0026-895X/09/7502-415–428$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 75:415–428, 2009

Vol. 75, No. 2
51227/3435049
Printed in U.S.A.

Relaxin Family Peptide Receptor (RXFP1) Coupling to $G\alpha_{i3}$ Involves the C-Terminal Arg^{752} and Localization within Membrane Raft Microdomains

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Received August 8, 2008; accepted November 24, 2008

ABSTRACT

The relaxin family peptide receptors (RXFP) 1 and 2 are targets for the relaxin family peptides relaxin and insulin-like peptide 3 (INSL3), respectively. Although both receptors and peptides share a high degree of sequence identity, the cAMP signaling pathways activated by the two systems are quite distinct. Relaxin activation of RXFP1 initially results in accumulation of cAMP via $G\alpha_s$, but this is modulated by inhibition of cAMP through $G\alpha_{oB}$. Over time, RXFP1 recruits coupling to $G\alpha_{i3}$, causing additional cAMP accumulation via a $G\alpha_{i3}$-$G\beta\gamma$-phosphoinositide 3-kinase (PI3K)-protein kinase C (PKC)ζ pathway. In contrast, INSL3 activation of RXFP2 results in accumulation of cAMP only via $G\alpha_s$, modulated by cAMP inhibition through $G\alpha_{oB}$. Thus, the aim of this study was to identify the cause of differential G-protein coupling between these highly similar receptors. Construction of chimeric receptors revealed that $G\alpha_{i3}$ coupling is dependent upon the transmembrane region of RXFP1 and independent of the receptor ectodomain or ligand bound. Generation of C-terminal truncated receptors identified the terminal 10 amino acids of the RXFP1 C terminus as essential for $G\alpha_{i3}$ signaling, and point mutations revealed an obligatory role for Arg^{752}. RXFP1-mediated $G\alpha_{i3}$, but not $G\alpha_s$ or $G\alpha_{oB}$, signaling was also found to be dependent upon membrane rafts, and RXFP1 coupled to $G\alpha_{i3}$ after only 3 min of receptor stimulation. Therefore, RXFP1 coupling to the $G\alpha_{i3}$-$G\beta\gamma$-PI3K-PKCζ pathway requires the terminal 10 amino acids of the RXFP1 C terminus and membrane raft localization, and the observed delay in this pathway occurs downstream of $G\alpha_{i3}$.

The relaxin family peptides are two-chain hormones that diverged from insulin during early vertebrate evolution (Hsu, 2003; Wilkinson et al., 2005). The family encompasses seven peptides in humans: relaxin-1, relaxin (equivalent to other species' relaxin-1), relaxin-3, and the insulin/relaxin-like peptides INSL3, INSL4, INSL5, and INSL6. All peptides within this family evolved from an ancestral *RLN3* (relaxin-3) gene (Hsu, 2003; Wilkinson et al., 2005), but have distinct functional profiles: relaxin was initially identified as a hormone of pregnancy (Hisaw, 1926), but is now credited with roles in the cardiovascular, renal and central nervous systems and in allergic responses. Relaxin-3 is principally expressed in the brain with roles in appetite regulation and anxiety; INSL3 is responsible for testis descent in men and for germ cell survival in both men and women (for review, see van der Westhuizen et al., 2008).

There are four recently deorphanized receptors for relaxin family peptides: relaxin family peptide receptor 1 (RXFP1; LGR7), RXFP2 (LGR8), RXFP3 (GPCR135; SALPR), and RXFP4 (GPCR142; GPR100) (Bathgate et al., 2006). The cognate ligands for these receptors have been identified as relaxin (Hsu et al., 2002), INSL3 (Kumagai et al., 2002), relaxin-3 (Liu et al., 2003), and INSL5 (Liu et al., 2004), respectively, although there is a degree of cross-reactivity

This work was supported in part by the National Health and Medical Research Council [Block Grant Reg Key 983001, Project Grants 300012 and 436713] and ARC Linkage Grant LP0560620.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.108.051227.

ABBREVIATIONS: INSL, insulin-like peptide; RXFP, relaxin family peptide receptor; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PTX, pertussis toxin; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride; $mG\alpha_{i3}$, $G\alpha_{i3}$ with Cys^{351}-to-Ile mutation (PTX-insensitive); $mG\alpha_{oB}$, $G\alpha_{oB}$ with Cys^{351}-to-Ile mutation (PTX-insensitive); βARK-ct, β-adrenergic receptor kinase 1 C terminus; PCR, polymerase chain reaction; bp, base pair(s); GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; ANOVA, analysis of variance; FBS, fetal bovine serum; HEK, human embryonic kidney; MES, 2-[*N*-morpholino]ethanesulfonic acid; MBS, MES-buffered saline; TBS-T, Tris-buffered saline with 0.1% Tween 20; TM, transmembrane helix.

between the relaxin family peptides. Although the peptides themselves are quite similar, the receptors can be divided into two distinct groups. RXFP1 and RXFP2 are family C leucine-rich repeat-containing GPCRs, distinguished by large extracellular domains containing an N-terminal low-density lipoprotein class A module and 10 leucine-rich repeats (Hsu, 2003). RXFP3 and RXFP4 have much smaller extracellular domains, and are similar to the small peptide GPCRs for somatostatin and angiotensin II (Liu et al., 2003).

The signaling pathways mediated by RXFP1 and RXFP2 are not yet clearly elucidated. To date, much research has focused upon the cAMP signaling of these two receptors, because increases in cAMP are observed after the generation of constitutively active receptor mutants (Hsu et al., 2000, 2002). Despite this, for RXFP1 at least, there is evidence for activation of a number of other signaling cascades depending upon the target tissue under investigation. Activation of RXFP1 can cause phosphorylation of extracellular signal-regulated kinase 1/2 (Zhang et al., 2002), activate tyrosine kinase signaling (Bartsch et al., 2001; Palejwala et al., 2001), and increase nitric oxide (Nistri et al., 2003; Conrad and Novak, 2004); relaxin may interact with the glucocorticoid receptor (Dschietzig et al., 2004). RXFP2 signaling is much less well defined, with only cAMP signaling studied in any detail.

Of all the signaling pathways, the cAMP accumulation responses to RXFP1 and RXFP2 are the most extensively studied. When expressed in HEK293T cells, RXFP1 couples to $G\alpha_s$ to increase cAMP accumulation, to $G\alpha_{oB}$ to negatively modulate the accumulation of cAMP, and to $G\alpha_{i3}$ to increase cAMP accumulation in a delayed manner through a pathway mediated by $G\beta\gamma$-PI3K-PKCζ-adenylate cyclase (Nguyen et al., 2003; Nguyen and Dessauer, 2005; Halls et al., 2006). Activation of this PI3K-PKCζ pathway has been demonstrated in THP-1, MCF-7, pregnant human myometrial, and mouse mesangial cells (Nguyen and Dessauer, 2005), indicating a functional role for this pathway in vivo. In contrast, when RXFP2 is expressed in HEK293T cells, the receptor can only couple to $G\alpha_s$ to increase cAMP accumulation and $G\alpha_{oB}$ to negatively modulate increases in cAMP (Halls et al., 2006). The ability of RXFP2 to either increase or decrease cAMP accumulation has been demonstrated in cells that endogenously express this receptor. When gubernacular cells are stimulated with INSL3, increased cAMP accumulation is observed (Kumagai et al., 2002), whereas pertussis toxin (PTX; G_i/G_o inhibitor)-sensitive decreases in cAMP accumulation occur in testicular germ cells and oocytes in response to the same ligand (Kawamura et al., 2004).

Thus, it is interesting that despite 60% amino acid sequence identity between RXFP1 and RXFP2, only RXFP1 is able to activate an additional pathway leading to cAMP accumulation. This study sought to identify the functional domain of RXFP1 that allows coupling to the $G\alpha_{i3}$-$G\beta\gamma$-PI3K-PKCζ pathway, using a receptor mutagenesis approach. By using a number of receptor chimeras, truncated receptors, and point mutants, we have shown that the final 10 amino acids of the RXFP1 C terminus (and specifically residue Arg^{752}) is an absolute requirement for coupling to the $G\alpha_{i3}$-$G\beta\gamma$-PI3K-PKCζ pathway and that coupling of the receptor to this pathway is dependent upon membrane raft microdomains. In addition, we demonstrate coupling of RXFP1 to $G\alpha_{i3}$ as early as 3 min after receptor activation, suggesting that the delay in cAMP production from this pathway occurs downstream of $G\alpha_{i3}$.

Materials and Methods

Hormones and Reagents. Recombinant relaxin was kindly provided by Corthera, Inc. (San Mateo, CA). Human INSL3 was chemically synthesized by Dr. John D. Wade (Howard Florey Institute, VIC, Australia). PTX, wortmannin, and filipin III were purchased from Sigma Aldrich (Castle Hill, NSW, Australia); LY294002 was purchased from Calbiochem (Clayton, VIC, Australia); and chelerythrine chloride was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Immobilized protein A/G was purchased from Pierce (Rockford, IL).

$G\alpha_o$ (K-20) rabbit polyclonal antibody, $G\alpha_s$ (A-16) goat polyclonal antibody, and anti-goat IgG-HRP antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, MA). Anti-rabbit IgG HRP antibody and DYKDDDDK tag (FLAG tag) rabbit polyclonal antibody were purchased from Cell Signaling Technology, Inc. (Danvers, MA). $G\alpha_{i3}$ chicken polyclonal antibody was purchased from Millipore (North Ryde, NSW, Australia), and the anti-chicken IgY HRP antibody was purchased from Abcam (Cambridge, UK). The anti-FLAG M2 mouse monoclonal antibody (for FLAG-tag binding assays) was purchased from Sigma Aldrich, and the ^{125}I-goat anti-mouse IgG antibody was purchased from PerkinElmer Life and Analytical Sciences (Melbourne, VIC, Australia).

The $G\alpha_{i/o}$-subunit antibodies used are specific for their targets with little cross-reactivity with other $G\alpha$-subunits. Anti-$G\alpha_o$ was raised against a divergent internal region of this G-protein corresponding to amino acids 105 to 124 (in sequence, KMVCDVVSRMEDTEPFSAEL) and is therefore specific to $G\alpha_o$, with no cross-reactivity with $G\alpha_{i1}$, $G\alpha_{i2}$, or $G\alpha_{i3}$ (Menco et al., 2001; Masudo et al., 2003). Anti-$G\alpha_{i3}$ was raised against the final 10 amino acids of the $G\alpha_{i3}$ C terminus (in sequence, KNNLKECGLY) and shows only weak cross-reactivity with $G\alpha_o$ (although not observed in this study) but no cross-reactivity with $G\alpha_{i1}$ or $G\alpha_{i2}$ (Diehl et al., 1996).

Constructs. βARK-ct (Koch et al., 1994) was obtained from Dr. Walter G. Thomas (University of Queensland, Brisbane, QLD, Australia) with the kind permission of Dr. Robert J. Lefkowitz (Duke University Medical Center, Durham, NC). The construct encodes a segment of the C-terminal end of bovine β-adrenergic receptor kinase that acts to sequester $G\beta\gamma$ subunits. PTX-insensitive G_i/G_o α-subunit mutants have a Cys(351)Ile mutation (Bahia et al., 1998) that renders them insensitive to ADP-ribosylation by PTX.

