitutively active, sub-picomolar relaxin response mediated by an RXFP1-specific GPCR signalosome. RXFP1 is pre-coupled to AC2, and this coupling depends on an inter-action with AKAP79, which is mediated by helix 8 of the receptor C-terminus. Sub-picomolar relaxin activates AC2 further through $G\alpha_s$ and $G\beta\gamma$. This activation is tonically opposed by the activity of PKA upon a scaffolded PDE4D3, tethered to RXFP1 through a β-arrestin 2 interaction that requires Ser^{704}. This elaborate pre-assembled complex that affords great sensitivity to relaxin provides a signalling mechanism for the low circulating levels of this pleiotropic hormone, which should open the door to a greater under-standing of the physiological effects of relaxin in target tissues, such as the normal heart, that do not locally produce the peptide. A ligand-independent GPCR signalosome, which is sensitive to attomolar concentrations of ligand, is a novel paradigm in GPCR signal transduction, and may presage a new facet of receptor signalling.

Results

Sub-picomolar concentrations of relaxin increase cAMP

The aim of this study was to search for a cAMP response to low concentrations of relaxin in single cells, using cytosolic and targeted Epac2-based FRET biosensors (Nikolaev *et al*, 2004; Wachten *et al*, 2010). Stimulation of RXFP1 with increasing concentrations of relaxin revealed a biphasic cAMP concentration–response curve, with EC_{50} values of 10.93 ± 0.92 aM and 0.35 ± 0.10 nM (Figure 1A–D; Table I; Supplementary Figure S1). The low EC_{50} demonstrates for the first time a cellular response to sub-picomolar relaxin, whereas the second EC_{50} reflects previously published values (Table I; Halls *et al*, 2005). There was no detectable cAMP response in cells co-expressing RXFP1 and a sensor that cannot bind cAMP (pmEpac2 R297E; Figure 1D), or in cells expressing the pmEpac2 sensor alone (Figure 1A). Note that stimulation of RXFP1 with a maximal relaxin concentration (100 nM) did not saturate the pmEpac2 sensor, as a combina-tion of forskolin, 3-isobutyl-1-methylxanthine (IBMX) and prostaglandin E_1 (PGE_1) yields higher stimulation (Figure 1B). Additionally, in cells co-expressing RXFP2 and pmEpac2, there was no evidence of a cellular response to sub-picomolar relaxin or INSL3 (Figure 1E and F); thus sub-picomolar relaxin signalling is unique to RXFP1.

To establish a physiological relevance for signalling by low concentrations of relaxin, the cAMP response was assessed in two cell types that endogenously express RXFP1: HeLa cells (Dschietzig *et al*, 2004) and primary cultures of rat cardiac fibroblasts (Samuel *et al*, 2004). To observe an increase in cAMP following relaxin stimulation, it was necessary to 'prime' endogenous AC with forskolin (1 μM). Only the glEpac2 sensor detected a cAMP response to relaxin in HeLa cells; sub-picomolar relaxin caused a significant concentration-dependent increase in cAMP, which was main-tained within the classical relaxin concentration range

(Figure 1G and I). As RXFP1 can couple to $G\alpha_{i/o}$ (Halls *et al*, 2006), we assessed the effect of the $G\alpha_{i/o}$ inhibitor pertussis toxin (PTX); this revealed the biphasic concentra-tion–response curve initially observed in HEK293 cells (EC_{50}: 7.10 ± 0.94 aM; 0.18 ± 0.09 nM; Table I), and indicates that the classical relaxin response in HeLa cells involves the inhibition of AC through $G\alpha_{i/o}$.

In cardiovascular disease models, activation of endogen-ous RXFP1 in cardiac fibroblasts by relaxin results in im-portant cardioprotective effects, including inhibition of hypertrophy and fibrosis (Du *et al*, 2010); thus this is an important model in which to study relaxin-stimulated signal-ling. Rat cardiac fibroblasts are poorly transfected, so it was necessary to infect the cells with an adenoviral version of the cytosolic Epac1-camps sensor (Ad-glEpac1; Nikolaev *et al*, 2005; Figure 1H and I). Sub-picomolar concentrations of relaxin induced a significant increase in cAMP, which then declined within the classical concentration range. The latter phase is consistent with cell population assays, which describe an inhibitory cAMP response curve (Halls ML, unpublished data). Pre-treatment of fibroblasts with PTX also revealed a biphasic, stimulatory concentration–response curve (EC_{50}: 5.11 ± 1.10 aM; 0.30 ± 0.08 nM; Table I).

Sub-picomolar relaxin signalling: $G\alpha_s$ and $G\beta\gamma$ activate AC2

To determine the mechanism whereby sub-picomolar concentrations of relaxin activate cAMP, we inhibited G-protein modulators of the classical relaxin response: $G\alpha_s$, $G\alpha_{i/o}$ and $G\beta\gamma$ (Figure 2; Halls *et al*, 2006). Inhibition of $G\alpha_{i/o}$ using PTX did not affect the cAMP response to sub-picomolar concentrations of relaxin; however, the max-imal cAMP response to relaxin was enhanced (Figure 2A and B). Thus, the $G\alpha_{i3}$-$G\beta\gamma$-PI3K-PKCζ pathway does not generate the cAMP detected by the pmEpac2 sensor. In contrast, inhibition of $G\alpha_s$ by NF449 (Hohenegger *et al*, 1998) signifi-cantly inhibited the 10 fM relaxin response (Figure 2C and D). To confirm this finding, $G\alpha_s$ was primed using a low concentration of cholera toxin (200 ng/ml). This treatment potentiated basal and relaxin-stimulated cAMP accumulation, with the response to 10 fM relaxin reaching the maximal relaxin response (Figure 2C and D). To examine any involve-ment of $G\beta\gamma$ subunits, the inhibitors gallein (Lehmann *et al*, 2008) and mSIRK (Scott *et al*, 2001; Goubaeva *et al*, 2003) were used (Figure 2E and F); both completely abolished the cAMP accumulation stimulated by 10 fM relaxin. Thus, in-creased cAMP elicited by sub-picomolar concentrations of relaxin requires both $G\alpha_s$ and $G\beta\gamma$.

Increased cAMP can occur by either activation of AC or inhibition of PDE. An AC inhibitor, 2′,5′-dd-3′-AMP-bis(*t*BuSATE) (ddAB; Laux *et al*, 2004), completely abolished the response to 10 fM relaxin (Figure 3A and B). Of the nine membrane-bound AC isoforms, only three are activated by both $G\alpha_s$ and $G\beta\gamma$: AC2, AC4 and AC7; of these, only AC2 and AC7 are expressed in HEK293 cells (Hellevuo *et al*, 1993; Figure 3C and D). Overexpression of AC2 significantly in-creased cAMP accumulation under all conditions. The en-hanced basal cAMP observed following AC2 overexpression depended upon co-expression of RXFP1: in the absence of the receptor, overexpression of AC2 had no effect upon basal cAMP (Figure 3E and F). In contrast, overexpression of AC7 abolished the cAMP elicited by 10 fM relaxin; overexpression
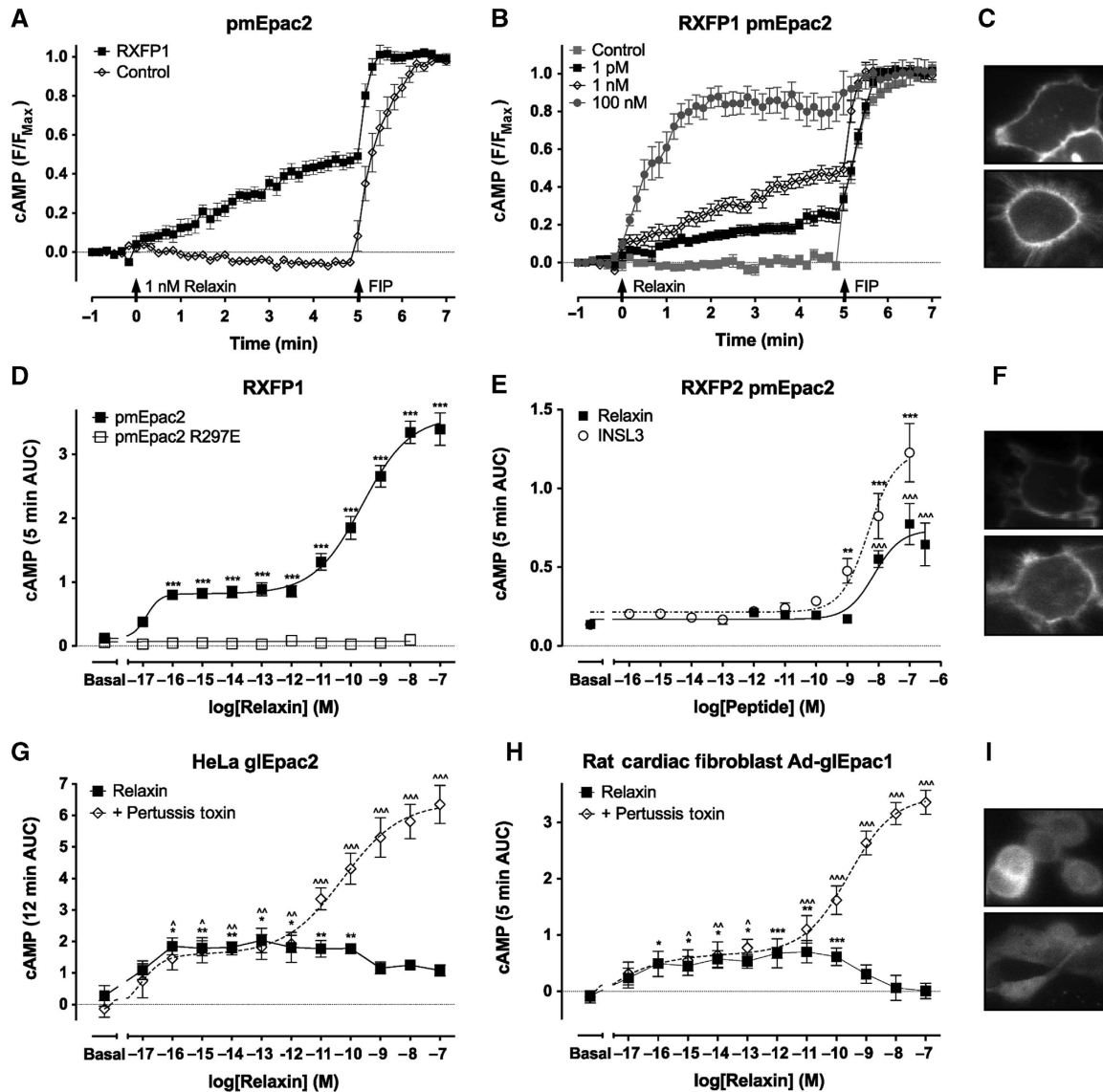
Figure 1 A sub-picomolar relaxin response. cAMP biosensors in single cells revealed a response to sub-picomolar concentrations of relaxin ($n = 10$–74). (**A**) Stimulation of HEK293 cells expressing pmEpac2 or co-expressing pmEpac2 and RXFP1 with a sub-maximal concentration of relaxin (1 nM) over 5 min. (**B**) HEK293 cells co-expressing RXFP1 and pmEpac2 were stimulated with vehicle (0.001 % trifluoroacetic acid, TFA), or a low (1 pM), sub-maximal (1 nM) or high (100 nM) concentration of relaxin over 5 min. All cells were stimulated at 0 min, a maximal cAMP response (FIP; 10 μM forskolin, 100 μM IBMX, 100 nM PGE$_1$) was induced at 6 min. Note that saturation of the probes is not achieved with relaxin stimulation, as FIP gives a greater response. (**C**) HEK293 cells expressing pmEpac2. (**D**) Relaxin (10 aM–100 μM) stimulation of RXFP1 co-expressed with pmEpac2 or pmEpac2 R297E (a sensor that cannot bind cAMP) in HEK293 cells. (**E**) Relaxin (1 pM–1 μM) or INSL3 (100 aM–100 μM) stimulation of RXFP2 co-expressed with pmEpac2 in HEK293 cells. (**F**) HEK293 cells expressing pmEpac2. (**G**) Relaxin (10 aM–100 μM) stimulation of HeLa cells (endogenously express RXFP1) transfected with glEpac2, in the presence of forskolin (1 μM) and the Gα$_{i/o}$ inhibitor pertussis toxin (PTX; 100 ng/ml, 16 h). (**H**) Relaxin (10 aM–100 μM) stimulation of primary rat cardiac fibroblasts (endogenously express RXFP1) infected with Ad-glEpac1, in the presence of forskolin (1 μM) and PTX. (**I**) HeLa cells (top) and rat cardiac fibroblasts (bottom) expressing glEpac2 or Ad-glEpac1, respectively. Data are expressed as the 5 or 12 min area under the curve (AUC). Symbols represent means, error bars, s.e.m. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ relaxin versus basal; ^$P < 0.05$, ^^$P < 0.01$ and ^^^$P < 0.001$ INSL3 or relaxin with PTX versus basal; two-way ANOVA with Bonferroni post tests.

