Multiple binding sites revealed by interaction of relaxin family peptides with native and chimeric relaxin family peptide receptors 1 and 2 (LGR7 & LGR8).

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Running Title: Characterisation of two binding sites for RXFP1 (LGR7) and RXFP2 (LGR8)

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Number of text pages: 29

Number of tables: 4

Number of figures: 7

Number of references: 38

Number of words in abstract: 260

Number of words in introduction: 938

Number of words in discussion: 1,729

Abbreviations: INSL3, insulin-like peptide 3; GPCR, G-protein-coupled receptor; RXFP1, relaxin family peptide receptor 1 (LGR7); RXFP2, relaxin family peptide receptor 2 (LGR8); RXFP1/2, relaxin family peptide receptor chimera 1/2(LGR7/8); RXFP2/1, relaxin family peptide receptor chimera 2/1(LGR8/7); RXFP1-BP, relaxin family peptide 1 receptor ectodomain (LGR7-BP); RXFP2-BP, relaxin family peptide receptor 2 ectodomain (LGR8-BP); RXFP1-TM, relaxin family peptide 1 receptor transmembrane domain; RXFP2-TM, relaxin family peptide 2 receptor transmembrane domain.

Recommended section assignment: Cellular & Molecular

Abstract

Relaxin family peptide 1 (RXFP1) receptor (LGR7) and the RXFP2 receptor (LGR8) were recently identified as the receptor-targets for H2 relaxin and INSL3 respectively. In this study we define the pharmacology of these two receptors by utilising a number of receptor chimeras and relaxin family peptides. We have identified two binding sites on these receptors: one primary, high affinity site within the ectodomain and a secondary, lower affinity site within the transmembrane region. The primary site was found to dictate receptor binding characteristics, although the lower affinity site also exerts some influence and modulates ligand affinity for the primary site in a manner dependent upon the peptide in question. Not all relaxin peptides were able to bind to the RXFP2 receptor, indicating that the relaxin-RXFP2 receptor interaction is species-specific. INSL3 was found to exhibit characteristics of a partial agonist at the RXFP2 and chimeric RXFP1/2 receptors, with low maximal cAMP responses but high potency in coupling to this pathway. cAMP accumulation studies also revealed that the binding sites couple to cAMP signalling pathways with differing efficiency: the high affinity site signals with high efficiency, while the lower affinity site signals with little to no efficiency. Comparisons between RXFP1, RXFP2, the chimeric receptors and the truncated receptors revealed that the interaction between receptor sites is critical for optimal ligand binding and signal transduction and that the ectodomain is essential for signaling. Evidence obtained in this study supports a two-stage binding model of receptor activation: binding to the primary site allows a conformational change and interaction with the low affinity transmembrane site.

Introduction

Relaxin is a two-chain peptide that was discovered following the observation that serum from pregnant guinea pigs caused relaxation of the pubic ligament (Hisaw, 1926). It is structurally closely related to insulin: both peptides have an A and B chain joined by two interchain disulphide bonds, and one intra-A-chain disulphide bond. This discovery established the concept of the insulin-relaxin superfamily (Schwabe and McDonald, 1977). The high degree of similarity between insulin and relaxin precipitated a search for additional members of the insulin-relaxin superfamily with the same structural motif. To date this peptide family includes insulin-like growth factor-1 (IGF-I) and IGF-II (Humbel, 1990), INSL3 (Adham et al., 1993), INSL4 (Koman et al., 1996), INSL5 (Conklin et al., 1999) and INSL6 (Lok et al., 2000).

Relaxin itself has many paralogs: in humans three non-allelic genes produce H1 relaxin (Hudson et al., 1983), H2 relaxin (Hudson et al., 1984) and the recently identified H3 relaxin (Bathgate et al., 2002). H2 relaxin is the major circulating form of relaxin in the human, and has equivalent orthologs in other species, including Porcine relaxin (Hudson et al., 1981; Haley et al., 1982), Rhesus monkey relaxin (Crawford et al., 1989) and Rat relaxin (Hudson et al., 1981). Like humans, rodents have more than one relaxin gene: one ortholog of relaxin equivalent to H2 relaxin, and another equivalent to H3 relaxin and found principally within the brain (Burazin et al., 2002). Relaxin orthologs equivalent to H1 relaxin are found only in higher primates and are thought to be the product of a gene duplication event (Ivell and Einspanier, 2002).