Receptor Mutagenesis. Wild-type human RXFP1 and RXFP2 receptor cDNAs were cloned into the BamHI (5′) and XhoI (3′) sites of the vector pcDNA3.1/Zeo(+) (Invitrogen, Mount Waverley, VIC, Australia), and included an N-terminal bovine prolactin signal peptide followed by a FLAG epitope tag. Mutant receptors were generated by PCR using high-fidelity Platinum Pfx DNA polymerase (Invitrogen) or by QuikChange mutagenesis (Stratagene, La Jolla, CA) and are summarized in Table 1. The chimeric receptors RXFP1/2 and RXFP2/1 have been described previously (Sudo et al., 2003; Halls et al., 2005).

tRXFP1–703. The construct encoding tRXFP1–703 was made by amplifying the truncated receptor from the full-length cDNA using PCR. The forward primer encoded a BamHI site (underlined) followed by KOZAK sequence and the first 13 nucleotides of the bovine prolactin signal peptide (5′-CATCATGGATCCGCCACCATGGACAGCAAAG-3′). The reverse primer had a XhoI restriction site (underlined), stop codon (bold underlined), and 23 nucleotides from residue 703 of the RXFP1 protein sequence (5′-GAGAGCTCGAG**TCA**TTTTCTTTGTCTGTAGTTATACC-3′). The PCR fragment was gel purified followed by digestion with BamHI and XhoI and ligation into pcDNA3.1/Zeo(+). The resulting construct was checked by DNA sequencing on both strands (Micromon; http://www.micromon.monash.org/).

tRXFP1–747. The construct encoding the truncated receptor tRXFP1–747 was made by replacing a 786-bp HpaI-XhoI cassette

from the wild-type sequence with a PCR fragment incorporating the coding sequence of the altered C-terminal tail (-TYPCEMSLI*). The forward primer (RXFP1-HpaI) was 67 bp upstream of a Hpa I site present in the wild-type RXFP1 cDNA (5′-AGAGTACTCATTGTCAGCTTGTAGGATCTTTGG-3′). The reverse primer was 5′-TTGACTCGAGTTGATTG**TCA**AATCAGTGACATTTCACAGGGGTATGTG-3′ (XhoI site underlined, stop codon bold underlined).

The PCR fragment was recovered from an agarose gel and digested with HpaI and XhoI, then ligated into wild-type RXFP1 plasmid from which the corresponding HpaI-XhoI fragment had been removed. The complete tRXFP1–747 insert and junctions with pcDNA3.1/Zeo(+) were checked by DNA sequencing on both strands (Micromon).

RXFP1 S755A and RXFP1 R752A. The mutants RXFP1 S755A and RXFP1 R752A were made by the same cassette strategy as that used for tRXFP1–747. PCR was done using wild-type RXFP1 plasmid as the template and the same forward primer (RXFP1-HpaI). The reverse primers for RXFP1 S755A and RXFP1 R752A were 5′-TGCCTCGAGATGAATTTCAGAGTCAGTCATGAATAGG**C**ATTGAGTC-3′ and 5′-TGCCTCGAGATGAATTTCAGAGTCAGTCATGAATAGGAATTGAGT**GC**CGTT GATTGAG-3′, respectively (XhoI sites underlined, mutated nucleotides bold underlined). The complete RXFP1 S755A and RXFP1 R752A inserts and junctions with pcDNA3.1/Zeo(+) were checked by DNA sequencing on both strands (Micromon).

RXFP2–10ct1. To test whether the last 10 amino acids of the RXFP1 C-terminal tail were sufficient to confer RXFP1-type signaling properties on RXFP2, we generated the mutant RXFP2–10ct1. The relevant C-terminal amino acid sequences are as follows: wild-type RXFP1, LMKPDLFTYPCEMSLISQSTRLNSYS*; wild-type RXFP2, GVLNKITLGDSIMKPVS*; and RXFP2–10ct1, GVLNKITLGDSIM**SL**VSQSTRLNSYS*. Two further residues present in RXFP2 (KP, underlined) were also changed to the corresponding RXFP1 amino acids (bold underlined), because lysine and proline are frequently represented in consensus sites for post-translational modification and protein-protein interactions and may have altered the behavior of the RXFP1 C terminus.

A 1.1-kilobase-pair fragment was generated using wild-type RXFP2 plasmid as the template for PCR with the following primers: forward, 5′-TCCTATGCTCCCCATGTCCGAATATGTATG-3′; reverse, 5′-CTAGACTCGAGTGATCCAAAATGATTGCTA**TGAATAGGAATTGAGTCTCGTTGATTGAGA**AACTA**GAC**TCATTATACTGTCTCC-3′ (bold underlined nucleotides correspond to the RXFP1 sequence). After recovery from an agarose gel, the PCR fragment was digested with HpaI and XhoI, then a 265-bp fragment corresponding to the 3′ end was recovered and ligated into wild-type RXFP2 plasmid from which the corresponding HpaI-XhoI fragment had been removed. The complete RXFP2–10ct1 insert and junctions with pcDNA3.1/Zeo(+) were checked by DNA sequencing on both strands (Micromon).

Cell Culture. HEK293T cells (American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 medium supplemented with heat-inactivated fetal bovine serum [10% (v/v)], penicillin (100 units/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM; all Trace Biosciences, Sydney, NSW, Australia). All tissue culture plates were coated with poly-L-lysine (0.1 mg/ml; Sigma-Aldrich) before use. Cells were maintained at 37°C in a CO_2 water-jacket incubator (Forma Scientific, Marietta, OH) in 5% CO_2 and 85% humidity.

Transient transfections were performed using Lipofectamine (Invitrogen) according to the manufacturer's instructions as described previously (Halls et al., 2005). Cells transiently expressing stated constructs were seeded into cell culture plates 24 h after transfection and used 48 h after transfection.

FLAG-Tag Binding for Cell Surface Expression. FLAG-tag binding was performed to confirm cell surface expression for all receptor mutants. Cells were transiently transfected with the receptor of interest as described above, then replated at 3.5×10^5 cells/well into 12-well plates precoated with poly-L-lysine. Cells were rinsed once with binding buffer [50 mM Tris-HCl, 100 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1% (w/v) fraction V bovine serum albumin, and 0.1% paraformaldehyde, pH 7.7], then incubated with binding buffer for 20 min at 4°C. Buffer was removed from the cells, and cells incubated with 0.5 μg/well anti-FLAG M2 monoclonal antibody diluted in binding buffer for 2 h at 4°C. Cells were washed three times with binding buffer, then incubated with 3×10^5 cpm/well ^{125}I-goat anti-mouse IgG antibody diluted in binding buffer, for 2 h on ice. Cells were washed three times with binding buffer, before solubilization with 0.5 M NaOH. Samples were counted on a RiaCalc WIZ 1470 γ-counter (PerkinElmer Life and Analytical Sciences). Results are expressed as the mean ± S.E.M. of n experiments as stated.

Competition Binding Assays. Whole-cell binding assays were performed as described previously (Halls et al., 2005) to confirm correct receptor pharmacology. Competition binding studies used 100 pM [^{33}P]relaxin (B33) that was generated as described previously (Tan et al., 1999). Nonspecific binding was determined using unlabeled relaxin (0.1 μM). The data were analyzed using a Prism (GraphPad Software, Inc., San Diego, CA) one-site competition binding model to obtain pIC_{50} values. Results are expressed as the mean ± S.E.M. of n experiments as stated.

Membrane Preparation. Membranes were prepared from transiently transfected HEK293T cells for [^{35}S]GTPγS immunoprecipitation experiments as described previously (Hutchinson et al., 2002) with modifications. Cells were grown as a monolayer before membranes were harvested. Cells were washed with phosphate-buffered

TABLE 1

Mutagenesis of the RXFP1 and RXFP2 receptors did not affect binding, cell surface expression, or functional responses

All receptor constructs were expressed at the cell surface, bound relaxin (B29) with correct affinity, and stimulated cAMP accumulation with correct potency, as determined by FLAG-tag binding (cpm/3.5×10^5 cells), competition binding with 100 pM [^{33}P]relaxin (B33) (pIC_{50} values) and cAMP accumulation to increasing concentrations of relaxin (pEC_{50} values), respectively. There was no significant difference between receptor mutants and the appropriate receptor control (RXFP1 or RXFP2) in terms of pIC_{50} and pEC_{50} values, except for the chimeric receptors as described previously (Halls et al., 2005). Values are expressed as mean ± S.E.M. of (n) experiments.

Construct	Description	Relative Expression	pIC_{50}	pEC_{50}
RXFP1	RXFP1	1039.43 ± 279.67 (3)	9.23 ± 0.17 (4)	10.01 ± 0.95 (4)
RXFP2	RXFP2	1255.07 ± 440.04 (3)	8.39 ± 0.18 (4)	9.49 ± 0.56 (5)
wtRXFP1	RXFP1 with extra Bam site removed	1115.98 ± 404.61 (3)	8.90 ± 0.24 (4)	10.81 ± 0.52 (4)
tRXFP1–703	RXFP1 with C-terminal truncated at residue 703	876.45 ± 354.33 (3)	9.40 ± 0.19 (5)	9.89 ± 0.76 (5)
tRXFP1–747	wtRXFP1 with C-terminal truncated at residue 747	952.32 ± 87.84 (3)	9.27 ± 0.43 (4)	9.90 ± 0.65 (4)
RXFP1 S704A	wtRXFP1 with Ser^{704} mutated to Ala	1359.39 ± 228.00 (4)	9.53 ± 0.33 (4)	10.95 ± 0.35 (4)
RXFP1 R752A	wtRXFP1 with Arg^{752} mutated to Ala	1263.80 ± 296.90 (3)	9.26 ± 0.26 (4)	10.85 ± 0.45 (4)
RXFP1 S755A	wtRXFP1 with Ser^{755} mutated to Ala	773.70 ± 92.48 (3)	9.00 ± 0.31 (4)	10.82 ± 0.48 (4)
RXFP1/2	RXFP1 ectodomain with RXFP2 transmembrane region	1247.52 ± 167.38 (3)	8.97 ± 0.07 (4)*	10.37 ± 0.53 (4)
RXFP2/1	RXFP2 ectodomain with RXFP1 transmembrane region	1148.48 ± 100.33 (3)	8.84 ± 0.11 (4)†	10.18 ± 0.84 (4)
RXFP2–10ct1	Final 10 amino acids of RXFP1 C-terminus added to C-terminus of RXFP2	491.79 ± 40.08 (4)	8.70 ± 0.33 (4)	9.47 ± 0.92 (4)

* $P < 0.05$ versus RXFP1 (unpaired t test).
† $P < 0.01$ versus RXFP2 (unpaired t test).

saline, then scraped from the flasks using a cell scraper and ice-cold buffer A (20 mM Tris, pH 7.5, 2 mM EDTA, 0.4 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, and 1 μg/ml leupeptin). Cells were homogenized with a Dounce homogenizer (10 strokes per pestle) and centrifuged at low speed (800*g*, 10 min). The supernatant was retained, and the pellet was rehomogenized and centrifuged again at low speed (800*g*, 10 min). Supernatants were pooled and centrifuged (39,000*g*, 15 min, 4°C) before the pellet was homogenized in ice-cold buffer B (50 mM Tris pH 7.5, 1 mM EDTA, 10 mg/ml bacitracin, 10 mg/ml pepstatin, 10 mg/ml leupeptin, and 0.5 mg/ml aprotinin), and protein concentration was determined using a protein assay according to the manufacturer's instructions (Bio-Rad Laboratories, Regents Park, NSW, Australia). Membranes were frozen at −20°C for later use.