of AC7 may hinder the activity of endogenous AC2, thus causing inhibition of the cAMP response. Nevertheless, this result demonstrates an absolute requirement for AC2 in sub-picomolar relaxin signalling.

Activation of AC2 requires an AKAP

Two immediate possibilities are suggested for the sustained cAMP stimulated by sub-picomolar concentrations of relaxin: (1) that this pool of cAMP is tightly controlled by a

PDE and (2) that AC2 is tonically inhibited by an AKAP (Piggott *et al*, 2008; Efendiev *et al*, 2010). We first investigated AKAP involvement by utilizing an inhibitor of AKAP/PKA interactions, St-Ht31 (Hundsrucker *et al*, 2006; Figure 3G and H). St-Ht31 completely abolished the cAMP accumulation stimulated by 10 fM relaxin, and there was no effect of the negative control, St-Ht31-P. Thus, an AKAP is required for the increase in AC2 activity following RXFP1 stimulation.

Table I Comparison of EC_{50} values for cAMP generation by relaxin and INSL3 using different methodologies

Cell type	Sensor	Relaxin		INSL3
		EC_{50} 1 (aM)	EC_{50} 2 (nM)	EC_{50} (nM)
HEK293-expressing RXFP1	α-Screen assay[a]	Undetectable	0.47 ± 0.21	NA
	glEpac2	140.34 ± 20.73	0.62 ± 0.12	NA
	pmEpac2	10.93 ± 0.92	0.35 ± 0.10	NA
HeLa	glEpac2	7.10 ± 0.94	0.18 ± 0.09	NA
Cardiac fibroblasts	Ad-glEpac1	5.11 ± 1.10	0.30 ± 0.08	NA
HEK293-expressing RXFP2	glEpac2	NA	57.70 ± 6.94	7.74 ± 1.67
	pmEpac2	NA	18.09 ± 3.84	1.42 ± 0.92
HEK293-expressing RXFP1/2	pmEpac2	NA	1.05 ± 0.21	NA
HEK293-expressing RXFP2/1	pmEpac2	7.86 ± 1.30	0.33 ± 0.08	0.26 ± 0.04

The average EC_{50} values for cAMP accumulation in response to relaxin or INSL3 stimulation of RXFP1 are shown. NA indicates that the ligand does not activate the response described by the EC_{50} value.
[a]EC_{50} values were obtained from Halls *et al* (2005), using a Perkin Elmer α-screen cAMP kit (measuring cAMP accumulation in cell populations).
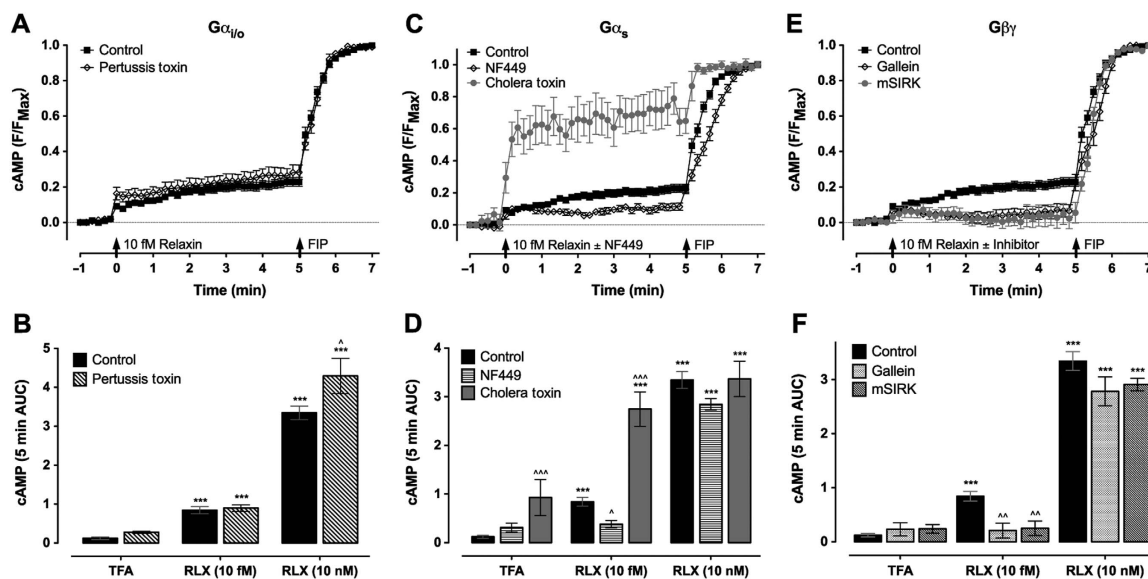

Figure 2 A sub-picomolar relaxin response requires $G\alpha_s$ and $G\beta\gamma$. Sub-picomolar relaxin signalling was examined at the G-protein level in HEK293 cells co-expressing RXFP1 and pmEpac2, and stimulated with vehicle (0.001 % TFA), 10 fM or 10 nM relaxin ($n = 43–51$). (**A**) Cells were pre-incubated with the $G\alpha_{i/o}$ inhibitor PTX (100 ng/ml, 16 h) and stimulated with 10 fM relaxin. (**B**) The 5 min area under the curve (AUC) from (**A**) for TFA, 10 fM and 10 nM relaxin. (**C**) Cells were pre-incubated with the $G\alpha_s$-specific antagonist NF449 (10 μM, 30 min), or the $G\alpha_s$-activator cholera toxin (200 ng/ml, 90 min) and stimulated with 10 fM relaxin. (**D**) The 5 min AUC from (**C**) for TFA, 10 fM and 10 nM relaxin. (**E**) $G\beta\gamma$ subunits were inhibited by gallein (10 μM, 30 min) or mSIRK (5 μM, 15 min) and stimulated with 10 fM relaxin. (**F**) The 5 min AUC from (**E**) for TFA, 10 fM and 10 nM relaxin. Data are expressed relative to the maximal cAMP response (FIP; 10 μM forskolin, 100 μM IBMX, 100 nM PGE₁), or as the 5 min AUC. Bars represent means, error bars s.e.m. ***$P < 0.001$ versus own basal; ^$P < 0.05$, ^^$P < 0.01$ and ^^^$P < 0.001$ versus response to relaxin alone; two-way ANOVA with Bonferroni post tests.

β-Arrestin 2 and PDE4 negatively regulate sub-picomolar relaxin signalling

As cAMP can only be degraded by PDE activity, we examined the effect of a PDE inhibitor, IBMX, upon the concentration–response to relaxin (Supplementary Figure S2). Inhibition of PDE activity abolished the biphasic concentration–response curve, by increasing basal cAMP levels such that no further increases were observed at sub-picomolar relaxin concentrations. We then compared the effect of IBMX upon basal cAMP levels in HEK293 cells transiently expressing RXFP1, RXFP2 or empty vector, pcDNA (Figure 4A and B). IBMX significantly increased basal cAMP only in cells expressing RXFP1. Thus, there is a constitutive negative regulation of cAMP accumulation in HEK293 cells that is induced by RXFP1 expression.