Although relaxin was initially thought to be important solely for the function of reproductive tissues (Hisaw, 1926), much recent research has revealed equally important functions in other organs including the heart (Kakouris et al., 1992), kidney (Novak et al., 2001) and brain (Weisinger et al., 1993). Relaxin has also been found to play a role in prevention of the tissue remodelling observed in fibrosis with a conservation of endogenous tissue structure (Unemori et al., 1996; Garber et al., 2001), and herein lies its likely potential as a therapeutic.

Despite the time since the discovery of relaxin, it was only recently demonstrated that two orphan leucine rich repeat (LRR)-containing GPCRs (LGRs), LGR7 and LGR8 were capable of mediating the action of relaxin through a G_S-cAMP-dependent pathway (Hsu et al., 2002). More recently it has been established that although H2 and porcine

relaxin can bind to and activate both LGR7 and LGR8, LGR7 is the elusive relaxin receptor (Hsu et al., 2002) while

LGR8 is the receptor for the related peptide, INSL3 (Kumagai et al., 2002). The identical phenotypes of the relaxin

and LGR7 as well as the INSL3 (Nef and Parada, 1999; Zimmermann et al., 1999) and LGR8 (Overbeek et al., 2001;

Gorlov et al., 2002) knockout animals, highlights these ligand receptor pairings. (Kumagai et al., 2002). These

receptors are now referred to as relaxin family peptide 1 (RXFP1 - LGR7) and RXFP2 (LGR8) receptors.

These ligand-receptor classifications have been further influenced by the recent discovery that H3 relaxin is the

specific ligand for two additional orphan GPCRs, the RXFP3 (GPCR135, somatostatin- and angiotensin-like peptide

receptor) and RXFP4 (GPCR142, GPR100) receptors (Liu et al., 2003a; Liu et al., 2003b). The relaxins or INSL3

peptides did not affect binding or cause functional responses at either of these two new relaxin family peptide

receptors. Stimulation of either the RXFP3 or RXFP4 receptor by H3 relaxin results in signalling through G_i causing

an inhibition of cAMP production. This is in contrast to stimulation of the RXFP1 and RXFP2 receptors which

results in an increase in cAMP through G_s. As a result of the identification of receptors specifically for H3 relaxin,

H2 relaxin is now considered the primary ligand for the RXFP1 receptor. Interestingly, INSL5 has only recently

been identified as the true ligand for the RXFP4 receptor (Liu et al., JBC, in press), leaving the RXFP3 receptor as

the true H3 relaxin receptor.

The RXFP1 and RXFP2 receptors (LGRs) have homology with the gonadotropin and thyrotropin receptors, with

distinctively large extracellular domains containing LRRs, important for the binding of glycoprotein hormones

(Kobe and Deisenhofer, 1993). The RXFP1 and RXFP2 receptors have 60% sequence identity (Hsu et al., 2002) and

are further distinguished from other glycoprotein hormone receptors by a unique low density lipoprotein receptor-

like cysteine-rich motif (LDL class A module) at the amino terminus (Hsu, 2003). The high degree of sequence

similarity between the two receptors has allowed the construction of RXFP1/RXFP2 receptor chimeras, enabling the

identification of functional receptor domains (Sudo et al., 2003). Specific areas of these receptors have been

postulated to be involved in the binding, and mediation of action, of relaxin family peptides (Sudo et al., 2003).

Recent research has since cemented this supposition: optimal interaction of H3 relaxin with the RXFP1 receptor was

found to require the presence of both the ectodomain and the exoloop 2 of the transmembrane region (Sudo et al.,

2003).

Here, we aimed to further define the pharmacological characteristics of the RXFP1 and RXFP2 receptors through the use of chimeric receptor constructs and a variety of relaxin family peptides. We have exploited the promiscuous nature of relaxin peptides for the RXFP1 and RXFP2 receptor to demonstrate that both the ectodomain and transmembrane regions of the receptors are required for optimal binding and signal transduction. Importantly the studies reveal for the first time a two-site binding model for the RXFP2 receptor, and confirm a two-site model at the RXFP1 receptor. The binding and cAMP-signalling characteristics of relaxin family peptides at the RXFP1, RXFP2 and chimeric receptor constructs are also defined.