[^{35}S]GTPγS Immunoprecipitation. [^{35}S]GTPγS immunoprecipitation was performed to demonstrate G_i/G_o isoform activation more directly. Reaction tubes were maintained at 30°C and contained assay buffer (10 mM HEPES, 100 mM NaCl, and 10 mM $MgCl_2$, pH 7.4), GDP (100 μM for $G\alpha_o$ immunoprecipitation; 1 μM for $G\alpha_{i3}$ immunoprecipitation), relaxin (30 nM), and [^{35}S]GTPγS (10 nM). Equal volumes of frozen membranes diluted to 200 μg per tube in assay buffer were added to start the reaction. Reactions were incubated at 30°C with shaking for 3 or 30 min. The reaction was terminated by placing tubes on ice.

An equal volume of immunoprecipitation buffer (100 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 1.25% Nonidet P40, and 0.1% SDS, pH 7.4) was added, together with 1% (v/v) immune serum (rabbit serum for $G\alpha_o$ immunoprecipitation; chicken serum for $G\alpha_{i3}$ immunoprecipitation) and 10% (v/v) protein A-Sepharose suspension to preclear the samples. Samples were rolled at 4°C for 90 min, before centrifugation (1000*g*, 2 min, 4°C). The supernatant was transferred to a new tube containing appropriate anti-Gα subunit antibody (both $G\alpha_o$ and $G\alpha_{i3}$ antibodies were used at 1:50 dilution), vortexed, and rolled overnight at 4°C. Protein A-Sepharose suspension was added to each sample [45% (v/v)], and samples were rolled for 90 min at 4°C. Beads were washed three times in ice-cold immunoprecipitation buffer without SDS. Samples were centrifuged at 1000*g* for 2 min at 4°C, then resuspended in immunoprecipitation buffer without SDS. After the final wash, the beads were resuspended in immunoprecipitation buffer, added to scintillation cocktail, and counted using a Wallac Winspectral 1414 liquid scintillation counter (PerkinElmer Life and Analytical Sciences). Data were normalized by expressing the results as a percentage of the basal activity. Results are expressed as the mean ± S.E.M. of four individual experiments. Statistical analyses were performed using a Prism (GraphPad Software) one-way ANOVA with Dunnett's multiple comparison post-test, with statistical significance accepted at $p < 0.05$.

cAMP Accumulation Assay. cAMP accumulation assays were used to initially confirm correct receptor pharmacology (concentration-response studies), and then in conjunction with inhibitors to dissect the receptor cAMP signaling pathway profiles (time course and single-point studies). cAMP responses were determined using the AlphaScreen cAMP accumulation assay (PerkinElmer Life and Analytical Sciences). Cells were seeded into 96-well plates (5×10^4 cells/well) in 10% (v/v) FBS/RPMI 1640 medium for 8 h, then partially serum-starved overnight [0.5% (v/v) FBS RPMI 1640 medium]. Inhibitors were preincubated with the cells for 30 min in 0.5% (v/v) FBS RPMI 1640 medium, except filipin III and pertussis toxin, which were incubated with the cells for 1 and 16 h, respectively.

cAMP assays were performed in duplicate as described previously (Halls et al., 2005). In brief, cells were incubated with stimulation buffer containing relaxin or INSL3 (concentrations ranging from 0.1 pM to 1 μM), forskolin (0.1 mM; Sigma-Aldrich, NSW, Australia) or blank for stated time periods at 37°C. After removal of stimulation buffer, cells were frozen in lysis buffer at −80°C to terminate the reaction and lyse cells. Samples were transferred to a 384-well white OptiPlate (PerkinElmer Life and Analytical Sciences) after thawing, and anti-cAMP acceptor beads then donor beads with biotinylated cAMP were added to all wells.

After overnight incubation, plates were read using a Fusion-α Microplate Reader (PerkinElmer Life and Analytical Sciences) and the data were analyzed against a standard curve using Prism software. Samples were normalized for cell number by expressing the results as a percentage of the 0.1 mM forskolin response, except in the case of filipin III pretreatment, where results are expressed as a percentage of the basal response as a result of effects of the inhibitor on forskolin-induced cAMP signaling. Each experiment was performed in duplicate and results are expressed as the mean ± S.E.M. of *n* separate experiments (as stated in figure legends). Statistical analyses were performed on the data using either a Prism two-way ANOVA with Bonferroni post-tests (time course studies), or a one-way ANOVA with Newman-Keuls multiple comparison test (single-point studies), with statistical significance accepted at $p < 0.05$.

Basic Lipid Fractionation. Transiently transfected HEK293T cells were grown in a monolayer in 10-cm dishes. Cells were washed with MBS buffer (25 mM MES, pH 6.5, 150 mM NaCl, 10 mg/ml bacitracin, 10 mg/ml pepstatin, 10 mg/ml leupeptin, 0.5 mg/ml aprotinin, and 0.2 mg/ml phenylmethylsulfonyl fluoride) at room temperature, before addition of 1 ml of MBS containing 1 mM EDTA and incubation for 10 min on ice. Cells were harvested, then centrifuged (1400*g*, 5 min, 4°C) before resuspension in 500 μl of MBS containing 1% Triton X-100. Samples were incubated on ice for 20 min and then homogenized using a Dounce homogenizer (20 strokes). A sample of the unfractionated homogenate was retained, and the remainder was centrifuged (16,000*g*, 5 min, 4°C). Supernatant was collected as the Triton-soluble fraction, and the pellet was resuspended in MBS containing 1% Triton X-100 by sonication to give the Triton-insoluble fraction. Protein concentration of samples was determined using a Bio-Rad protein assay according to the manufacturer's instructions.

Samples containing equivalent amounts of protein (approximately 100 μg/lane) were separated on a 10% polyacrylamide gel and electrotransferred to a polyvinylidene difluoride membrane (Millipore). After transfer, the membrane was blocked (blocking buffer: 5% nonfat dry milk and 0.1% Tween 20 in Tris-buffered saline; 1 h at room temperature), washed in TBS-T (Tris-buffered saline with 0.1% Tween 20; three 5-min washes at room temperature), and then incubated with primary antibody (diluted as stated in Tris-buffered saline with 5% bovine serum albumin; overnight at 4°C). After incubation, the membrane was washed in TBS-T (three 5-min washes at room temperature) and then incubated with secondary antibody (diluted as stated in blocking buffer; 1 h at room temperature). The membrane was washed again (TBS-T; three 5-min washes at room temperature), and peroxidase activity was observed by chemiluminescence using the Millipore Immobilon Western substrate and exposure to film. Experiments were performed on four separate occasions and representative blots are shown.

Results

Only RXFP1, and Not RXFP2, Can Couple to the $G\alpha_{i3}$-$G\beta\gamma$-PI3K-PKCζ Pathway to Increase cAMP Accumulation and Does So within 3 Min of Receptor Stimulation. Previous studies (Halls et al., 2006) used HEK293T cells stably expressing RXFP1 or RXFP2. Here, we assessed activation of the delayed pathway in HEK293T cells transiently expressing the receptors of interest (Figs. 1 and 2). Time-course studies in the presence and absence of PTX (G_i/G_o inhibitor; 100 ng/ml, 16-h preincubation) and wortmannin (PI3K inhibitor; 100 nM, 30-min preincubation) showed, as in the stably expressing cells, a complex response after relaxin (30 nM) stimulation of RXFP1 over a 40-min period. Initially, there was no effect of either inhibitor; after 10 min, however, both inhibitors reduced cAMP accumulation compared with relaxin treatment alone (Fig. 1A).

We showed previously that preincubation with inhibitors of the Gα_{i3}-G$\beta\gamma$-PI3K-PKCζ pathway significantly reduced the maximum cAMP response but had no effect on pEC$_{50}$ values obtained from concentration-response curves (Halls et al., 2006). This was confirmed for the transient expression system used in this study (data not shown). Thus, to clearly characterize the cAMP response of a number of receptor constructs, we chose a concentration of peptide (30 nM for both relaxin and INSL3) that produced a maximal response and assessed the effect of pathway inhibitors on this response after 30-min stimulation with relaxin or INSL3. Preincubation of HEK293T cells expressing RXFP1 with PTX, caused significant inhibition of the cAMP response after relaxin stimulation, consistent with removal of the Gα_{i3} pathway (Fig. 1B). Replacement of endogenous G$_i$/G$_o$ proteins with mutants that were PTX-insensitive confirmed coupling of RXFP1 to Gα_{i3} at this time point, because only mGα_{i3} (not mGα_{oB}) restored signaling in the presence of PTX, with a significant increase in response compared with PTX treatment alone. In addition, transfection with βARK-ct (G$\beta\gamma$ scavenger; 57 ng/cm^2) or preincubation with wortmannin, LY294002 (PI3K inhibitor; 10 μM, 30-min preincubation), or chelerythrine chloride (PKC inhibitor; 1 μM, 30-min preincubation) all significantly inhibited the relaxin response, confirming the requirement for these signaling components in the Gα_{i3} pathway activated by RXFP1 in transiently transfected cells.

To examine receptor/G-protein isoform coupling in a more direct manner, [^{35}S]GTPγS immunoprecipitation was also performed (Fig. 1, C and D). Coupling of receptors to G-proteins involves the exchange of GDP for GTP to facilitate Gα-subunit activation. The [^{35}S]GTPγS protocol uses GTPγS, a stable analog of GTP that binds irreversibly to activated Gα-subunits. When combined with immunoprecipitation,