To identify the PDE isoform that negatively regulates the cAMP accumulation stimulated by sub-picomolar relaxin, we used PDE3- and PDE4-selective inhibitors; PDE3 and PDE4 are the main isoforms expressed in HEK293 cells (Lynch *et al*, 2005; Figure 4C and D; Supplementary Figure S2). Both IBMX and the PDE4-specific Ro 20-1724 significantly increased basal cAMP, and there was no further increase following stimulation with 10 fM relaxin. In contrast, there was no significant effect of the PDE3 inhibitor, cilostamide, upon basal, and cAMP accumulation remained responsive to stimulation with 10 fM relaxin. Thus, PDE4 regulates the cAMP generated following AC2 stimulation by RXFP1. Further, vacant receptors exert a low level of constitutive cAMP signalling, which is tightly controlled by PDE4 activity;
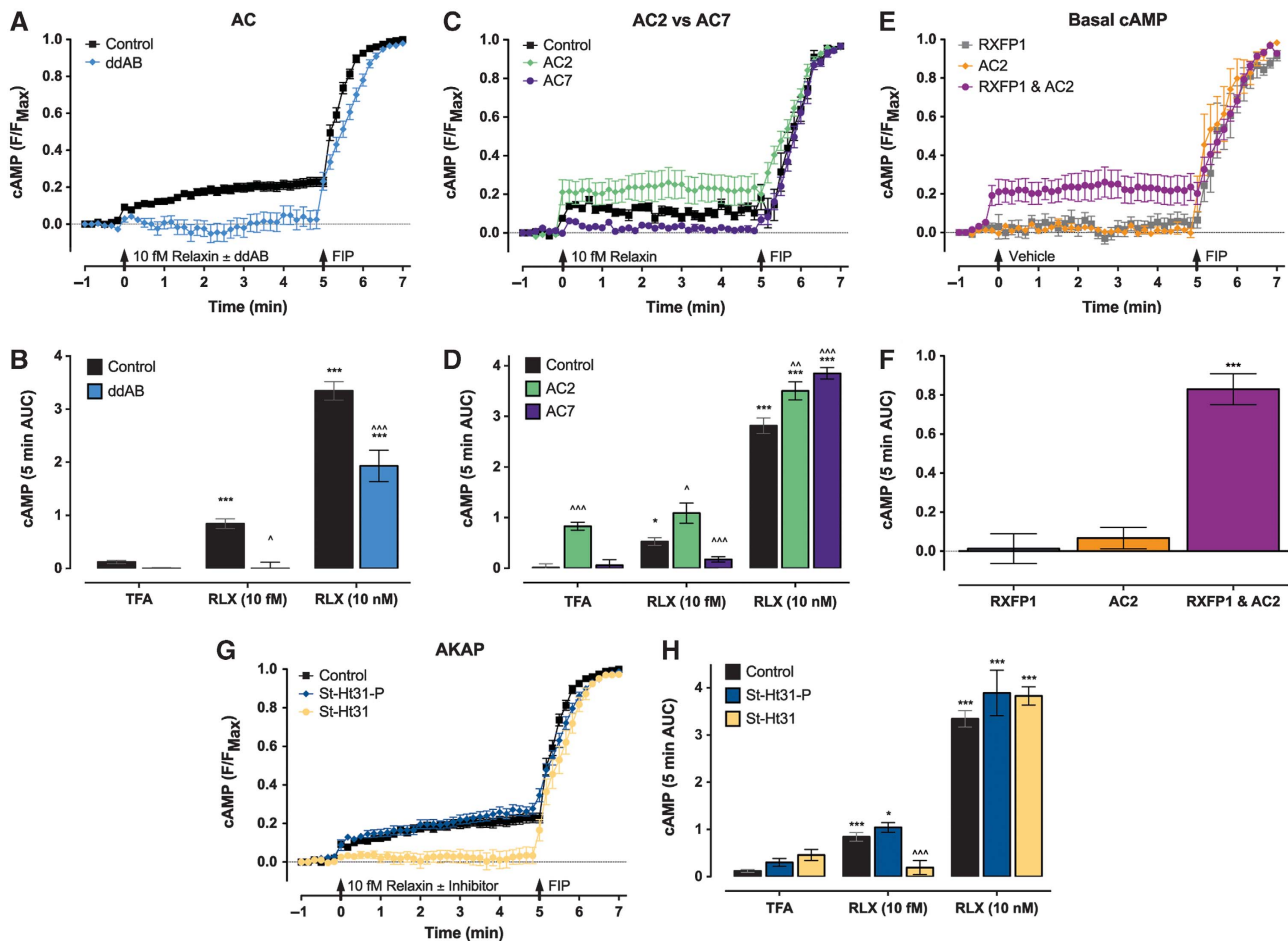
Figure 3 A cellular response to sub-picomolar relaxin requires AC2 and an AKAP. Sub-picomolar relaxin signalling was examined downstream of $G\alpha_s$ and $G\beta\gamma$, in HEK293 co-expressing RXFP1 and pmEpac2, and stimulated with vehicle (0.001% TFA), 10 fM or 10 nM relaxin ($n = 11-52$). (**A**) AC activity was inhibited by 2′, 5′-dd′-3′-AMP-bis(t-BuSATE) (ddAB; 1 μM, 30 min) and cells were stimulated with 10 fM relaxin. (**B**) The 5 min area under the curve (AUC) from (**A**) for TFA, 10 fM and 10 nM relaxin. (**C**) The effect of overexpression of AC2 or AC7 on the response to 10 fM relaxin. (**D**) The 5 min AUC from (**C**) for TFA, 10 fM and 10 nM relaxin. (**E**) The effect of AC2 overexpression with and without co-expression of RXFP1 upon basal cAMP. (**F**) The 5 min AUC from (**E**) for TFA, 10 fM and 10 nM relaxin. (**G**) AKAP/PKA binding was inhibited using St-Ht31 or control peptide St-Ht31-P (both 20 μM, 45 min) and cells were stimulated with 10 fM relaxin. (**H**) The 5 min AUC from (**G**) for TFA, 10 fM and 10 nM relaxin. Data are expressed relative to the maximal cAMP response (FIP; 10 μM forskolin, 100 μM IBMX, 100 nM PGE₁), or as the 5 min AUC. Bars represent means, error bars s.e.m. $*P < 0.05$ and $***P < 0.001$ versus basal; $^P < 0.05$, $^{^}P < 0.01$ and $^{^^}P < 0.001$ versus relaxin/RXFP1; two-way ANOVA with Bonferroni post tests.

activation of RXFP1 with sub-picomolar concentrations of relaxin drives further cAMP production by AC2, overcoming the tonic regulation exerted by PDE4.

Activation of the long isoforms of PDE4 (PDE4D3 and PDE4D5) occurs by PKA-mediated phosphorylation (MacKenzie *et al*, 2002), thus we tested the effect of two PKA inhibitors, H89 and KT5720 (Figure 4E and F). Inhibition of PKA had the same effect as inhibition of PDE4: basal cAMP levels were significantly increased, and stimulation with 10 fM relaxin caused no further increase in cAMP. There was no additive effect if both PKA and PDE4 were simultaneously inhibited (Supplementary Figure S2), which suggests that PKA is upstream of PDE4. Therefore, activation of the PDE4-mediated negative regulation of cAMP depends on PKA.

β-Arrestin-mediated scaffolding of PDE often accompanies GPCR activation, and is an important mechanism whereby GPCRs are both desensitized and internalized. Thus, we assessed the effect of knockdown of β-arrestin 1 or β-arrestin

2 using siRNA (Figure 4G and H), which resulted in a significant reduction of β-arrestin protein (Figure 4I). Expression of β-arrestin 2 siRNA, but not β-arrestin 1 or scrambled siRNA, significantly increased basal cAMP accumulation, and there was no further increase in cAMP induced by 10 fM relaxin. There was also no effect of siRNA upon the classical cAMP response to relaxin, which agreed with previous reports (Callander *et al*, 2009).

Negative regulation by β-arrestin 2 and PDE4 requires Ser[704] of RXFP1

We wished to determine whether the $G\alpha_s$-mediated stimulation of cAMP elicited by different concentrations of relaxin acting on RXFP1 could be separated by manipulation of receptor structure—that is could we preserve one set of effects while eliminating the other (Figure 5). Application of receptor chimeras developed earlier (Sudo *et al*, 2003; Halls *et al*, 2005) revealed the necessity of the RXFP1-transmembrane/C-terminus for sub-picomolar signalling,
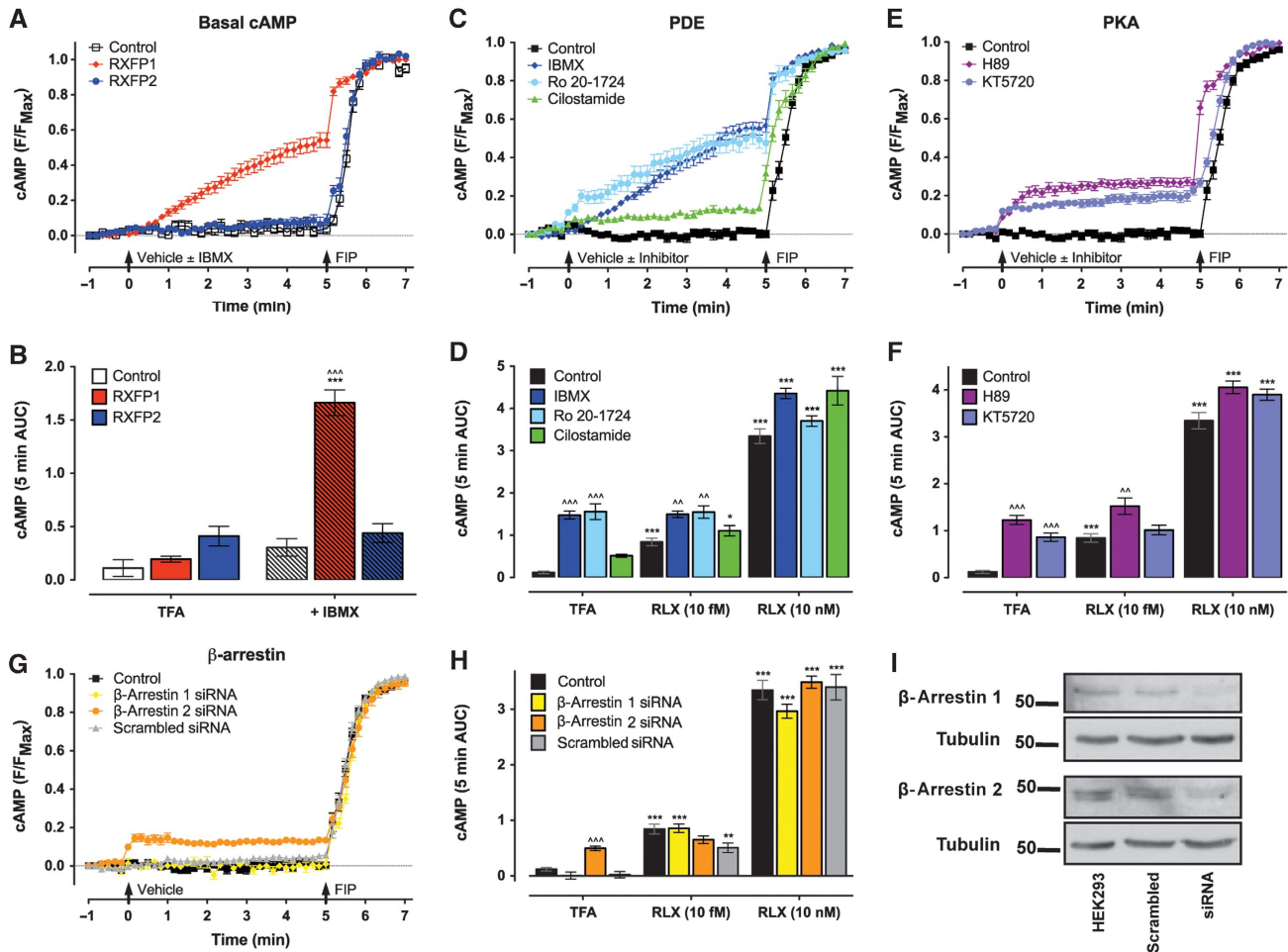
Figure 4 Negative regulation of sub-picomolar relaxin signalling requires PKA, PDE4 and β-arrestin 2. Negative regulation was examined in HEK293 co-expressing RXFP1 or RXFP2, and pmEpac2, and stimulated with vehicle (0.001 % TFA), 10 fM or 10 nM relaxin ($n = 44–51$). (**A**) The effect of PDE inhibition (IBMX, 100 μM, 30 min) on basal cAMP in cells expressing RXFP1 or RXFP2. (**B**) The 5 min area under the curve (AUC) from (**A**) for TFA, 10 fM and 10 nM relaxin. (**C**) The effect of IBMX (general inhibitor), Ro 20-1724 (PDE4 specific; 10 μM, 30 min) or cilostamide (PDE3 specific; 10 μM, 30 min) on the basal cAMP. (**D**) The 5 min AUC from (**C**) for TFA, 10 fM and 10 nM relaxin. (**E**) PKA was inhibited by H89 (10 μM, 30 min) or KT5720 (1 μM, 30 min) and the basal cAMP measured. (**F**) The 5 min AUC from (**E**) for TFA, 10 fM and 10 nM relaxin. (**G**) The effect of β-arrestin 1, β-arrestin 2 or scrambled siRNA (all 100 nM) on the basal cAMP response. (**H**) The 5 min AUC from (**G**) for TFA, 10 fM and 10 nM relaxin. (**I**) Western blots showing specific knockdown of β-arrestin 1 or β-arrestin 2 by targeted siRNA compared with controls. Blots were re-probed with tubulin to ensure equal protein loading (Supplementary data). Data are expressed relative to the maximal cAMP response (FIP; 10 μM forskolin, 100 μM IBMX, 100 nM PGE$_1$), or as the 5 min AUC. Bars represent means, error bars s.e.m. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ versus basal; ^^$P < 0.01$ and ^^^$P < 0.001$ versus relaxin/pcDNA; two-way ANOVA with Bonferroni post tests.