Materials and Methods

Hormones and Reagents

Recombinant H2 relaxin was kindly provided by BAS Medical (San Mateo, California, USA); Porcine relaxin (native) was obtained from the Howard Florey Institute; Rhesus monkey relaxin, Rat relaxin and human INSL3 were chemically synthesised by Dr John Wade at the Howard Florey Institute, Melbourne, Australia.

Receptor Constructs

Native RXFP1 and RXFP2 receptors were examined in parallel with a number of chimeric, ectodomain only and transmembrane only receptors. Chimeric receptor constructs utilised included RXFP1/2 (ectodomain of RXFP1 fused with transmembrane region of RXFP2) and RXFP2/1 (ectodomain of RXFP2 fused with the transmembrane region of RXFP1), as previously described (Sudo et al., 2003). Ectodomain-only receptor constructs included RXFP1-BP (7BP as previously described by Hsu 2002) and an ectodomain only RXFP2 receptor, RXFP2-BP, which was constructed in an identical manner to the RXFP1-BP construct (Hsu et al., 2002). In brief, the ectodomain of human LGR8 was fused to the transmembrane and cytoplasmic region of CD8 through a stretch of the thrombin receptor sequence containing the thrombin cleavage site. Furthermore, the signal peptide was replaced with the bovine prolactin signal peptide fused to a FLAG epitope at the N-terminus and a six Histidine Tag was added between the thrombin cleavage site and the C-terminus of the LGR8 ectodomain. The transmembrane-only constructs RXFP1-TM and RXFP2-TM were made starting with the GISS sequence just outside the first transmembrane domain. For each construct the bovine prolactin signal peptide fused to a FLAG epitope was inserted onto the N-terminus using overlap PCR. Cell surface expression was tested using the FLAG epitope (Sudo et al., 2003).

Cell Culture

HEK293T cells (ATCC #CRL-1573, ATCC, Manassas, VA) were maintained in RPMI 1640 supplemented with heat-inactivated foetal bovine serum (10% v/v), penicillin (100 units/mL), streptomycin (100µg/mL), L-glutamine (2mM, all Trace Biosciences, Sydney, Australia), and zeocin (2mg/mL for stable cell lines, Invitrogen, Vic, Australia). All tissue culture plates and flasks were coated with poly-L-lysine (0.1 mg/mL, Sigma-Aldrich, Sydney,

Australia) prior to 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.

HEK293T cells stably expressing the RXFP1 and RXFP2 receptors were used as previously described (Sudo et al., 2003).   Transient transfections were performed using LipofectAmine (Invitrogen, Vic, Australia) as per manufacturer's instructions. Cells transiently expressing the receptors were used 48hrs after transfection.

96-Well Plate Ligand Binding Assay

Whole-cell ligand binding assays were conducted in 96-well plates pre-coated with Sigmacote™ (Sigma-Aldrich, Sydney, Australia) to prevent the non-specific binding of relaxin to the plate surface. [^{33}P]-H2 relaxin (Sudo et al., 2003), unlabelled peptides and whole cell re-suspension, was made up to 100µL/well with binding buffer (20mM HEPES, 1.5mM $CaCl_2$, 50mM NaCl, 1% BSA, 0.1mg/mL lysine, 0.01% NaN_3, pH 7.5). After 90min incubation at 25°C, cells were harvested onto GF/C filter plates (Skudtek, Vic, Australia) and dried prior to addition of Microscint O (PerkinElmer, Vic, Australia). Filters were counted on a Topcount Scintillation Counter (PerkinElmer, Vic, Australia) and the data analysed using a GraphPad PRISM (GraphPad Software, San Diego, CA) non-linear regression one-site binding model to obtain pK_i (competition binding) and pK_D and B_{max} (saturation binding) values. Values were expressed as means ± standard error of the mean of 4-5 observations.