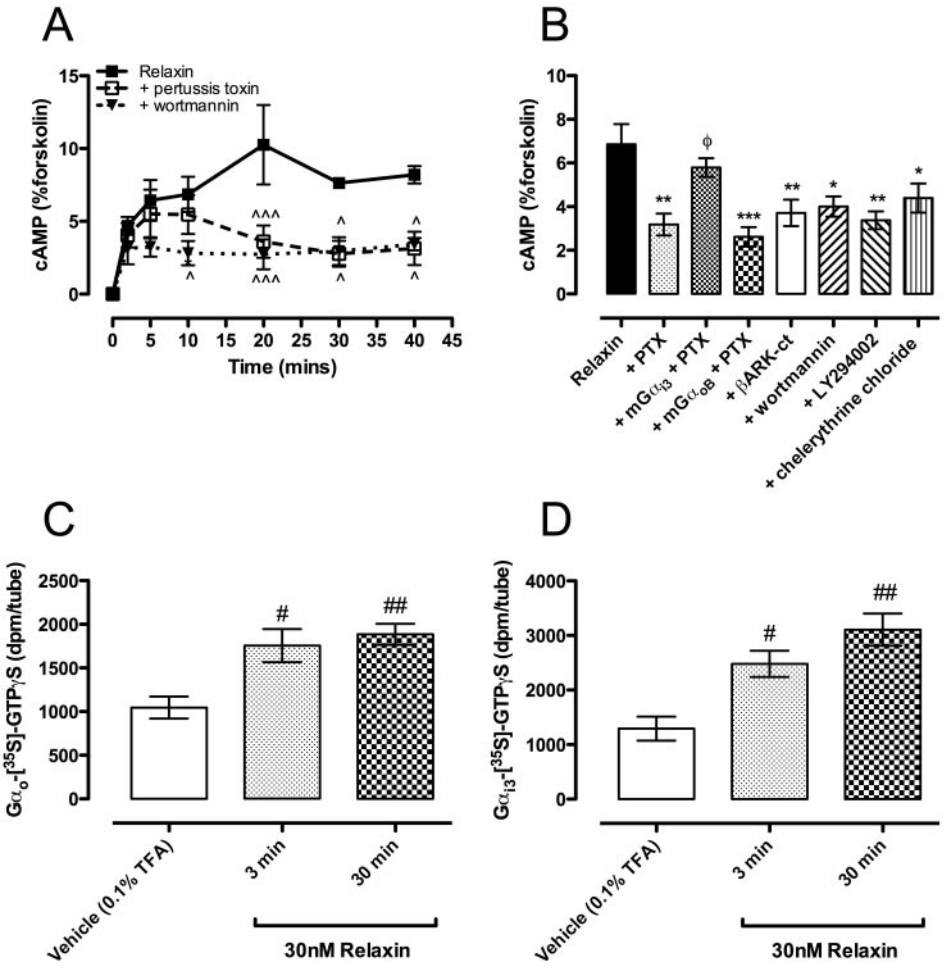

Fig. 1. RXFP1 initially couples to Gα_s, Gα_{oB}, and Gα_{i3}, although increased cAMP generated by the Gα_{i3}-G$\beta\gamma$-PI3K-PKCζ pathway is delayed. The G-protein coupling (C and D) and activation profiles of cAMP accumulation (A and B) were examined in HEK293T cells transiently expressing RXFP1. Time-course studies (A) were performed over a 40-min period of relaxin (30 nM) stimulation of RXFP1 (transient expression, 114 ng/cm^2 culture area) with or without PTX (G$_i$/G$_o$ inhibitor; 100 ng/ml, 16-h preincubation) or wortmannin (PI3K inhibitor; 100 nM, 30-min preincubation) pretreatment. The effect of a single concentration of relaxin (B; 30 nM) at RXFP1 was also assessed at 30 min with or without PTX pretreatment, Gα_{i3}(C351I) with PTX (mGα_{i3}), Gα_{oB}(C351I) with PTX (mGα_{oB}), βARK-ct (G$\beta\gamma$ scavenger), wortmannin, LY294002 (PI3K inhibitor, 10 μM, 30-min pretreatment) or chelerythrine chloride (PKC inhibitor; 1 μM, 30-min pretreatment). RXFP1, mGα_{i3}, mGα_{oB}, and βARK-ct were all transiently transfected at 57 ng/cm^2 culture area. In both cases cAMP was expressed as a percentage of the response to forskolin (0.1 mM) after 30-min incubation. G-protein coupling of RXFP1 after 3- and 30-min stimulation with relaxin (30 nM) was assessed using a [^{35}S]GTPγS assay with immunoprecipitation for Gα_o (C; Gα_o (K-20) rabbit polyclonal antibody, 1:50 dilution) or Gα_{i3} (D; Gα_{i3} chicken polyclonal antibody, 1:50 dilution). Symbols or columns represent means, and vertical bars S.E.M. of three to nine different experiments performed in duplicate. $\wedge$, $p < 0.05$; $\wedge\wedge\wedge$, $p < 0.001$ versus relaxin alone, two-way ANOVA with Bonferroni post-tests; *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ versus relaxin alone; ϕ, $p < 0.05$ versus relaxin with PTX pretreatment, one-way ANOVA with Newman-Keuls multiple comparison test; #, $p < 0.05$ and ##, $p < 0.01$ versus vehicle, one-way ANOVA with Dunnett's multiple comparison post-test.

this allows specific identification of activated Gα-subunits. Stimulation of RXFP1 with relaxin caused significantly increased activation of $G\alpha_o$ at both 3 and 30 min of relaxin stimulation. RXFP1-mediated $G\alpha_{i3}$ activation was increased significantly after 30 min of relaxin stimulation and, interestingly, also after only 3 min of stimulation.

In accord with previous work (Halls et al., 2006), the RXFP2 response to INSL3 stimulation involved only $G\alpha_s$ and $G\alpha_{oB}$ signaling: PTX preincubation significantly enhanced cAMP accumulation after 10-min INSL3 stimulation, whereas wortmannin had no effect (Fig. 2A). Likewise, assessment of single point INSL3 (30 nM) stimulation in the presence and absence of $G\alpha_{i3}$ pathway inhibitors (Fig. 2B) confirmed that RXFP2 coupled to one PTX-sensitive G-protein, $G\alpha_{oB}$, whereas the other inhibitors had no effect on the cAMP response. The G-protein coupling profile of RXFP2 was confirmed with [^{35}S]GTPγS immunoprecipitation. There was significant activation of $G\alpha_o$, but not $G\alpha_{i3}$, subunits above basal levels.

Transiently transfected RXFP1 and RXFP2 were both expressed at the cell surface (as determined by FLAG-tag binding), and displayed similar pIC_{50} (as determined by [^{33}P]relaxin 100 pM competition binding) and pEC_{50} (as determined by cAMP relaxin concentration response curve) values to those previously reported for RXFP1 and RXFP2 (Halls et al., 2005) (Table 1).

Activation of the $G\alpha_{i3}$-Gβγ-PI3K-PKCζ Pathway by RXFP1 Is Not Dependent upon the Ectodomain of the Receptor. The high degree of amino acid sequence homology between RXFP1 and RXFP2 has allowed the generation of receptor chimeras (Sudo et al., 2003) that have previously been used to identify the location of multiple binding sites upon these receptors (Sudo et al., 2003; Halls et al., 2005). RXFP1/2 (ectodomain of RXFP1 fused to the transmembrane/C-terminal domain of RXFP2), when stimulated with relaxin, increased accumulation of cAMP over time (Fig. 3A), and PTX enhanced cAMP accumulation. Single-point experiments (Fig.

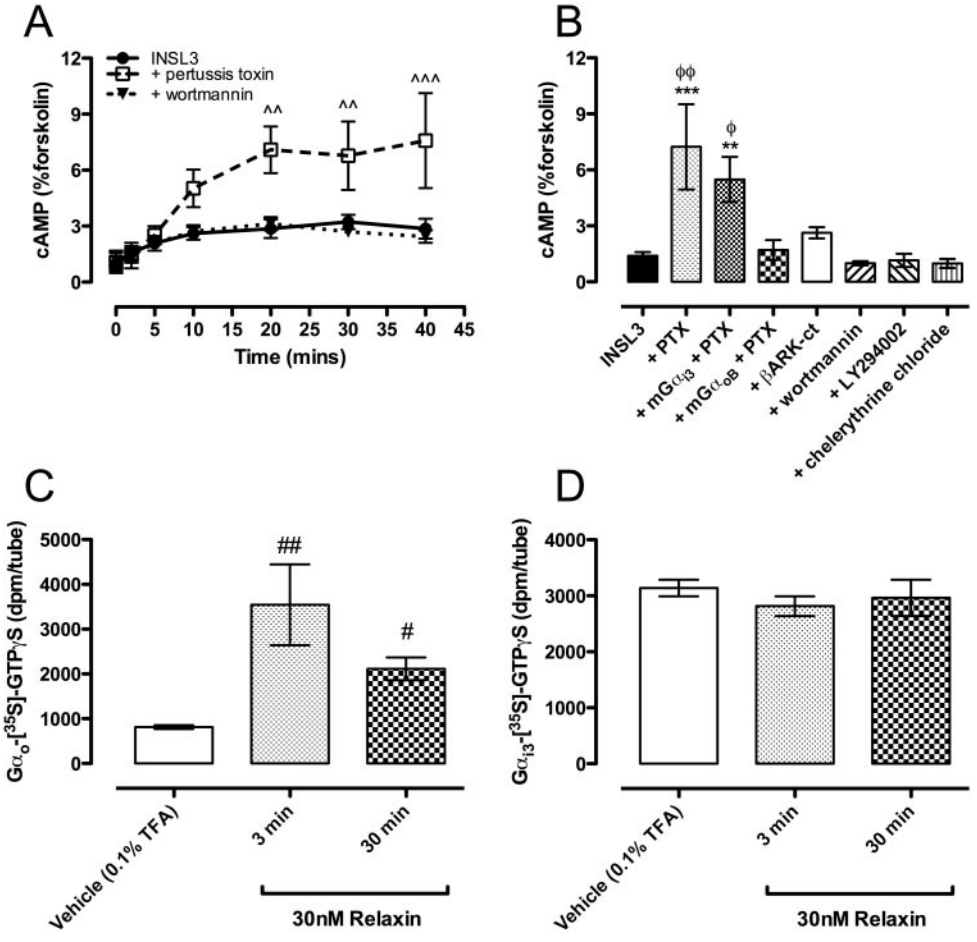

Fig. 2. RXFP2 couples only to $G\alpha_s$ and $G\alpha_{oB}$ and is unable to recruit coupling to $G\alpha_{i3}$. The G-protein coupling (C and D) and activation profiles of cAMP accumulation (A and B) were examined in HEK293T cells transiently expressing RXFP2. Time-course studies (A) were performed over a 40-min period of INSL3 (30 nM) stimulation of RXFP2 (transient expression, 114 ng/cm^2 culture area) with or without pertussis toxin (PTX; G_i/G_o inhibitor; 100 ng/ml, 16-h preincubation) or wortmannin (PI3K inhibitor; 100 nM, 30-min preincubation) pretreatment. The effect of a single concentration of INSL3 (B; 30 nM) at RXFP2 was also assessed at 30 min in the presence or absence of PTX, $G\alpha_{i3}$(C351I) with PTX ($mG\alpha_{i3}$), $G\alpha_{oB}$(C351I) with PTX ($mG\alpha_{oB}$), βARK-ct (Gβγ scavenger), wortmannin, LY294002 (PI3K inhibitor, 10 μM, 30 min pretreatment), or chelerythrine chloride (PKC inhibitor; 1 μM, 30 min pretreatment). RXFP2, $mG\alpha_{i3}$, $mG\alpha_{oB}$, and βARK-ct were all transiently transfected at 57 ng/cm^2 culture area. In both cases, cAMP was expressed as a percentage of the response to forskolin (0.1 mM) after 30-min incubation. The G-protein coupling of RXFP2 after 3- and 30-min stimulation with relaxin (30 nM) was assessed using a [^{35}S]GTPγS assay with immunoprecipitation for $G\alpha_o$ [C; $G\alpha_o$ (K-20) rabbit polyclonal antibody, 1:50 dilution] or $G\alpha_{i3}$ (D; $G\alpha_{i3}$ chicken polyclonal antibody, 1:50 dilution). Symbols or columns represent means, and vertical bars S.E.M. of three to nine different experiments performed in duplicate. ∧∧, $p < 0.01$ and ∧∧∧, $p < 0.001$ versus INSL3 alone, two-way ANOVA with Bonferroni post-tests; **, $p < 0.01$ and ***, $p < 0.001$ versus INSL3 alone, ϕ, $p < 0.05$ and $\phi\phi$, $p < 0.01$ versus $mG\alpha_{oB}$ with PTX, one-way ANOVA with Newman-Keuls multiple comparison test; #, $p < 0.05$ and ##, $p < 0.01$ versus vehicle alone, one-way ANOVA with Dunnett's multiple comparison post-test.