while maintaining the classical response mediated by Gα$_s$. Thus, a biphasic response was evident upon expression of RXFP2/1 (RXFP2-ectodomain, RXFP1-transmembrane/C-terminus), but not RXFP1/2 (RXFP1-ectodomain, RXFP2-transmembrane/C-terminus; Figure 5A and B; Table I). Furthermore, only relaxin, but not the closely related peptide INSL3, increased cAMP accumulation at sub-picomolar concentrations. Thus, the biphasic concentration–response is not only specific for RXFP1, but also for relaxin.

The involvement of the RXFP1 C-terminus was then explored further using a number of previously described receptor mutants (Halls et al, 2009). The glEpac2 sensor was used for these experiments because of very low cAMP levels detected by the pmEpac2 sensor following activation of some of the mutants. Truncation of the receptor C-terminus beyond helix 8 (tRXFP1-703; Figure 5C and D) removed the negative regulation of cAMP signalling. In contrast to the effects of PDE4 or PKA inhibition, or β-arrestin 2 knockdown,

tRXFP1-703 remained responsive to stimulation with sub-picomolar concentrations of relaxin. Thus, truncation of the RXFP1 C-terminus eliminates the regulation of sub-picomolar signalling but not G$_s$-mediated activation of AC2. The maximal cAMP response to relaxin was also reduced following truncation, because of loss of activation of the Gα$_{i3}$ pathway (Halls et al, 2009).

Overlapping the end of helix 8 and the first few residues of the C-terminus is a putative phosphorylation motif (residues 700–708; RQRK**S**MDSK), which if basally phosphorylated could potentially provide an interaction site for β-arrestin 2. Mutation of the target serine to alanine (RXFP1 S704A; Figure 5C and D) had the same effect as inhibition of PKA and PDE4, and knockdown of β-arrestin 2: basal cAMP accumulation was significantly increased. Furthermore, there was no additional effect of PKA inhibition in response to relaxin stimulation of RXFP1 S704A (Supplementary Figure S3). Thus, regulation by β-arrestin 2, PKA and PDE4 requires Ser[704],
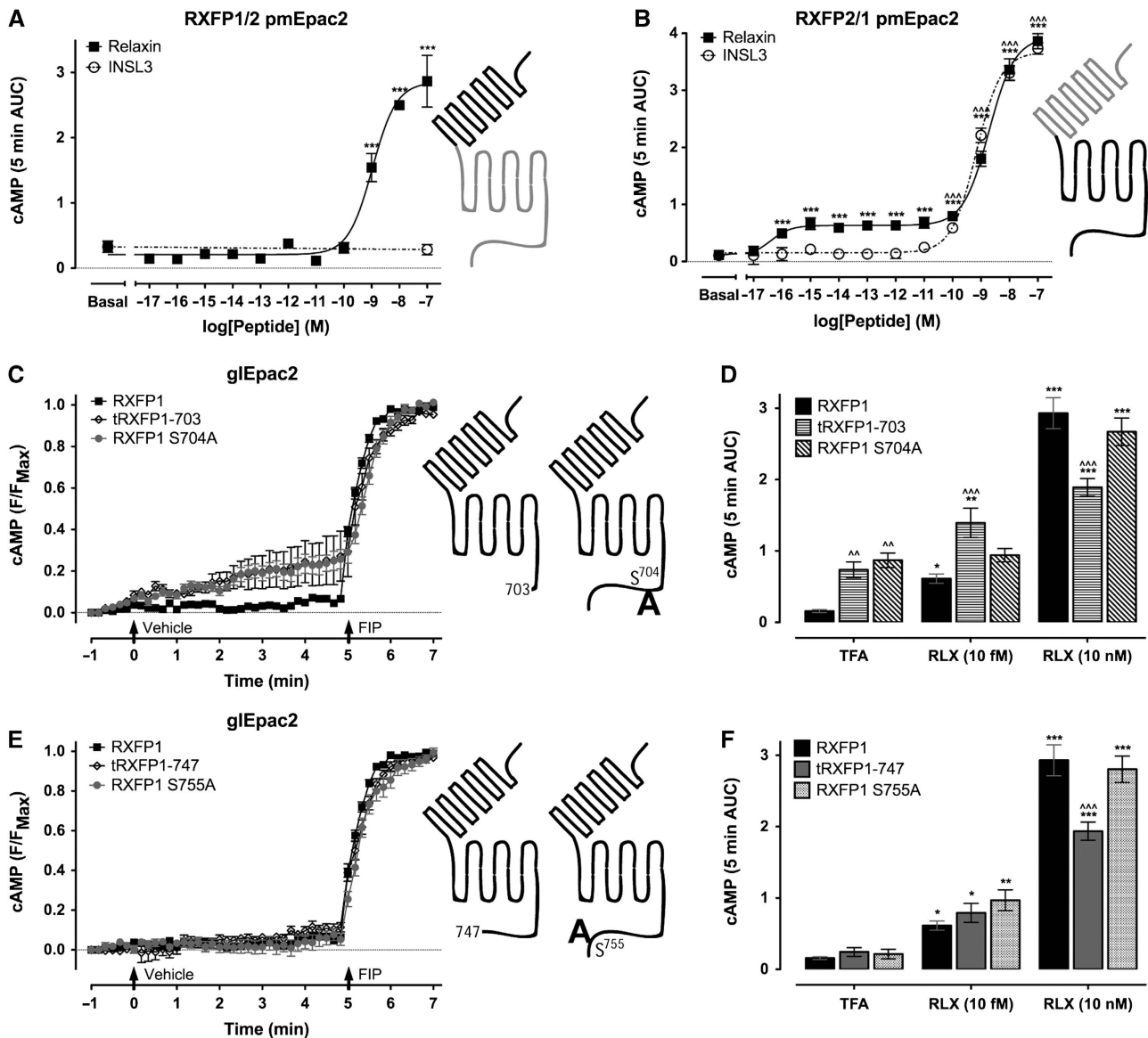
Figure 5 Sub-picomolar relaxin signalling requires the RXFP1-transmembrane/C-terminus, negative regulation requires Ser[704]. The region of RXFP1 controlling sub-picomolar relaxin signalling was examined using mutant receptors in HEK293 cells expressing pmEpac2 or glEpac2 ($n = 10$–45). (**A**) Relaxin (10 aM–100 μM) or INSL3 (100 μM) stimulation of RXFP1/2 (RXFP1-ectodomain, RXFP2-transmembrane/C-terminus) co-expressed with pmEpac2. (**B**) Relaxin or INSL3 (10 aM–100 μM) stimulation of RXFP2/1 (RXFP2-ectodomain, RXFP1-transmembrane/C-terminus) co-expressed with pmEpac2. (**C**) The basal cAMP response in cells co-expressing RXFP1, tRXFP1-703 (truncated following helix 8; left) or RXFP1 S704A (potential phosphorylation site mutation; right) and glEpac2. (**D**) The 5 min area under the curve (AUC) from (**C**) for TFA, 10 fM and 10 nM relaxin. (**E**) The basal cAMP response in cells co-expressing RXFP1, tRXFP1-747 (truncation of final 10 residues; left) or RXFP1 S755A (potential phosphorylation site mutation; right) and glEpac2. (**F**) The 5 min AUC from (**E**) for TFA, 10 fM and 10 nM relaxin. Data are expressed relative to maximal cAMP (FIP; 10 μM forskolin, 100 μM IBMX, 100 nM PGE$_1$), or as the 5 min area AUC. Bars represent means, error bars s.e.m. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ relaxin versus basal; $^{\wedge\wedge}P < 0.01$ and $^{\wedge\wedge\wedge}P < 0.001$ INSL3/mutant versus basal/RXFP1 respectively; two-way ANOVA, Bonferroni post tests.

and AKAP-dependent stimulation of AC2 does not depend on the receptor C-terminus beyond helix 8.

To exclude a function for the remainder of the C-terminus, we examined two additional mutant receptors (Figure 5E and F): a receptor lacking the final 10 amino acids of the C-terminus (tRXFP1-747) and another phosphorylation mutant within a potential site encompassing the final 10 residues (residues 748–757; SQSTRLN**S**YS; RXFP1 S755A). There was no effect of either of these mutations upon the cAMP response to sub-picomolar concentrations of relaxin. The

maximal cAMP response to relaxin was reduced through tRXFP1-747, because of loss of activation of the Gα$_{i3}$ pathway as previously reported (Halls *et al*, 2009). On the basis of this series of studies, we would conclude that there are specific properties within the molecular nature of RXFP1 that are necessary for regulation of the high-affinity response. In later experiments, we would exploit these apparent characteristics in order to determine whether a different cadre of molecular components would continue to associate with appropriately modified receptor elements.