Saturation binding studies used [^{33}P]-H2 relaxin (50pM – 2.5nM) to determine pK_D and B_{max} values. Non-specific binding was defined by H2 relaxin (10µM). Duplicate counts were normalised for protein content (Lowry et al., 1951) in each whole-cell re-suspension. Competition binding studies used [^{33}P]-H2 relaxin (100pM) for RXFP1, RXFP1/2 and RXFP2/1, and 100pM or 1nM for RXFP2.   Competition studies with the transmembrane-only constructs RXFP1-TM and RXFP2-TM used a radioligand concentration of 5nM. Non-specific binding was determined using H2 relaxin (10µM) for RXFP1, RXFP1/2 and RXFP2/1, and INSL3 (10µM) for RXFP2. Relaxin family peptides (H2 relaxin, Porcine relaxin, Rhesus monkey relaxin, Rat relaxin and INSL3) were used within the range of 1pM – 100nM.

24-Well Plate Ligand Binding Assay

Whole-cell binding assays were also conducted in a 24-well plate format. Media was removed, and cells washed with phosphate buffered saline (PBS) before pre-incubation in binding buffer. Binding studies were performed with [^{33}P]-H2 relaxin and competitor peptides or blank in binding buffer at 25°C for 60min (final volume 200μL). [^{33}P]-H2 relaxin concentrations, non-specific binding and binding buffer were as for the 96-well plate ligand binding assay above. After incubation, cells were washed with PBS, digested with 500μL 1M NaOH, transferred to scintillation vials and counted in a liquid scintillation counter (Packard1900 TR). Data are expressed as mean ± standard error of the mean of the percentage of specific binding of triplicate determinations performed 3-5 times. Data were analyzed using a GraphPad PRISM (GraphPad Software, San Diego, CA) one-site binding model.

cAMP Accumulation Assay

Functional cAMP responses were determined using the AlphaScreen^{TM} cAMP Accumulation Assay (PerkinElmer, Vic, Australia). Cells were seeded into 96-well plates (5x10^4/well), incubated with stimulation buffer (Hank's Balanced Salt Solution - HBSS: 13mM NaCl, 5.1mM KCl, 0.77mM MgSO$_4$.7H$_2$O, 1.23mM CaCl$_2$.2H$_2$O, 0.32mM Na$_2$HPO$_4$.2H$_2$O, 0.42mM KH$_2$PO$_4$, 5.27mM D-glucose; 1mg/mL BSA, 0.5mM IBMX, 5mM HEPES; pH 7.4) containing peptides, forskolin (100μM, Sigma-Aldrich, Sydney, Australia) or blank for 30mins at 37°C. All reactions were performed in duplicate. Stimulation buffer was removed and the cells frozen in lysis buffer (1mg/mL BSA, 0.3% Tween-20, 5mM HEPES; pH 7.4) at -80°C. Samples were transferred to a 384-well white plate (PerkinElmer, Vic, Australia). Anti-cAMP acceptor beads were added to all samples in the dark and allowed to incubate for 30mins at room temperature before addition of donor beads-biotinylated cAMP. Plates were detected the following day using a Fusion^{TM}-α Microplate Reader (PerkinElmer, Vic, Australia) and the data analysed against a cAMP standard curve using GraphPad PRISM (GraphPad Software, San Diego, CA). Samples were normalized for cell number and the results expressed as a percentage of the 100μM forskolin response. Each value was performed in duplicate and expressed as means ± standard error of the mean of 4-5 observations.

Results

RXFP1 and RXFP2 Receptor Two-Site Binding

Saturation binding studies indicated two binding sites at the RXFP2 receptor, as determined by iterative curve fitting and shown as Scatchard plots (figure 1A). This was confirmed by analysis of the binding characteristics of the ectodomain-only receptor, RXFP2-BP, which revealed the presence of only one site illustrated as a Scatchard plot in figure 1B. The two RXFP2 receptor sites had significantly different affinities, with pK_D values (table 1) of 9.24±0.30 (higher affinity site) and 8.35±0.10 (lower affinity binding site) ($p<0.001$). Although saturation studies failed to identify two binding sites at the RXFP1 receptor, the pK_D (9.26±0.22; table 1) resembled that of the high affinity RXFP2 receptor site (9.24±0.30). The two chimeric receptors displayed similar pK_D values (table 1): The RXFP1/2 receptor with a pK_D of 9.57±0.18 and the RXFP2/1 receptor with a value of 9.33±0.12. The ectodomain-only constructs exhibited pK_D values that closely resembled those of the high affinity sites displayed by the parent receptor and in addition, RXFP1-BP had a pK_D very similar to the RXFP1/2 chimera, while RXFP2-BP had a pK_D value very similar to the RXFP2/1 chimera.