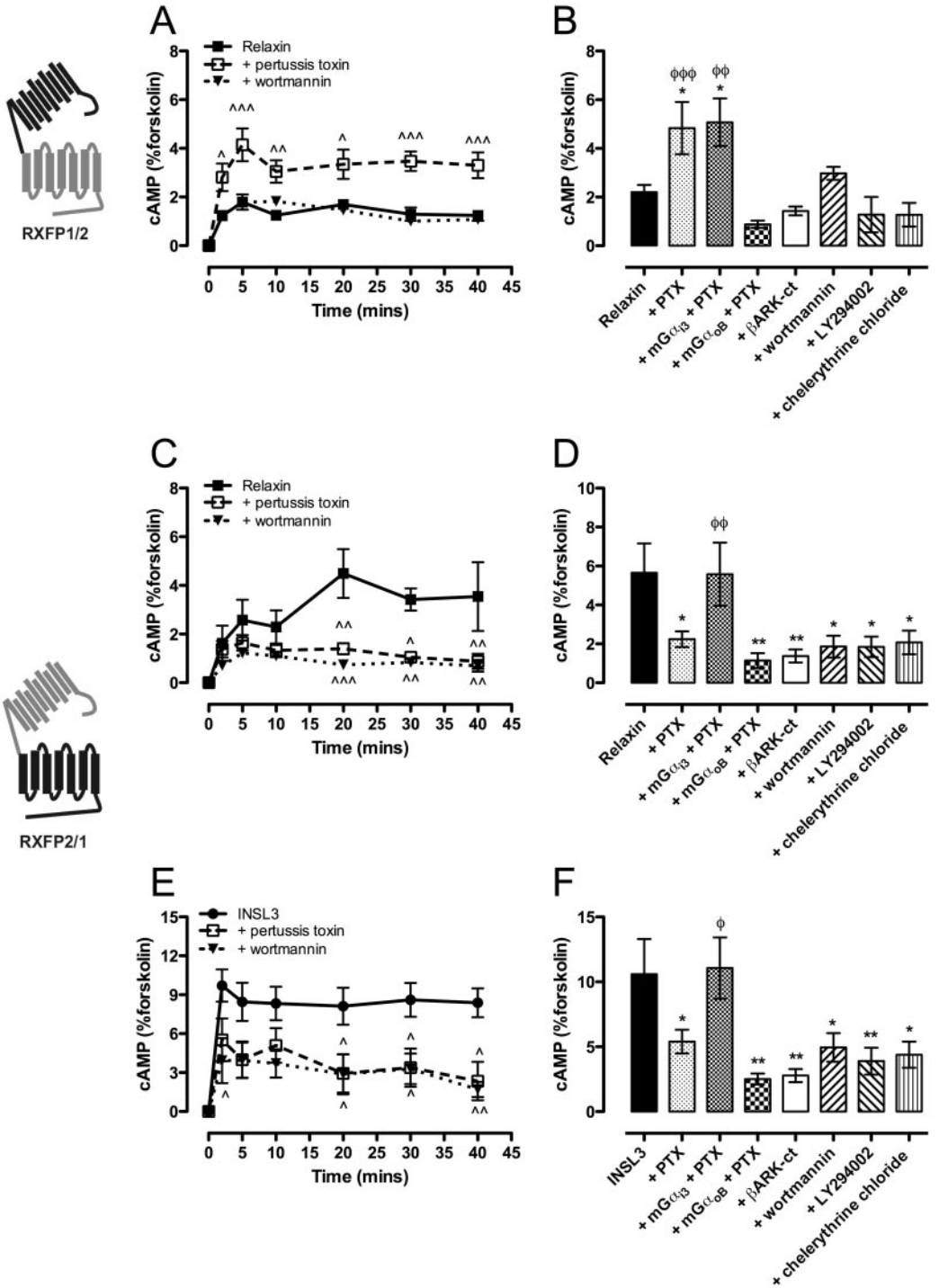

Fig. 3. Coupling of RXFP1 to the Gα_{i3}-G$\beta\gamma$-PI3K-PKCζ pathway is dependent upon the transmembrane/C-terminal domain of the receptor and is independent of both the ectodomain and the ligand. The activation profile of cAMP accumulation was examined in HEK293T cells transiently expressing either RXFP1/2 (A and B; chimeric receptor consisting of the ectodomain of RXFP1 fused to the transmembrane/C-terminal domain of RXFP2) or RXFP2/1 (C, D, E, and F; chimeric receptor with the ectodomain of RXFP2 fused to the transmembrane/C-terminal domain of RXFP1). For each receptor time course (A, C, and E; both receptors transiently expressed, 114 ng/cm^2 culture area), studies were performed over a 40-min period of relaxin (A and C; 30 nM) or INSL3 (E; 30 nM) stimulation, in the presence and absence of pertussis toxin (PTX; G_i/G_o inhibitor; 100 ng/ml, 16-h preincubation) or wortmannin (PI3K inhibitor; 100 nM, 30-min preincubation). The effect of a single concentration of relaxin (B and D; 30 nM) or INSL3 (F; 30 nM) stimulation was also assessed at 30 min in the presence and absence of PTX, Gα_{i3}(C351I) with PTX (mGα_{i3}), Gα_{oB}(C351I) with PTX (mGα_{oB}), βARK-ct (G$\beta\gamma$ scavenger), wortmannin, LY294002 (PI3K inhibitor; 10 μM, 30-min pretreatment), or chelerythrine chloride (PKC inhibitor; 1 μM, 30-min pretreatment). In B, D, and F, RXFP1 and RXFP2, mGα_{i3}, mGα_{oB}, and βARK-ct were all transiently transfected at 57 ng/cm^2 culture area. cAMP accumulation is expressed as a percentage of the response to forskolin (0.1 mM) after 30-min incubation. Symbols or columns represent means and vertical bars represent S.E.M. of four to eight different experiments performed in duplicate. $\wedge$, $p < 0.05$; $\wedge\wedge$, $p < 0.01$; and $\wedge\wedge\wedge$, $p < 0.001$ versus peptide alone, two-way ANOVA with Bonferroni post-tests; $*$, $p < 0.05$; and $**$, $p < 0.01$ versus peptide alone, ϕ, $p < 0.05$; $\phi\phi$, $p < 0.01$; and $\phi\phi\phi$, $p < 0.001$ versus mGα_{oB} with PTX, one-way ANOVA with Newman-Keuls multiple comparison test.

3B) confirmed that this chimera couples to $G\alpha_{oB}$ and that there was no effect of βARK-ct transfection or preincubation with wortmannin, LY294002, or chelerythrine chloride. Thus RXFP1/2 exhibits an "RXFP2-like" signaling profile.

RXFP2/1 (ectodomain of RXFP2, with a transmembrane/C-terminal domain of RXFP1), when stimulated with relaxin, showed activation of the $G\alpha_{i3}$ pathway (Fig. 3C), with inhibition of the cAMP response after approximately 10 min by both PTX and wortmannin. Single point experiments (Fig. 3D) showed that the receptor could couple to $G\alpha_{i3}$ and that the cAMP response was significantly inhibited by βARK-ct transfection and preincubation with wortmannin, LY294002, or chelerythrine chloride. Thus RXFP2/1 exhibited an "RXFP1-like" signaling profile. Therefore the ligand-recognition properties of the chimeric receptors reflected the ectodomain, but the signaling behavior depends upon the "origin" of their transmembrane and associated C-terminal domains.

To determine whether activation of the $G\alpha_{i3}$-$G\beta\gamma$-PI3K-PKCζ pathway was specific for relaxin activation of the receptor, we tested the effect of INSL3 stimulation on the two receptor chimeras, RXFP1/2 and RXFP2/1. Although INSL3 has been shown to cause small amounts of cAMP accumulation via RXFP1/2 (through binding to the RXFP2 low-affinity transmembrane site; Halls et al., 2005), the response was too small to accurately test the effect of signaling inhibitors (data not shown).

At RXFP2/1, the INSL3 time course response was found to involve the $G\alpha_{i3}$ pathway (Fig. 3E); PTX and wortmannin both inhibited cAMP accumulation after approximately 10 min. In single-point experiments (Fig. 3F), INSL3 stimulation was also found to cause coupling to $G\alpha_{i3}$, and the INSL3 response was significantly inhibited by transfection with βARK-ct or pretreatment with wortmannin, LY294002, or chelerythrine chloride. Thus, activation of the $G\alpha_{i3}$ pathway is not a ligand-dependent event but is instead directed wholly by the transmembrane and associated C-terminal domain of RXFP1.

RXFP1/2 and RXFP2/1 were both expressed at the cell surface (as determined by FLAG-tag binding) and displayed similar pIC_{50} (as determined by [^{33}P]relaxin 100 pM competition binding) and pEC_{50} (as determined by cAMP relaxin concentration response curve) values to those described previously (Halls et al., 2005) (Table 1).

Sequential Truncation of the RXFP1 C Terminus Removes Coupling to the $G\alpha_{i3}$-$G\beta\gamma$-PI3K-PKCζ Pathway; Coupling to This Pathway Is Dependent upon the Final 10 Amino Acids Of the C-Terminal Tail. To further define the precise region of the RXFP1 transmembrane and C-terminal domain that directs coupling to the $G\alpha_{i3}$ pathway, C-terminal truncates of RXFP1 were generated. Truncation of RXFP1 immediately after helix 8 (tRXFP1–703) removed the inhibitory effects of PTX and wortmannin, PTX now consistently enhancing cAMP accumulation over the 40-min period (Fig. 4A). When the single-point profile was examined (Fig. 4B), only replacement of $mG\alpha_{oB}$ restored the pattern of cAMP signaling, and neither transfection of βARK-ct nor pretreatment with wortmannin, LY294002, or chelerythrine chloride affected the cAMP response. Thus tRXFP1–703 is unable to couple to the $G\alpha_{i3}$ pathway and has an RXFP2-like signaling profile, indicating that the C terminus is essential for directing the coupling of RXFP1 to the $G\alpha_{i3}$ pathway.

Alignment of the RXFP1 and RXFP2 transmembrane regions shows high similarity within the transmembrane α helices but greater disparity within the C termini. In particular, RXFP2 lacks the C-terminal 10 amino acids of RXFP1 (Halls et al., 2007b). Thus, we assessed the effect of truncation of the final 10 amino acids of the RXFP1 C terminus. As with the initial truncated receptor, truncation of the final 10 amino acids of the C terminus (tRXFP1–747) removed the inhibitory effects of PTX and wortmannin, PTX consistently enhancing cAMP accumulation over 40 min (Fig. 4C). Single-point analysis (Fig. 4D) confirmed that this receptor was able to couple to $G\alpha_{oB}$ and that there was no effect of transfection of βARK-ct or pretreatment with wortmannin, LY294002, or chelerythrine chloride. Thus tRXFP1–747 cannot couple to the $G\alpha_{i3}$ pathway, and instead responds in an RXFP2-like manner, showing that coupling to the $G\alpha_{i3}$ pathway is dependent upon the final 10 amino acids of the RXFP1 C terminus.

To determine whether other regions of RXFP1 might influence coupling to the delayed pathway, we spliced the final 10 amino acids of the RXFP1 C terminus onto the C-terminal end of RXFP2. This receptor, RXFP2–10ct1, was then tested with relaxin (data not shown) or INSL3 stimulation (Fig. 4, E and F). In both instances, PTX or wortmannin significantly inhibited cAMP accumulation after approximately 20 min. When the single point profile of this receptor was assessed, it revealed coupling to $G\alpha_{i3}$ and significant inhibition of cAMP accumulation after transfection with βARK-ct and pretreatment with wortmannin, LY294002, or chelerythrine chloride. The pattern of response was similar for both relaxin and INSL3 stimulation, reiterating the nonligand dependence of this response. Thus RXFP2–10ct1 behaves in an RXFP1-like manner and exhibits "gain-of-function" coupling to the $G\alpha_{i3}$ pathway.

tRXFP1–703, tRXFP1–747 and RXFP2–10ct1 were all expressed at the cell surface (as determined by FLAG-tag binding), and had similar pIC_{50} (as determined by [^{33}P]relaxin 100 pM competition binding) and pEC_{50} (as determined by cAMP relaxin concentration response curve) values to the normal RXFP1 (tRXFP1–703 and tRXFP1–747) or RXFP2 (RXFP2–10ct1) receptors (Table 1).