AKAP79 tethering of AC2 to RXFP1 occurs within helix 8, whereas β-arrestin 2, PDE4D3 and PKA associate with helix 8 and residues 704–725

The molecular components of the RXFP1 signalosome, and their sites of interaction with the C-terminus, were then directly examined using GST pull-down assays (Figure 6) and co-immunoprecipitation (co-IP) (Figure 7).

We generated GST fragments of the RXFP1 C-terminus (Figure 6A), and assessed pull down of candidate proteins from HEK293 cell lysate by immunoblotting. β-Arrestin 2 was pulled down by helix 8 and residues 704–725 (Figure 6B and D). As our HEK293 cells principally express PDE4D5, PDE4D3 and PDE4B2 (Willoughby et al, 2007), and only

the long isoforms of PDE4 (PDE4D3 and PDE4D5) are activated by PKA phosphorylation (MacKenzie et al, 2002), immunoreactivities from pull-down experiments were examined with a PDE4D-specific antibody (Figure 6B and D). Two bands were evident in the input, corresponding to the anticipated molecular weights of PDE4D5 (105 kDa; MacKenzie et al, 2002) and PDE4D3 (95 kDa; Terrenoire et al, 2009). As β-arrestin 2 can sequester PDE4 to GPCRs (Perry et al, 2002), we anticipated that the relevant PDE4 isoform would be pulled down by the same fragments of the RXFP1 C-terminus as β-arrestin 2: helix 8 and residues 704–725. Only one band was pulled down by the RXFP1 C-terminus, corresponding to the molecular weight of PDE4D3. This
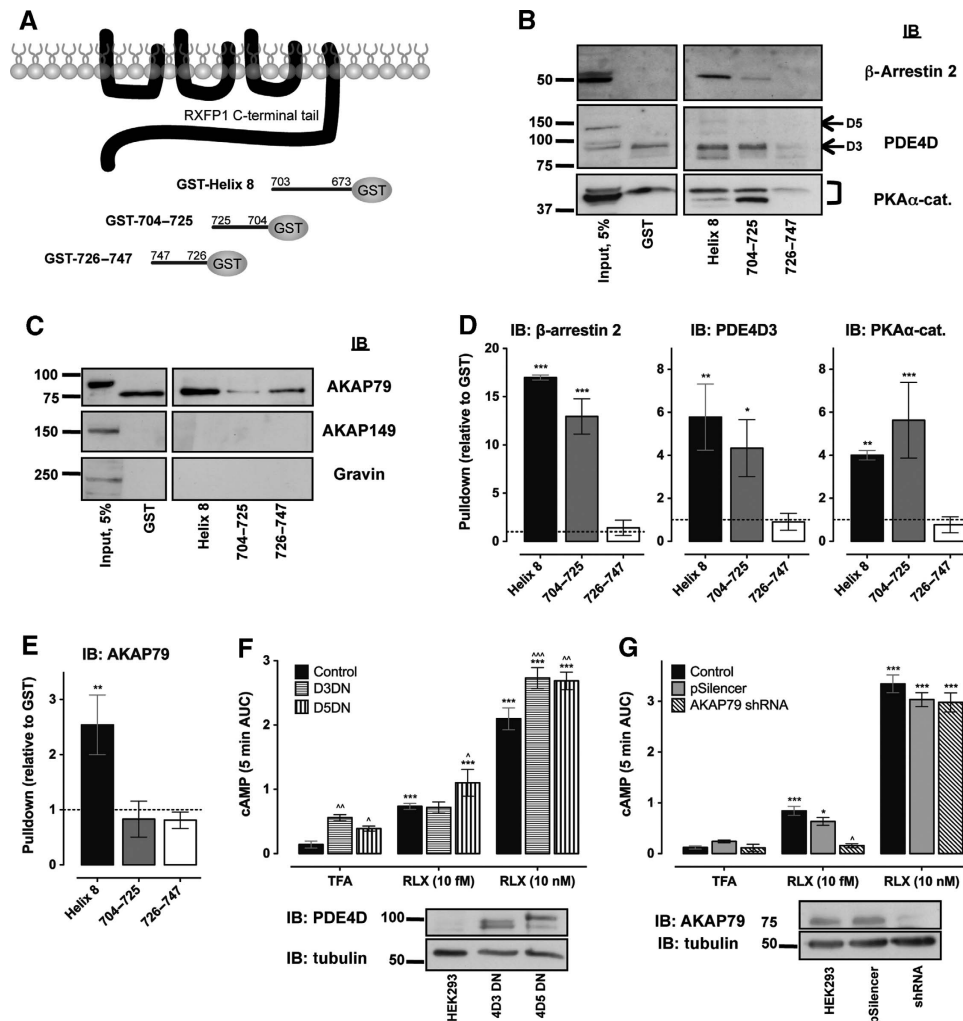

Figure 6 The RXFP1 signalosome: AC2, AKAP79, β-arrestin 2, PKA and PDE4D3. Potential interactions between RXFP1 and mediators of the sub-picomolar relaxin response were assessed by GST pull down from HEK293 lysate using fragments of the RXFP1 C-terminus ($n = 4$), and confirmed in single-cell FRET studies. (**A**) Fragments of the RXFP1 C-terminus were tagged with GST. (**B**) Representative blots of GST pull-down assays probed with β-arrestin 2, PDE4D and PKAα-catalytic subunit antibodies (Supplementary data). (**C**) Representative blots probed with AKAP79, AKAP149 and gravin antibodies (Supplementary data). (**D**) Densitometry was calculated for β-arrestin 2, PDE4D3 and PKA. (**E**) Densitometry was calculated for AKAP79. Data are expressed as band density relative to pull down by GST alone. Bars represent means, error bars s.e.m. *$P < 0.05$ and **$P < 0.01$ versus GST alone, two-way ANOVA with Bonferroni post tests. (**F**) Dominant negative PDE4D3 and PDE4D5 were overexpressed in HEK293 cells with RXFP1 and pmEpac2 for single-cell FRET studies. The 5 min area under the curve (AUC) was calculated for TFA, 10 fM and 10 nM relaxin ($n = 16–43$). Western blotting shows expression of the two isoforms with equal loading confirmed using a tubulin antibody (Supplementary data). (**G**) The effects of AKAP79 shRNA or pSilencer control were assessed in HEK293 cells co-expressing RXFP1 and pmEpac2 using single-cell FRET studies. The 5 min AUC was calculated for TFA, 10 fM and 10 nM relaxin ($n = 20–51$). Western blotting shows decreased AKAP79 protein following shRNA transfection with equal protein loading confirmed using a tubulin antibody (Supplementary data). Bars represent means, error bars s.e.m. *$P < 0.05$ and ***$P < 0.001$ versus own basal; ^$P < 0.05$ and ^^$P < 0.01$ versus control; two-way ANOVA with Bonferroni post tests.
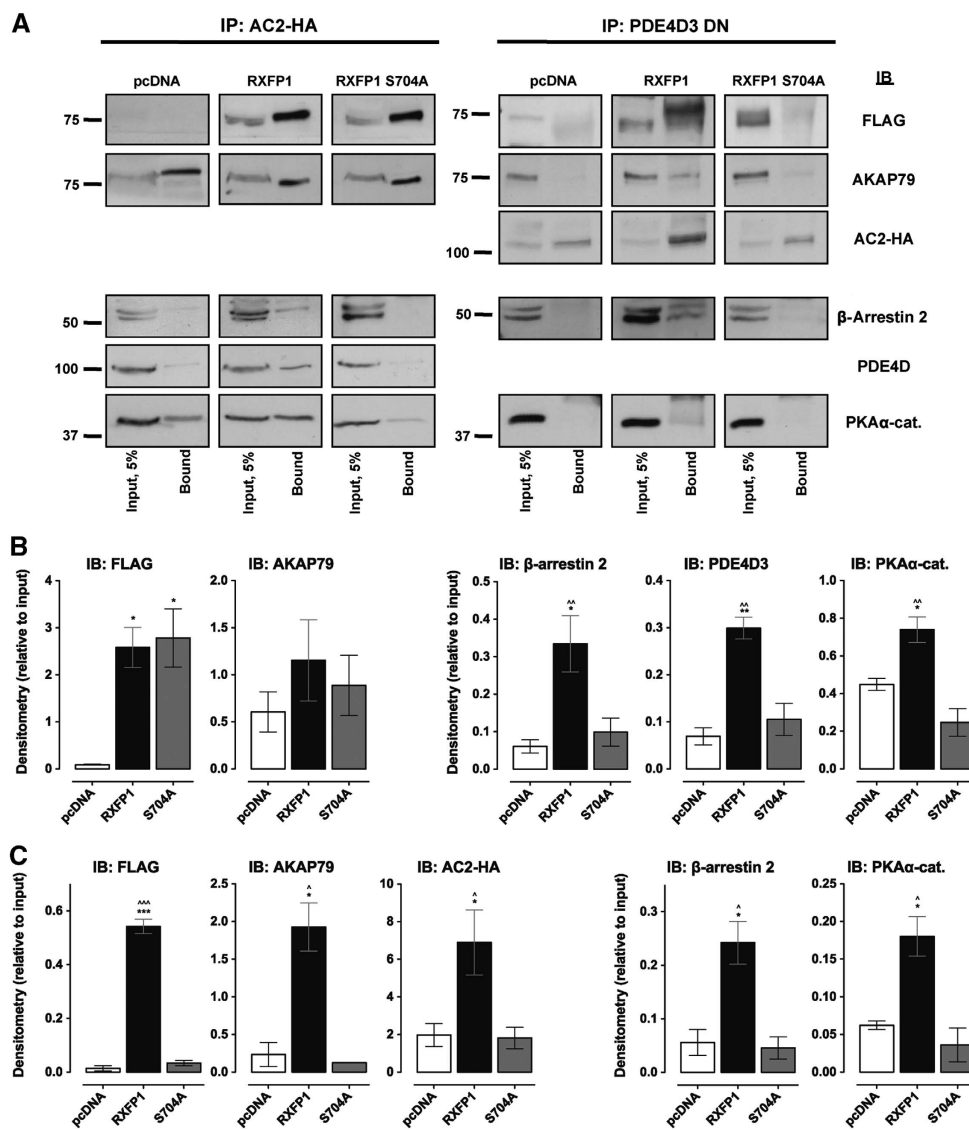
Figure 7 AKAP79 and AC2 associate with full-length RXFP1, and the interaction of RXFP1 with β-arrestin 2, PDE4D3 and PKA depends upon Ser[704]. The proteins that constitute the RXFP1 signalosome were assessed for interactions with the full-length receptor and RXFP1 S704A by immunoprecipitation (IP) of AC2-HA or dominant negative PDE4D3 ($n = 3$–4). (**A**) Representative blots of AC2-HA or dominant negative (DN) PDE4D3 IP from HEK293 lysate expressing pcDNA and AC2-HA, FLAG-RXFP1 and AC2-HA, or FLAG-RXFP1 S704A and AC2-HA, and probed with FLAG, AKAP79, HA, β-arrestin 2, PDE4D or PKAα-catalytic subunit antibodies (Supplementary data). (**B**) Average densitometry for AC2-HA IP. (**C**) Average densitometry for PDE4D3 DN IP. Data are expressed as band density relative to 5% input. Bars represent means, error bars s.e.m. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ versus pcDNA; ^$P < 0.05$, ^^$P < 0.01$ and ^^^$P < 0.001$ versus RXFP1 S704A; one-way ANOVA with Newman–Keuls multiple comparison test.

suggests that PDE4D3 mediates the negative regulation of cAMP stimulated by sub-picomolar relaxin. Immunoblotting with an antibody targeting the PKAα-catalytic domain also revealed an interaction with helix 8 and residues 704–725 (the antibody identifies two bands; Figure 6B and D).