Point Mutations of Potential Phosphorylation Sites within the C Terminus Reveal an Absolute Requirement for Arg^{752}, and a Partial Influence of Ser^{755}, upon Activation of the $G\alpha_{i3}$-$G\beta\gamma$-PI3K-PKCζ Pathway. Based upon the frequent association between receptor phosphorylation and activation of secondary signaling pathways, we analyzed the final 10 amino acids of the RXFP1 C-terminal tail for potential phosphorylation motifs based upon three criteria: 1) a score >0.5 derived from NetPhos 2.0 prediction (Blom et al., 1999), 2) the vicinity of basic or acidic residues, proline residues, or other phosphorylated residues that are generally required for protein kinase binding, and 3) conservation of target sequences across species homologues. This revealed a potential motif encompassing residues Arg^{752} and Ser^{755} (in sequence SQST**R**LN**S**YS). Interestingly, one of the residues, Arg^{752}, was completely conserved across 17 different species. Both of these residues were mutated to alanine to produce the constructs RXFP1 R752A (Arg^{752} to Ala) and RXFP1 S755A (Ser^{755} to Ala).

RXFP1 R752A (Fig. 5, A and B) displayed no coupling to the $G\alpha_{i3}$-$G\beta\gamma$-PI3K-PKCζ pathway. The time course of relaxin-induced cAMP accumulation suggested that coupling to

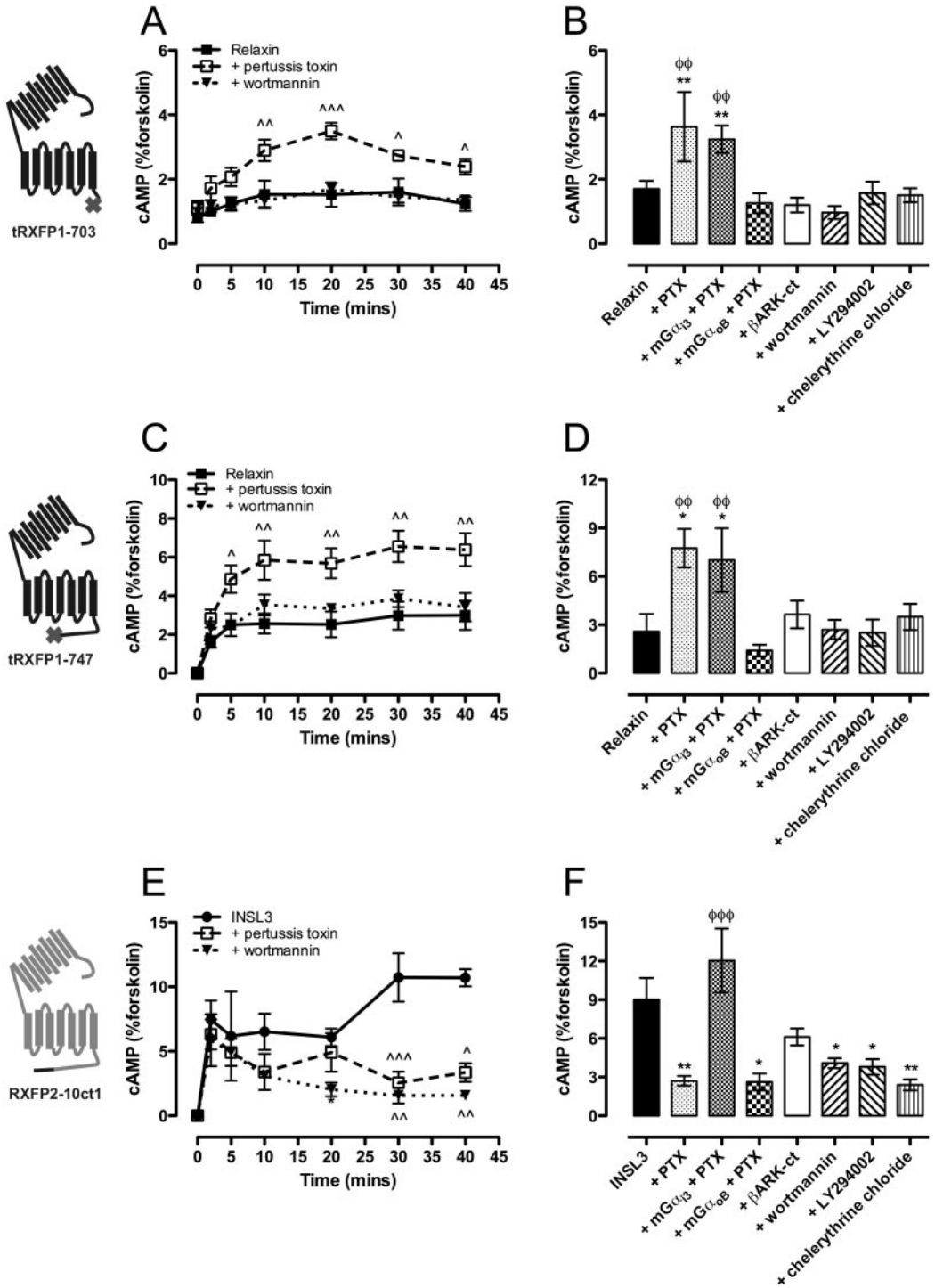

Fig. 4. Truncation of the RXFP1 C terminus removes coupling to the Gα_{i3}-G$\beta\gamma$-PI3K-PKCζ pathway: activation of this pathway depends upon the final 10 amino acids of the RXFP1 C terminus. cAMP accumulation was measured in HEK293T cells transiently expressing (transfected at 114 ng/cm^2 culture area) either tRXFP1–703 (A and B; RXFP1 receptor truncated at residue 703, immediately after helix 8), tRXFP1–747 (C and D; RXFP1 receptor truncated at residue 747, removing the final 10 amino acids of the C terminus), or RXFP2–10ct1 (E and F; RXFP2 with the C-terminal 10 amino acids of RXFP1 added to the C terminus of RXFP2). For each receptor, time-course studies (A, C, and E) were performed over a 40-min period of relaxin (A and C; 30 nM) or INSL3 (E; 30 nM) stimulation, with or without pertussis toxin (PTX; G_i/G_o inhibitor; 100 ng/ml, 16-h preincubation) or wortmannin (PI3K inhibitor; 100 nM, 30-min preincubation) pretreatment. The effect of a single concentration of relaxin (B and D; 30 nM) or INSL3 (F; 30 nM) stimulation was also assessed at 30 min in the presence and absence of PTX, Gα_{i3}(C351I) with PTX (mGα_{i3}), Gα_{oB}(C351I) with PTX (mGα_{oB}), βARK-ct (G$\beta\gamma$ scavenger), wortmannin, LY294002 (PI3K inhibitor; 10 μM, 30-min pretreatment), or chelerythrine chloride (PKC inhibitor; 1 μM, 30-min pretreatment). For B, D, and F, receptors and mGα_{i3}, mGα_{oB} and βARK-ct were all transiently transfected at 57 ng/cm^2 culture area. cAMP accumulation is expressed as a percentage of the response to forskolin (0.1 mM) after 30-min incubation. Symbols or columns represent means and vertical bars represent S.E.M. of four to ten different experiments performed in duplicate. $\wedge$, $p < 0.05$; $\wedge\wedge$, $p < 0.01$; and $\wedge\wedge\wedge$, $p < 0.001$ versus peptide alone, two-way ANOVA with Bonferroni post-tests; *, $p < 0.05$ and **, $p < 0.01$ versus peptide alone; ϕ, $p < 0.05$; $\phi\phi$, $p < 0.01$ versus mGα_{oB} with PTX (B and D) or versus INSL3 with PTX (F), one-way ANOVA with Newman-Keuls multiple comparison test.

the $G\alpha_{i3}$ pathway was lost, with PTX consistently enhancing cAMP accumulation over the 40 min (Fig. 5A). Single-point analysis confirmed that this receptor construct coupled to $G\alpha_{oB}$, and that there was no effect of βARK-ct, wortmannin, LY294002, or chelerythrine chloride on the cAMP response (Fig. 5B). In contrast, the serine mutant, RXFP1 S755A, showed a different phenotype to both RXFP1 and RXFP2: only wortmannin significantly inhibited the relaxin response, and there was no effect of PTX pretreatment (Fig. 5C). Single-point analysis confirmed this signaling profile (Fig. 5D); there was no effect of PTX or cotransfection of $mG\alpha_{i3}$ or βARK-ct. However, cotransfection of $mG\alpha_{oB}$ significantly decreased cAMP accumulation, as did pretreatment with wortmannin, LY294002 or chelerythrine chloride. Taken together, this probably indicates partial or inefficient coupling of RXFP1 S755A to the $G\alpha_{i3}$-Gβγ-PI3K-PKCζ pathway.

RXFP1 R752A and RXFP1 S755A were both expressed at the cell surface (as determined by FLAG-tag binding) and had similar pIC_{50} (as determined by [^{33}P]-relaxin 100 pM competition binding) and pEC_{50} (as determined by cAMP relaxin concentration response curve) values to the normal RXFP1 receptor (Table 1).

RXFP1 Coupling to the $G\alpha_{i3}$-Gβγ-PI3K-PKCζ Pathway Is Dependent upon Receptor and $G\alpha_{i3}$ Localization within Membrane Raft Signaling Domains. We have previously shown that β_3-adrenoceptor signaling is influenced by the localization of the receptor within caveolae (Sato et al., 2007). Thus, we tested the effect of membrane

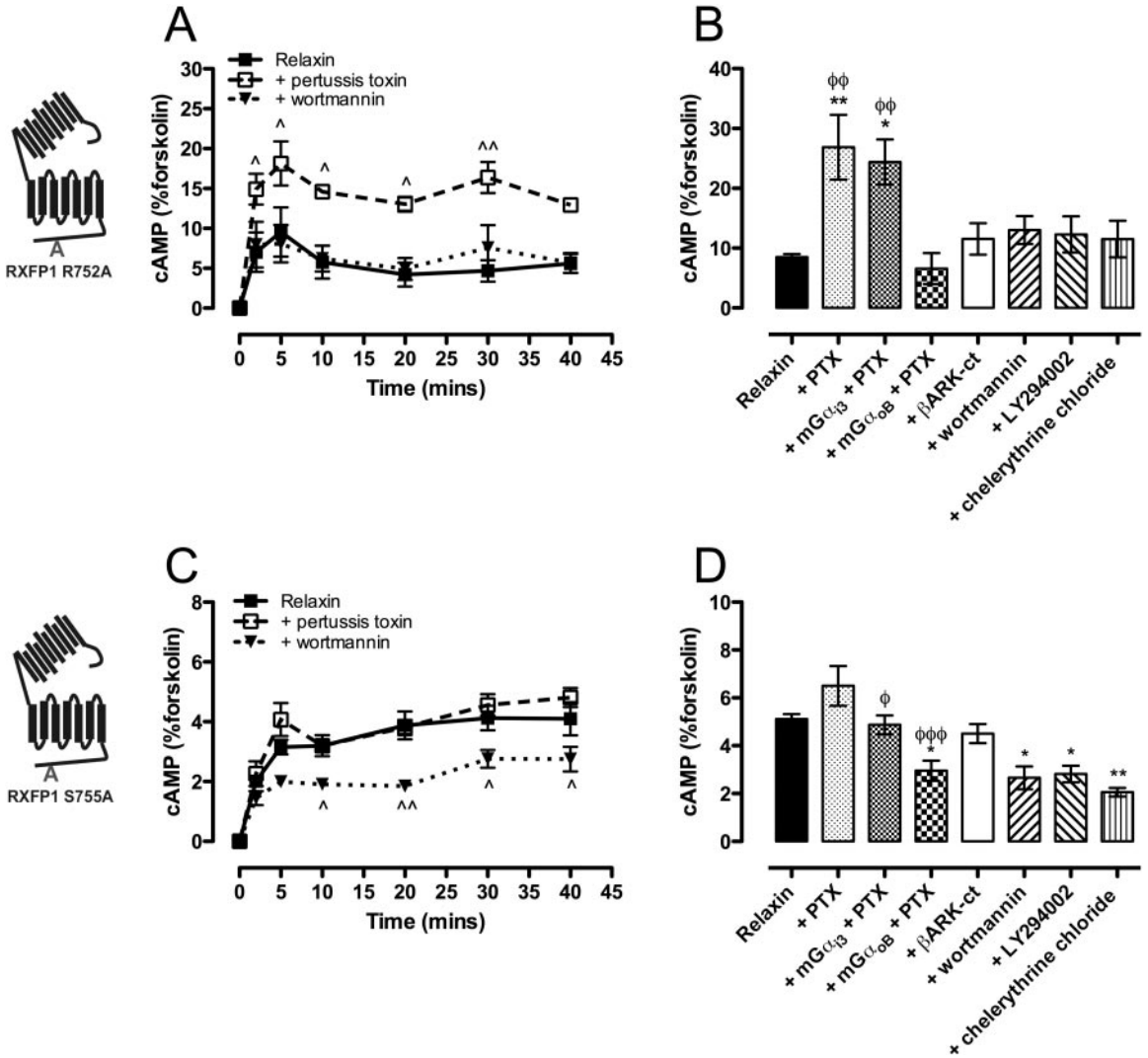

Fig. 5. Coupling of RXFP1 to $G\alpha_{i3}$ absolutely requires Arg^{752}. cAMP accumulation was measured in HEK293T cells transiently expressing either RXFP1 R752A (A and B; RXFP1 receptor with Arg^{752} replaced with Ala) or RXFP1 S755A (C and D; RXFP1 receptor with Ser^{755} replaced with Ala). For each receptor, time-course studies (A and C; both receptors transiently expressed, 114 ng/cm^2 culture area) were performed over a 40-min period of relaxin (30 nM) stimulation, with or without PTX (G_i/G_o inhibitor; 100 ng/ml, 16-h preincubation) or wortmannin (PI3K inhibitor; 100 nM, 30-min preincubation) pretreatment. The effect of a single concentration of relaxin (30 nM) was also assessed at 30 min (B and D) in the presence and absence of PTX, $G\alpha_{i3}$(C351I) with PTX ($mG\alpha_{i3}$), $G\alpha_{oB}$(C351I) with PTX ($mG\alpha_{oB}$), βARK-ct (Gβγ scavenger), wortmannin, LY294002 (PI3K inhibitor; 10 μM, 30-min pretreatment), or chelerythrine chloride (PKC inhibitor; 1 μM, 30-min pretreatment). For B and D, receptors and $mG\alpha_{i3}$, $mG\alpha_{oB}$, and βARK-ct were all transiently transfected at 57 ng/cm^2 culture area. cAMP accumulation is expressed as a percentage of the response to forskolin (0.1 mM) after 30 min incubation. Symbols or columns represent means, and vertical bars, S.E.M. of three to eight different experiments performed in duplicate. ∧, $p < 0.05$; and ∧∧, $p < 0.01$ versus relaxin alone, two-way ANOVA with Bonferroni post-tests; *, $p < 0.05$; and **, $p < 0.01$ versus relaxin alone; ϕ, $p < 0.05$; ϕϕ, $p < 0.01$; and ϕϕϕ, $p < 0.001$ PTX versus $mG\alpha_{oB}$ with PTX or $mG\alpha_{i3}$ with PTX, one-way ANOVA with Newman-Keuls multiple comparison test.

raft disruption upon RXFP1 signaling using filipin III (Fig. 6). Filipin III is a macrolide pentene polyene antibiotic that binds sterols such as cholesterol (Bolard, 1986), thereby disrupting membrane raft formation (Rothberg et al., 1990). Preincubation of cells with filipin III to disrupt membrane rafts completely removed signaling of RXFP1 via the Gα_{i3}-G$\beta\gamma$-PI3K-PKCζ pathway (Fig. 6A) but had no effect upon signaling of the receptor via Gα_s or Gα_{oB}. Likewise, membrane raft disruption removed the gain-of-function signaling of RXFP2–10ct1 via the Gα_{i3} pathway but had no effect upon either Gα_s or Gα_{oB} signaling (Fig. 6E). There was no effect of membrane raft disruption upon receptors unable to couple to the Gα_{i3} pathway: RXFP2, tRXFP1–747, and RXFP1 R752A signaling via Gα_s or Gα_{oB} was unaffected (Fig. 6, B, C, and D, respectively). To confirm the importance of membrane rafts for RXFP1-Gα_{i3} signaling, basic lipid fractionation using Triton X-100 was performed (Figs. 6F). Sphingolipid- and cholesterol-rich microdomains are insoluble in nonionic detergents (such as Triton X-100) at 4°C (Sargiacomo et al., 1993; Chun et al., 1994), thus Triton X-100-insoluble fractions may be distinguished from Triton X-100 soluble fractions. RXFP1 and Gα_{i3} were contained within Triton X-100 insoluble fractions, both under basal conditions and after 30-min relaxin stimulation. All other receptor constructs (RXFP2, tRXFP1–

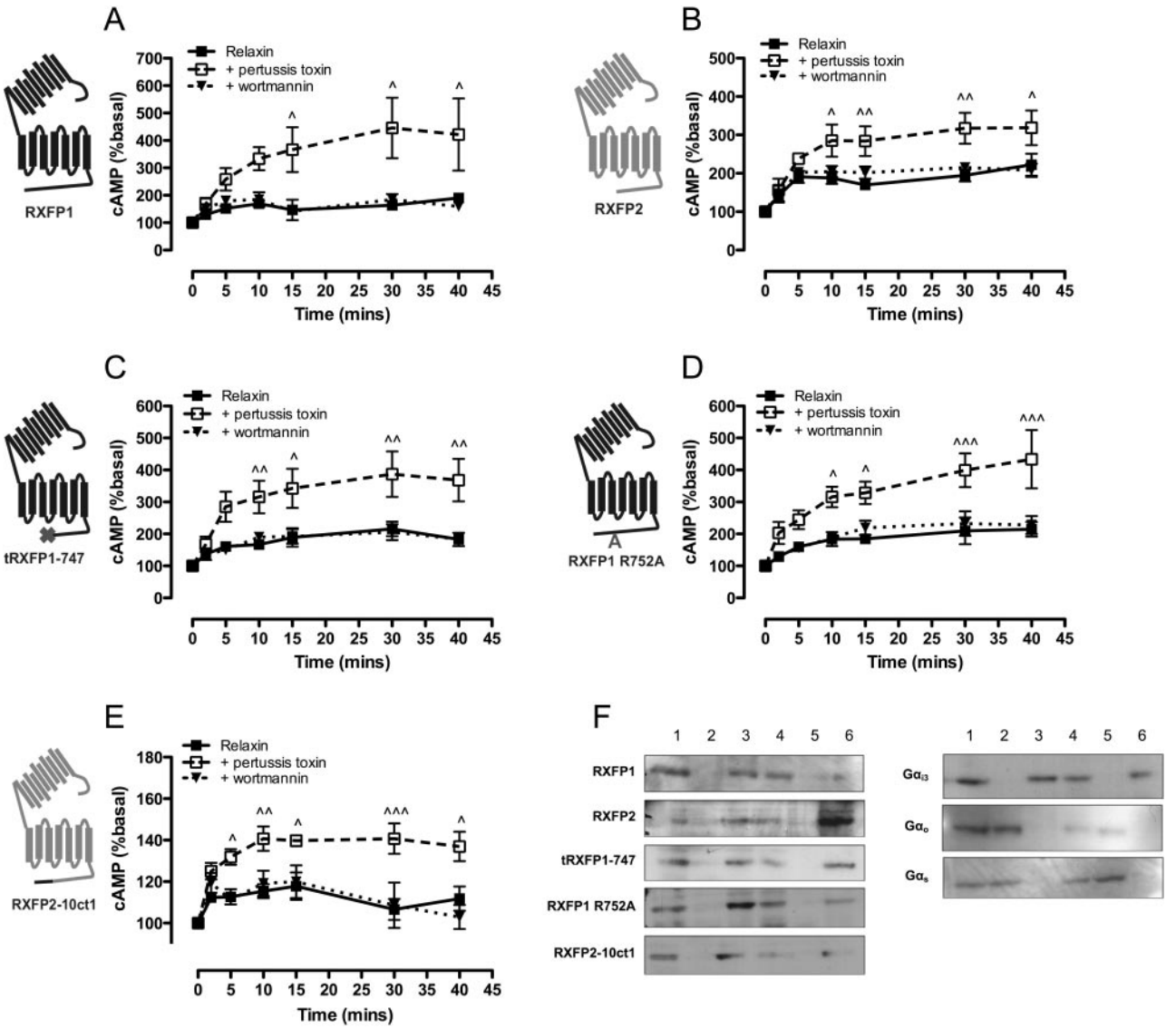

Fig. 6. Coupling of RXFP1 to Gα_{i3}, but not Gα_s or Gα_{oB}, depends upon receptor and Gα_{i3} localization within membrane rafts. The activation profile of cAMP accumulation of RXFP1 (A), RXFP2 (B), tRXFP1–747 (C), RXFP1 R752A (D), and RXFP2–10ct1 (E) was assessed after preincubation with filipin III (disrupts membrane rafts; 1 μg/ml, 1-h preincubation), and stimulation with relaxin (30 nM) in the presence or absence of PTX (G_i/G_o inhibitor; 100 ng/ml, 16-h preincubation) or wortmannin (PI3K inhibitor; 100 nM, 30-min preincubation). The relative location of RXFP1, RXFP2, tRXFP1–747, RXFP1 R752A, RXFP2–10ct1, Gα_s, Gα_{oB}, and Gα_{i3} was examined under basal conditions and after 30-min stimulation with relaxin (30 nM) by basic lipid fractionation, followed by protein separation and Western blotting with appropriate antibodies [DYKDDDDK tag (FLAG tag) rabbit polyclonal antibody, 1:1000 dilution, secondary 1:2000 dilution; Gα_s (A-16) goat polyclonal antibody, 1:500 dilution, secondary 1:50,000 dilution; Gα_o (K-20) rabbit polyclonal antibody, 1:500 dilution, secondary 1:50,000 dilution; Gα_{i3} chicken polyclonal antibody, 1:500 dilution, secondary 1:50,000 dilution]. Samples were loaded in equivalent volumes as follows: 1, total unfractionated homogenate at basal; 2, Triton-soluble fraction at basal; 3, Triton-insoluble fraction at basal; 4, total unfractionated homogenate with 30-min relaxin stimulation; 5, Triton-soluble fraction with 30-min relaxin stimulation; and 6, Triton-insoluble fraction with 30-in relaxin stimulation. $\wedge$, $p < 0.05$; $\wedge\wedge$, $p < 0.01$; and $\wedge\wedge\wedge$, $p < 0.001$ versus relaxin alone, two-way ANOVA with Bonferroni post-tests.