To confirm the specific involvement of PDE4D3 over PDE4D5, we overexpressed dominant negative mutants (which retain β-arrestin binding, but lack catalytic activity; Baillie et al, 2003; McCahill et al, 2005) of the two isoforms and examined the RXFP1-stimulated cAMP response using the pmEpac2 sensor (Figure 6F). Overexpression of dominant negative PDE4D3 significantly increased basal cAMP, but there was no further increase in the cAMP response to 10 fM relaxin. Conversely, overexpression of dominant negative PDE4D5 significantly increased the cAMP response to all

concentrations of relaxin, suggesting a global effect of PDE4D5, but a targeted action of PDE4D3 on the sub-picomolar relaxin response.

As HEK293 cells express three major AKAPs (AKAP79, AKAP149 and gravin; Gardner et al, 2006; Willoughby et al, 2006), we also probed GST pull-down blots with specific AKAP antibodies. There was no pull down of AKAP149 or gravin by RXFP1 (Figure 6C). AKAP79 was only pulled down by helix 8 (Figure 6C and E). To confirm a specific involvement of AKAP79 in the RXFP1 signalosome, we assessed the effect of AKAP79 knockdown upon the cAMP response detected by the pmEpac2 sensor (Figure 6G). Knockdown of AKAP79 using shRNA results in a significant reduction of AKAP79 protein (Figure 6G), and abolished the cAMP response to 10 fM relaxin.

To confirm that the proteins identified by GST pull down also interacted with full-length RXFP1, we performed two different co-IP experiments: one looking for proteins that co-IP with overexpressed AC2-HA, and a second to identify proteins that co-IP with overexpressed dominant negative PDE4D3 (Figure 7). Results showed co-IP of RXFP1 with AC2-HA, which was unaffected by mutation of Ser[704] (RXFP1 S704A; Figure 7A and B). AKAP79 associated with AC2-HA in the absence and presence of RXFP1, and this was also unaffected by the S704A mutation (Figure 7A and B). Conversely, β-arrestin 2, PDE4D3 and PKA all exhibited significantly increased co-IP with AC2-HA in the presence of RXFP1, which did not occur in lysate from cells expressing RXFP1 S704A (Figure 7A and B). Thus, AKAP79 and AC2-HA associate with full-length RXFP1, and the association of β-arrestin 2, PDE4D3 and PKA depends upon Ser[704]. The absolute requirement of Ser[704] was further established using co-IP of overexpressed dominant negative PDE4D3: while RXFP1, AKAP79, AC2-HA, β-arrestin 2 and PKA all associated with PDE4D3, these interactions were abolished if Ser[704] was mutated (Figure 7A and C). Note that there are some expected constitutive associations, for example between AC2 and AKAP79 (Efendiev *et al*, 2010), but there is a clear and specific increment in these (and other) associations upon RXFP1 expression in both biochemical and functional terms (Figures 4 and 6), confirming the targeted formation of an RXFP1-specific complex.

AC2 interacts with RXFP1 through AKAP79, whereas the association with PDE4D3 and PKA depends upon β-arrestin 2 binding to Ser[704]

The contact points between the proteins of the RXFP1 signalosome were examined by assessing the effect of knockdown of either AKAP79 or β-arrestin 2 upon GST pull-down assays (Figure 8) or co-IP studies (Figure 9).

Knockdown of β-arrestin 2 significantly decreased the pull down of β-arrestin 2, PDE4D3 and PKA by helix 8 and residues 704–725 (Figure 8A and B). There was no effect of knockdown on the non-specific pull down of these proteins by GST alone or residues 726–747. Thus, β-arrestin 2 is required for the interaction of both PDE4D3 and PKA with the RXFP1 C-terminus. Although a recent proteomics study confirmed a PDE4D3/β-arrestin 2 interaction, it failed to detect an association between overexpressed FLAG-tagged β-arrestin 2 and PKA (Xiao *et al*, 2007). However, that study also failed to find expected interactions between the overexpressed β-arrestins and any GPCRs (despite overexpression of the angiotensin 1A receptor). Thus, although potentially highly sensitive, proteomics approaches may still be susceptible to confounding factors such as detergent choice and the use of protein tags.

There was no effect of β-arrestin 2 knockdown on the pull down of AKAP79 by helix 8 (Figure 8A and C), confirming that the interaction between AKAP79 and RXFP1 is independent of β-arrestin 2. Similarly, knockdown of β-arrestin 2 did not significantly affect the association between AC2-HA and full-length RXFP1 or AKAP79 (Figure 9A and B). However, the interactions between AC2-HA and β-arrestin 2, PDE4D3 and PKA were significantly decreased (Figure 9A and B). Furthermore, knockdown of β-arrestin 2 significantly reduced the co-IP between PDE4D3 and all proteins within the RXFP1 signalosome (Figure 9A and C), thus the

interaction of these proteins with PDE4D3 depends on β-arrestin 2.

Knockdown of AKAP79 significantly decreased the co-IP between AC2-HA and all proteins within the RXFP1 signalosome (Figure 9A and B), which confirms that AKAP79 scaffolds AC2 to RXFP1. Conversely, knockdown of AKAP79 did not affect the association between PDE4D3 and β-arrestin 2, PKA or RXFP1, but significantly reduced the interaction between PDE4D3 and both AKAP79 and AC2-HA (Figure 9A and C).

Thus, the stimulatory (AC2 and AKAP79) and regulatory (β-arrestin 2, PDE4D3 and PKA) components of the RXFP1 signalosome interact with the receptor independently; AC2 associates with RXFP1 through AKAP79, and β-arrestin 2 binding to Ser[704] recruits PDE4D3 and PKA to the protein complex (Figure 10).

Discussion

Although a wide range of tissues locally produce high concentrations of relaxin that are effective at the traditional relaxin signalling pathways, the peptide only occurs at low concentrations in the circulation (Gedikli *et al*, 2009). To date, there has been no evidence of a physiological function for such low concentrations of circulating relaxin (Sherwood, 2004). The effects presented here of sub-picomolar relaxin upon a constitutively active RXFP1 provide, for the first time, a cellular response to low levels of the circulating hormone. This response was detectable in a wide range of cell types, and thus provides a mechanism whereby circulating levels of relaxin could affect physiological targets where there is minimal production of the hormone, such as the normal heart. Such sensitivity has been demonstrated in a few other physiological systems (Ying *et al*, 1986; Alleva *et al*, 1997; Ueda *et al*, 2001), but is unheard of for a GPCR and thus represents a novel paradigm for GPCR signalling.

The response that we have uncovered here is completely different from known RXFP1 signalling. The classical cAMP response generated by relaxin activation of RXFP1 is well characterized (Nguyen *et al*, 2003; Nguyen and Dessauer, 2005a, b; Halls *et al*, 2006, 2009), but of these components only Gα$_s$ and Gβγ are involved in sub-picomolar relaxin signalling (Supplementary Figure S2). Furthermore, neither AKAP79 nor β-arrestin 2 are involved in the classical relaxin response, as demonstrated here by a lack of effect of inhibitors upon the cAMP production stimulated by 10 nM relaxin. Even more significantly, stimulation of RXFP1 with 10 nM (but not 10 fM) relaxin reduces the association between RXFP1 and PDE4D3 or AC2 (Supplementary Figure S4), suggesting dissociation of the RXFP1 signalosome following activation of the classical relaxin response. Thus, a pathway that is completely independent of the classical cAMP response mediates the increase in cAMP stimulated by sub-picomolar concentrations of relaxin.

An unusual characteristic of the classical RXFP1 regulatory repertoire is the lack of significant receptor desensitization and internalization (Callander *et al*, 2009; Kern and Bryant-Greenwood, 2009). This is in contrast to more prototypical GPCRs, such as the β$_2$-adrenoceptor, and may potentially explain the toleration of constitutive activity documented here, in addition to the specificity of the RXFP1 complex for PDE4D3, over the preference of a β$_2$-adrenoceptor/AKAP79/
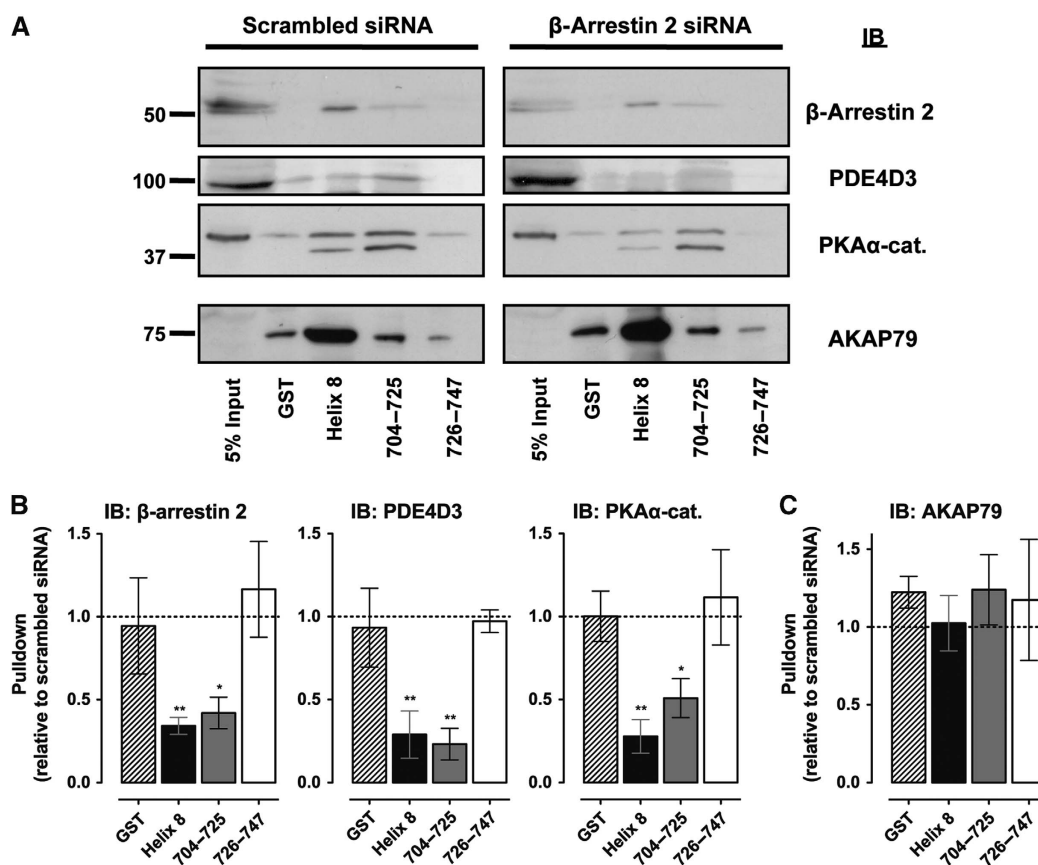
Figure 8 The pull down of PDE4D3 and PKA depends on β-arrestin 2. To assess the protein–protein interactions within the RXFP1 signalosome, the effect of knockdown of β-arrestin 2 upon the GST pull-down assay was assessed ($n = 4$). (**A**) Representative blots of GST pull-down assays from HEK293 lysate, transfected with scrambled or β-arrestin 2 siRNA, and probed with β-arrestin 2, PDE4D, PKAα-catalytic subunit or AKAP79 antibodies (Supplementary data). (**B**) Average densitometry for β-arrestin 2, PDE4D3 and PKA. (**C**) Average densitometry for AKAP79. Data are expressed as β-arrestin 2 siRNA band density relative to scrambled siRNA controls. Bars represent means, error bars s.e.m. *$P < 0.05$ and **$P < 0.01$ versus scrambled siRNA control, one-way ANOVA with Newman–Keuls multiple comparison test.

β-arrestin 2 complex for PDE4D5 (Bolger *et al*, 2003; Lynch *et al*, 2005; Willoughby *et al*, 2007). The lack of appreciable receptor desensitization and internalization may also rationalize another unique feature of this signalosome that opposes the accepted behavioural paradigm for GPCRs: the constitutive dual coupling of RXFP1 to both G-proteins and β-arrestin 2. These same characteristics have recently been demonstrated by the D4 dopamine receptor (Rondou *et al*, 2010; Spooren *et al*, 2010), and thus may represent an emerging and particular hallmark of receptors that constitutively associate with β-arrestins, but do not desensitize or internalize. However, an apparent contradiction is seen between the present results and those of Callander *et al* (2009) who reported cytosolic expression of GFP-tagged β-arrestin 2 in cells expressing RXFP1. This difference might reflect the high expression of GFP-β-arrestin 2 in that study, but is more likely due to the pre-formation of the complex that we are describing with endogenous β-arrestin 2 (precluding association with GFP-β-arrestin 2), or the possibility that RXFP1 cannot physically associate with GFP-β-arrestin 2.

Detailed examination of sub-picomolar relaxin signalling also revealed a novel constitutive activity of RXFP1. Only in cells expressing RXFP1, but not RXFP2, did inhibition of PDE significantly increase basal cAMP. Furthermore, overexpres-

sion of AC2 only increased basal cAMP upon co-expression of RXFP1. Thus at rest, a constitutive stimulation of AC2 by RXFP1 increases cAMP accumulation, which is tonically opposed by PDE4D3. Additional regulation of the activity of the RXFP1 signalosome may also be exerted by AKAP79, which can interact with AC2, AC3, AC5, AC6, AC8 and AC9. Although the interaction of AKAP79 with AC3 and AC9 is neutral, the same association causes inhibition of AC2, AC5, AC6 and AC8 activity (Bauman *et al*, 2006; Efendiev *et al*, 2010; Willoughby *et al*, 2010). Thus, inhibition of AC2 by AKAP79 may provide an additional means of dampening the Gα$_s$- and Gβγ-mediated activation, explaining the inability of 10 fM relaxin to elevate cAMP levels beyond those seen with PDE4 inhibition.

We have comprehensively dissected both the molecular and functional composition of the sub-picomolar relaxin response: stimulation of the receptor leads to Gα$_s$- and Gβγ-dependent activation of AC2, which depends on the interaction of AKAP79 with helix 8 of the receptor C-terminus (Figure 10). AKAP79 scaffolding of AC2 to RXFP1 likely facilitates efficient transduction of the cAMP signal. Negative regulation of the sub-picomolar relaxin response occurs through PKA activation of PDE4D3, both of which are tethered to the receptor by β-arrestin 2. Binding of this negative regulatory complex requires Ser[704], and thus the
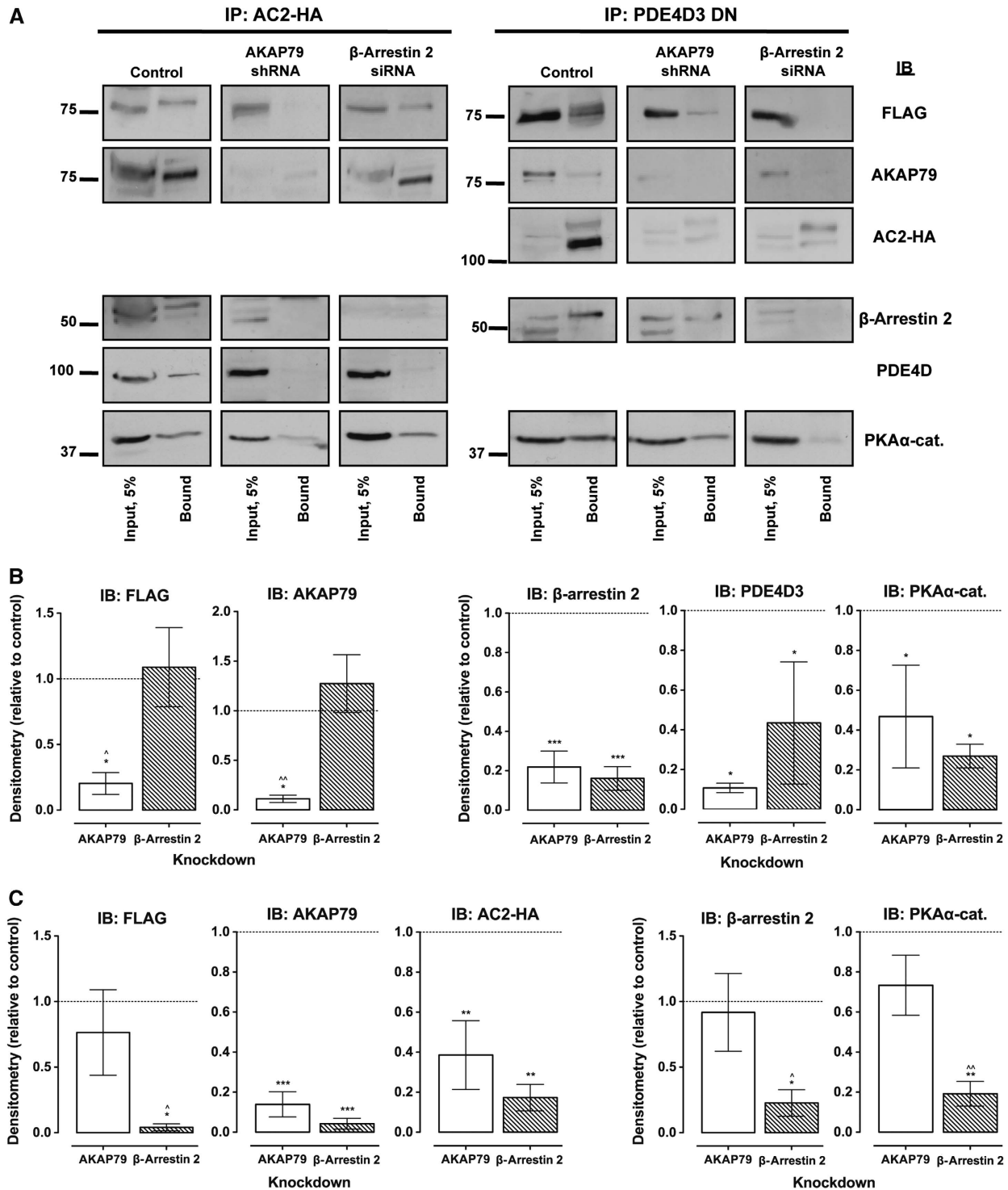
Figure 9 AKAP79 tethers AC2 to RXFP1 independently of the regulatory complex; β-arrestin 2 controls the association of RXFP1 with PDE4D3 and PKA. To examine the protein–protein interactions within the RXFP1 signalosome, the effect of knockdown of either AKAP79 or β-arrestin 2 upon AC2-HA IP and PDE4D3 DN IP was assessed ($n = 3$–4). (**A**) Representative blots of AC2-HA or PDE4D3 DN IP from lysate of cells co-expressing AC2-HA and FLAG-RXFP1, and transfected with AKAP79 shRNA or β-arrestin 2 siRNA, and probed with FLAG, AKAP79, HA, β-arrestin 2, PDE4D and PKAα-catalytic subunit antibodies (Supplementary data). (**B**) Average densitometry for AC2-HA IP. (**C**) Average densitometry for PDE4D3 DN IP. Data are expressed as band density relative to control. Bars represent means, error bars s.e.m. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ versus control; $^\wedge P < 0.05$ and $^{\wedge\wedge} P < 0.01$ versus β-arrestin 2 knockdown for AC2-HA IP or versus AKAP79 knockdown for PDE4D3 DN IP; one-way ANOVA with Newman–Keuls multiple comparison test.