747, RXFP1 R752A, and RXFP2–10ct1) were also localized within membrane raft regions, whereas $G\alpha_s$ and $G\alpha_o$ were found within nonmembrane raft domains of the cell.

Discussion

To determine the region of RXFP1 that enables coupling to the $G\alpha_{i3}$-$G\beta\gamma$-PI3K-PKCζ pathway, we used two paradigms to characterize cAMP responses generated by mutant receptors: time course studies over 40 min with or without PTX or wortmannin and the effect of inhibitors on a 30-min stimulation using a single peptide concentration (30 nM) that produces a maximal response. cAMP responses are characterized as either RXFP1-like or RXFP2-like based upon the response profile. RXFP1-like responses are characterized by a time course in which there is little effect of PTX (G_i/G_o inhibitor) or wortmannin (PI3K inhibitor) at times up to 10 min. With continued stimulation, PTX or wortmannin significantly inhibit the cAMP response compared with peptide alone. It is noteworthy that our study shows that the 10-min delay is unlikely to occur at the level of receptor-G-protein interaction: [^{35}S]GTPγS immunoprecipitation studies showed activation of $G\alpha_{i3}$ by RXFP1 within 3 min of relaxin stimulation. Thus RXFP1 couples to $G\alpha_{i3}$ almost immediately, suggesting that the observed delay in cAMP accumulation occurs downstream of this point, most likely involving PKCζ translocation to the cell membrane (Nguyen and Dessauer, 2005).

The second approach used to characterize the RXFP1 cAMP response involved more comprehensive inhibitor testing using a single peptide concentration at 30 min and mutant G-proteins. After PTX pretreatment, only m$G\alpha_{i3}$ (PTX-resistant $G\alpha_{i3}$ mutant) can restore signaling (indicating receptor coupling to this G-protein), and inhibition of G_i/G_o, G-$\beta\gamma$, PI3K, or PKCζ by PTX, βARK-ct, wortmannin/LY294002, or chelerythrine chloride, respectively, all significantly reduced the cAMP response to relaxin peptide. In contrast, RXFP2-like responses are characterized by an increase in cAMP accumulation over time that is enhanced by PTX pretreatment and not affected by wortmannin. In addition, after 30-min stimulation with a single peptide concentration, inhibition of G_i/G_o by PTX significantly increases cAMP accumulation; only m$G\alpha_{oB}$ restores cAMP signaling in the presence of PTX, and there is no effect of inhibition of PI3K or PKCζ. These signaling profiles were used to screen the effect of a number of receptor mutations.

Previously, we have exploited the high degree of similarity between RXFP1 and RXFP2 to generate receptor chimeras comprising the ectodomain of one receptor fused to the transmembrane and C terminus of the other (Sudo et al., 2003; Halls et al., 2005). Pharmacological analysis of these receptors revealed that the receptor ectodomain directs the binding profile and relative potency of relaxin peptides (Halls et al., 2005). It was therefore interesting that the transmembrane and intracellular regions of the receptors directed their G-protein coupling profile, because RXFP2/1 showed RXFP1-like Gα-isoform coupling ($G\alpha_s$, $G\alpha_{oB}$, and $G\alpha_{i3}$), whereas RXFP1/2 showed RXFP2-like Gα coupling ($G\alpha_s$ and $G\alpha_{oB}$) in response to both relaxin and INSL3. Thus, RXFP1 coupling to the $G\alpha_{i3}$-$G\beta\gamma$-PI3K-PKCζ pathway is dependent solely upon the transmembrane/C-terminal region of the receptor, and the receptor Gα-isoform profile is independent of the bound peptide and the receptor ectodomain.

Sequential truncations of the RXFP1 C terminus were then used to examine the role of this domain in coupling to the $G\alpha_{i3}$-$G\beta\gamma$-PI3K-PKCζ pathway. Truncation immediately after helix 8, at residue 703 (tRXFP1–703), produced an RXFP2-like receptor. Thus the presence of the RXFP1 C terminus is absolutely required (though not necessarily sufficient) for linkage to the $G\alpha_{i3}$-$G\beta\gamma$-PI3K-PKCζ cascade. In contrast, there was no effect of a similar truncation of RXFP2 (data not shown), suggesting that coupling of both RXFP1 and RXFP2 to $G\alpha_s$ and $G\alpha_{oB}$ does not involve the C terminus. The divergence of the C-terminal amino acid sequence between RXFP1 and RXFP2 is highlighted in the terminal 10 amino acids of the RXFP1 C terminus, which are lacking in RXFP2. A truncated RXFP1 lacking these 10 amino acids (tRXFP1–747), although RXFP1 in sequence, had an RXFP2-like signaling profile. It is noteworthy that when these 10 amino acids were added to the C terminus of RXFP2, there was a gain-of-function event such that the modified RXFP2 receptor then had an RXFP1-like signaling profile. This also suggests that these 10 C-terminal amino acids of RXFP1 are required for coupling to the $G\alpha_{i3}$-$G\beta\gamma$-PI3K-PKCζ pathway, because the C-terminal tails of these two receptors are otherwise quite different. To further define the residues involved in coupling to $G\alpha_{i3}$-$G\beta\gamma$-PI3K-PKCζ, we constructed point mutations within the C terminus. Only RXFP1 R752A completely switched signaling to an RXFP2-like profile, suggesting an absolute requirement for Arg^{752}. Mutation of Ser^{755} partially changed the signaling profile of RXFP1, perhaps suggesting that it forms part of a binding motif involving Arg^{752}.

To examine RXFP1-$G\alpha_{i3}$ signaling further, we disrupted membrane rafts using filipin III (Sato et al., 2007) and showed that RXFP1 coupling to $G\alpha_{i3}$ was dependent upon their presence, whereas RXFP2 signaling was insensitive to membrane raft disruption. Indeed, RXFP2–10ct1 coupling to the $G\alpha_{i3}$ pathway was also prevented by membrane raft disruption, whereas deletion of the final 10 amino acids of the RXFP1 C terminus or substitution of Arg^{752} for Ala removed the sensitivity of RXFP1 to membrane raft disruption. RXFP1 and $G\alpha_{i3}$ were located within detergent-resistant membrane fractions both under basal conditions and after 30-min relaxin exposure, as would be predicted by the effect of filipin III, but so too were the receptors unaffected by membrane raft disruption (RXFP2, tRXFP1–747, and RXFP1 R752A). This suggests that the membrane raft dependence of $G\alpha_{i3}$ cAMP signaling is not due merely to receptor localization within these domains but perhaps more importantly to formation of signaling complexes that facilitate activation of the $G\alpha_{i3}$ pathway by RXFP1. Furthermore, the dependence of $G\alpha_{i3}$ signaling upon membrane rafts suggests compartmentalization of relaxin-induced cAMP accumulation, supported by previous findings showing that cAMP signaling after RXFP1 activation, as determined by a CRE (cAMP response element) reporter gene, is localized within specific microdomains of HEK293T cells: CRE activity occurred only in response to cAMP downstream of $G\alpha_s$ and $G\alpha_{oB}$ signaling, but not the $G\alpha_{i3}$-cAMP pathway (Halls et al., 2007a). Thus cAMP signaling by RXFP1 seems highly organized into specific microdomains but occurs both within and outside membrane raft domains.

The precise mechanism of RXFP1-$G\alpha_{i3}$ signaling is not yet clear, although there are a number of intriguing possibilities. A phosphorylation event or cascade within the C terminus

(perhaps initiated within the final 10 amino acids) that enables coupling to the Gα_{i3} signaling pathway is an unlikely explanation, because preliminary studies from our laboratory suggest only very minimal phosphorylation of RXFP1 upon agonist activation of the receptor. Alternatively, the final 10 amino acids of the RXFP1 C terminus may contain a protein-protein binding site required to recruit a signaling complex activating the Gα_{i3}-G$\beta\gamma$-PI3K-PKCζ pathway. It is also possible that there is a direct interaction between the RXFP1 C terminus and Gα_{i3} itself. However, although this final possibility may facilitate 'precoupling' of RXFP1 and Gα_{i3}, it is highly unlikely to be the sole determinant of Gα_{i3} activation. Recent structural studies suggest that upon G-protein-coupled receptor activation, the C terminus of the Gα subunit binds within a cavity created by both the outward tilting of transmembrane helix 6 (TM6), thus altering the position of this helix relative to TM5, and a restructuring of the link between TM7 and helix 8 (Scheerer et al., 2008). In accord with this mechanism, recent evidence indicates that coupling of RXFP1 to Gα subunits involves the cytoplasmic end of TM6, because peptides derived from this region inhibit receptor-mediated cAMP production (Shpakov et al., 2006). Thus differential signaling by RXFP1 and RXFP2 is likely due to specific protein-protein interactions (or signaling complex formation) required for activation of the Gα_{i3}-G$\beta\gamma$-PI3K-PKCζ pathway by RXFP1. Such interactions must contribute to the coupling of RXFP1 to Gα_{i3} rather than to recruitment or activation of effectors downstream of Gα_{i3}, because RXFP2 cannot mediate Gα_{i3}-[^{35}S]GTPγS binding. Alternatively, although both receptors localize with Gα_{i3} in detergent-resistant membrane fractions, there may be further compartmentalization of receptors and signaling proteins in membrane "nanodomains," as described in studies on β_2-adrenoceptors (Pontier et al., 2008). Elucidation of the role of the C-terminal 10 amino acids of RXFP1 in Gα_{i3}-G$\beta\gamma$-PI3K-PKCζ activation will require comprehensive testing of these alternative hypotheses, perhaps using the corresponding peptide as bait in a pull-down assay of protein binding partners, or by conducting further biophysical studies on interactions between RXFP1 and membrane lipid components (Pontier et al., 2008).

In conclusion, it is now well documented that RXFP1 and RXFP2 display different cAMP signaling profiles; RXFP1 is able to activate a Gα_{i3}-G$\beta\gamma$-PI3K-PKCζ pathway that induces a substantial cAMP accumulation over time. Here we demonstrate that RXFP1 couples to Gα_{i3} within 3 min of stimulation, suggesting that the delay in cAMP observed with this pathway is due to signaling elements downstream of the G-protein. Using receptor mutagenesis, we have shown that the final 10 amino acids of RXFP1, and especially residue Arg^{752}, are essential for and direct the coupling of RXFP1 to the Gα_{i3}-G$\beta\gamma$-PI3K-PKCζ pathway and that this pathway is dependent upon the presence of membrane rafts.

Acknowledgments

We thank Corthera, Inc. (San Mateo, CA) for the supply of recombinant relaxin, Sharon Layfield for making the tRXFP1–703 and tRXFP2–712 receptor mutants, Maria Papaioannou for excellent technical assistance, and Dr. Sebastian Furness for help with the protocol for [^{35}S]GTPγS immunoprecipitation.

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