β-arrestin 2 association with RXFP1 probably occurs within a putative phosphorylation motif (700-RQRK**S**MDSK-708) (Figure 10). Importantly, the stimulatory (AKAP79 and AC2) and regulatory (β-arrestin 2, PDE4D3 and PKA) components of the signalosome appear to interact with spatially distinct areas of the RXFP1 C-terminus.
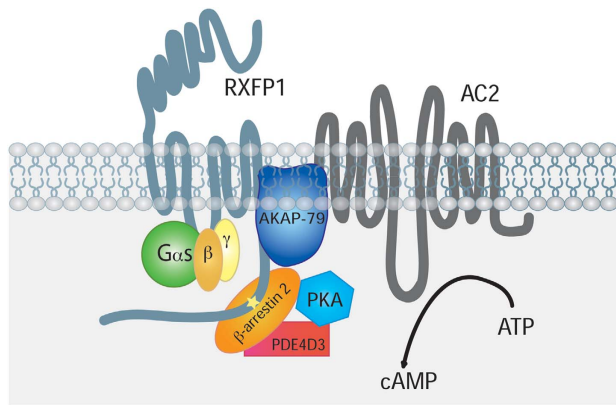
Figure 10 A cartoon illustrating the components of the constitutively assembled RXFP1 signalosome that can be activated by sub-picomolar concentrations of relaxin. The interaction of AKAP79 with helix 8 of the RXFP1 C-terminus allows the co-localization of the receptor with AC2. AC2 is stimulated by $G\alpha_s$ and $G\beta\gamma$, to increase cAMP accumulation. This increase in cAMP results in sequential activation of PKA and PDE4D3. β-Arrestin 2 interacting with Ser[704] (indicated with an asterisk) of the receptor C-terminal tail localizes the tonic opposition mediated by PKA and PDE4D3. Importantly, the stimulatory (AKAP79 and AC2) and regulatory (β-arrestin 2, PDE4D3 and PKA) components interact with distinctly defined regions of the C-terminal tail.

It is important to stress that the complex that we are proposing is not only supported by various biochemical strategies, but also that the complex is altered in a predictable manner when individual components are knocked down. For instance, β-arrestin 2 knockdown only prevents the association of AC2 (in AC2 co-IP) with PDE4D3 and PKA, but completely removes any protein interactions detected by PDE4D3 co-IP; similarly, knockdown of AKAP79 only prevents the association of PDE4D3 with AC2 (in PDE4D3 co-IP), but completely removes any protein interactions detected by AC2 co-IP. Thus, the consequences of targeted knockdown are not reflective of proteins that merely directly interact, but point to a defined and inter-dependent complex organization (Figure 10). Of even greater significance is the fact that the RXFP1 signalosome, as identified by these biochemical strategies, shows the same functional dependency for its composition; that is to say that perturbing individual components has outcomes that are predicted based upon the biochemical measurements. For instance, β-arrestin 2 knockdown mimics PDE4D3 or PKA inhibition; similarly, the knockdown of AKAP79 reflects inhibition of AC2, or indeed $G\alpha_s$ and $G\beta\gamma$. Finally, the experiments that effectively involved structure-functional analysis of the requirements of the receptor for participation in this complex are particularly valuable. A construct with a Ser[704] mutation pulled down only some (AKAP79 and AC2) but not all components of the complex, and simultaneously exhibited the anticipated functional outcomes. Furthermore, a chimeric receptor (RXFP1/2) preserved only the familiar $G\alpha_s$-dependent classical response and lost the novel signalling identified here. Thus, we would propose that the complex we have identified is clearly defined and exists in both molecular and functional terms.

The use of highly sensitive single-cell techniques has revealed a cellular response to sub-picomolar concentrations

of relaxin, which provides for the first time an important physiological relevance for circulating levels of this pleiotropic hormone. Furthermore, this approach has allowed the identification of a pre-assembled, ligand-independent and constitutively active RXFP1 signalosome, with well-defined stimulatory and regulatory components (Figure 10). The identification of a distinct cellular response to low rather than high concentrations of relaxin implies that investigators have previously only been examining one component of the relaxin effect. This new discovery provides opportunities for additional research of the function of this distinct pathway in the multiple physiological and pathological conditions regulated by relaxin. Further, this finding could have major ramifications for understanding the function of relaxin in various physiological systems, and has even greater implications for the use of relaxin or relaxin derivatives as therapeutic agents in pathologies including heart failure and cancer metastases. Finally, the sensitivity of this pre-assembled signalosome to attomolar concentrations of ligand represents a novel paradigm in GPCR signalling, and may facilitate the identification of similar responses in other receptor systems.

Materials and methods

Peptides
Recombinant human relaxin was provided by Corthera, Inc. Human INSL3 was synthesized by Professor John D Wade (Howard Florey Institute, Australia).

Constructs
All RXFP receptor constructs (Halls *et al*, 2009), β-arrestin 1, β-arrestin 2 and scrambled siRNA (Lynch *et al*, 2005; Willoughby *et al*, 2007), AKAP79 shRNA and pSilencer (Hoshi *et al*, 2005; Willoughby *et al*, 2007), dominant negative PDE4D3 and PDE4D5 (Baillie *et al*, 2003; McCahill *et al*, 2005), and Ad-glEpac1, glEpac2, pmEpac2 and pmEpac2 R297E have been described previously (Nikolaev *et al*, 2004, 2005; Mironov *et al*, 2009; Wachten *et al*, 2010). β-Arrestin 1, β-arrestin 2, scrambled siRNA and dominant negative PDE4D3 and PDE4D5 were kindly provided by Professor Miles D Houslay (University of Glasgow, UK); AKAP79 shRNA and pSilencer were kindly provided by Professor John D Scott (University of Seattle); Ad-glEpac1 and glEpac2 were kindly provided by Professor Martin J Lohse (University of Würzburg, Germany).

GST-tagged fragments of the RXFP1 C-terminus were generated by amplifying the required region from the full-length cDNA using PCR, and cloning into pGEX-4T1. AC2-HA was generated by adding a HA-tag to the C-terminus of rat AC2 by PCR, and cloned into pcDNA3.0.

Cell culture
Cells were maintained as described in Supplementary data. Primary rat cardiac fibroblasts were prepared as described previously (Woodcock *et al*, 2002). Transient transfections were performed using Lipofectamine 2000 (Invitrogen). For siRNA, shRNA, pSilencer and dominant negative PDE4D3 or PDE4D5, cells were transfected with the relevant construct followed 24 h later by receptor and biosensor DNA (single-cell cAMP measurements) or receptor and AC2-HA (co-IP studies). Adenoviral infection of rat cardiac fibroblasts used a multiplicity of infection of 200 PFU/well.

Single-cell cAMP measurements
Cells were seeded onto 18 mm poly-L-lysine-coated coverslips 24 h following transfection, or 6 h following adenoviral infection, in 0.5% (v/v) FBS-culture medium, and used the following day. Fluorescent imaging was performed using an Andor Ixon+ CCD camera and Optosplit (505DC) (Cairn Research) as described

previously (Wachten *et al*, 2010) and analysed using Metamorph software (Molecular Devices).

Data were analysed by expressing the change in CFP (470 nm) versus YFP (535 nm) emission ratio relative to a maximal stimulus (10 μM forskolin, 100 μM IBMX and 100 nM PGE$_1$). The area under the curve (AUC) for the first 5 or 12 min (HeLa cells only) was calculated using GraphPad Prism, and takes into account changes in both the rate and magnitude of the cAMP response. The biphasic concentration-dependent increase in cAMP in response to relaxin was also evident when data were analysed by 'peak-response' or 'rate of response' (Supplementary Figure S1); as increasing concentrations of relaxin amplify both the rate and magnitude of the cAMP response, the AUC analyses allows incorporation of the most information. There was no difference in the cAMP response to relaxin or the associated effects of inhibitors over a longer time course of 20 min (Supplementary Figure S1). Biphasic concentration–response curves were fit using a biphasic non-linear regression analysis; classical concentration–response curves were fit using a log[agonist] versus response non-linear regression analysis (GraphPad Prism).

GST pull-down assay
GST fragments of the RXFP1 C-terminus were expressed in BL21-Rosetta cells at 30°C following induction with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were lysed by sonication in lysis buffer (10 μM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM benzamidine, protease inhibitors, 1 μg DNase in phosphate-buffered saline (PBS)) supplemented with 200 μg/ml lysozyme. Homogenates were centrifuged (27 000g, 4°C, 15 min), supernatant passed through a glutathione-Sepharose 4B resin, and washed until no protein remained in the eluate. An equal volume of PBS (containing 0.02% NaN$_3$) to resin was added to create a 50% slurry.

HEK293 cells were lysed in GST-Fish buffer (10% v/v glycerol, 100 mM NaCl, 50 mM Tris, pH 7.4, 0.5% Tween-20, 2 mM dithiothreitol, 1 mM PMSF, 1 mM benzamidine, protease inhibitors, 10 mM β-glycerophosphate, 2 mM sodium orthovanadate) by rotating for 30 min at 4°C, before centrifugation (12 000g, 4°C, 15 min). The cell lysate was incubated with GST beads for 4 h at 4°C with rotation. GST beads were washed twice in GST-Fish buffer, and bound proteins eluted in Laemmli buffer and boiled for 5 min prior to immunoblotting (Supplementary data).

HA-IP
HEK293 cells co-expressing pcDNA and AC2-HA, AC2-HA and FLAG-RXFP1, or AC2-HA and FLAG-RXFP1 S704A were washed with PBS and lysed in solubilization buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.1 mM EDTA, 0.3% (v/v) NP-40, 100 μM EGTA, protease inhibitors, 10 mM β-glycerophosphate and 2 mM sodium orthovanadate) using a 21-gauge needle. The cell suspension was centrifuged (900g, 4°C, 5 min) before IP using anti-HA

affinity-agarose beads (Roche). Lysate was rotated with 100 μl pre-washed bead slurry (50%) for 4 h at 4°C. Beads were washed twice with wash buffer (solubilization buffer with 0.03% (v/v) NP-40), and proteins were eluted in wash buffer with 1% (w/v) SDS and DNase. Laemmli buffer was added and samples incubated at 37°C for 30 min prior to immunoblotting (Supplementary data).

PDE4D3-IP
HEK293 cells co-expressing dominant negative PDE4D3, and pcDNA and AC2-HA, AC2-HA and FLAG-RXFP1, or AC2-HA and FLAG-RXFP1 S704A were washed with PBS and lysed in lysis buffer (50 mM Tris, pH 7.4, 1 mM EDTA, 1 mM MgCl$_2$, 150 mM NaCl, 0.5% (v/v) Tween-20, protease inhibitors) using a 21-gauge needle. The cell suspension was centrifuged (200g, 4°C, 3 min) before IP using rabbit anti-PDE4D antibody (1:150; Abcam). Samples were rotated for 2 h at 4°C, protein G agarose (30 μl of a 50% slurry) was added, and samples rotated for 2 h at 4°C. Beads were washed twice with wash buffer (lysis buffer with 0.05% (v/v) Tween-20), and bound proteins eluted in Laemmli buffer with incubation at 37°C for 30 min prior to immunoblotting (Supplementary data).

Statistical analyses
Single-cell FRET data were analysed by two-way ANOVA with Bonferroni post tests and densitometric analyses, by one-way ANOVA with Newman–Keuls multiple comparison test (GraphPad Prism). Data are expressed as mean ± s.e.m of n cells/experiments, with statistical significance accepted at $P < 0.05$.

Supplementary data
Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Conflict of interest

The authors declare that they have no conflict of interest.

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