RXFP1 Receptor Characteristics

In HEK293T cells transiently expressing the RXFP1 receptor, H2 relaxin, Porcine relaxin, Rhesus monkey relaxin and Rat relaxin effectively competed for [^{33}P]-H2 relaxin binding (figure 2, table 2; not all data shown graphically). INSL3 bound only weakly to the RXFP1 receptor at high concentrations. The rank order of binding affinity at the RXFP1 receptor was H2 relaxin = Rhesus monkey relaxin > Porcine relaxin > Rat relaxin >>> INSL3. At the RXFP1/2 chimera, although the rank order of affinity was the same as the RXFP1 receptor, individual affinities of some peptides for the receptor changed. H2 relaxin had significantly decreased affinity for the RXFP1/2 receptor ($p<0.01$), as did Rhesus monkey relaxin ($p<0.001$), while Rat relaxin and INSL3 both had significantly increased affinity at the RXFP1/2 receptor ($p<0.05$; $p<0.01$ respectively).

Interestingly, when the affinities of H2 relaxin, H3 relaxin and INSL3 at the RXFP1 receptor are compared to the affinities of the same peptides for the RXFP1 ectodomain-only receptor (RXFP1-BP), differences are observed (figure 3, table 3). The affinity of H2 relaxin was significantly decreased at RXFP1-BP ($p<0.05$), H3 relaxin behaved in the same manner as at the RXFP1 receptor, while the affinity of INSL3 was significantly increased

10

(p<0.05). H2 relaxin and INSL3 behave in a similar manner at RXFP1/2 and RXFP1-BP but not at the native

RXFP1 receptor (Table 2). H2 relaxin binding was also examined in the RXFP1-TM construct. Unlabelled H2

relaxin (10^{-8}M) but not INSL3 (up to 3×10^{-8}M) competed with specific H2 relaxin binding (figure 4A).

The functional characteristics of the RXFP1 receptor were also examined (figure 5, table 4). The ability of the

peptides to produce cAMP was similar to their respective binding affinities, and the rank order of potency was

identical with H2 relaxin = Rhesus monkey relaxin > Porcine relaxin > Rat relaxin >>> INSL3. When the response

of the RXFP1/2 chimera was compared to that of the RXFP1 receptor, the rank order of potency was the same,

although Rhesus monkey relaxin and Rat relaxin had significantly decreased potency (p<0.01; p<0.001 respectively)

and INSL3 was able to produce a response at RXFP1/2. Cells expressing RXFP1-TM produced a normal cAMP

response to forskolin (10µM) but there was no detectable response to either H2 relaxin or INSL3 (10^{-10}-10^{-6}M).

RXFP2 Receptor Characteristics

In HEK293T cells transiently expressing the RXFP2 receptor, addition of H2 relaxin, Porcine relaxin, Rhesus

monkey relaxin and INSL3 caused concentration-dependent competition for [^{33}P]-H2 relaxin binding (figure 2, table

2; not all data shown graphically). The rank order of affinity was INSL3 > H2 relaxin > Porcine relaxin = Rhesus

monkey relaxin >> Rat relaxin (no binding). When the affinity of the peptides for the RXFP2 receptor was

compared to their affinity at the RXFP2/1 chimera, the rank order of affinity was the same. The affinities of H2

relaxin and INSL3 were significantly increased at the chimeric receptor compared to the RXFP2 receptor (p<0.001,

p<0.05 respectively).

In the presence of 1nM [^{33}P]-H2 relaxin (identifies more of the low affinity sites), the rank order of binding affinity

altered such that INSL3 > H2 relaxin = Porcine relaxin > Rhesus monkey relaxin >> Rat relaxin. Additionally, the

affinity of INSL3 for the receptor was significantly increased (p<0.01). Although affinity of H2 relaxin, Rhesus

monkey relaxin and Rat relaxin did not significantly change, Rhesus monkey relaxin competed to a lesser extent than

at the lower concentration of [^{33}P]-H2 relaxin (100pM), and Rat relaxin gained some ability to compete.

Competition experiments conducted at RXFP2-BP with H2 relaxin, Rhesus monkey relaxin and INSL3 revealed a profile that resembled the RXFP2 receptor identified with [^{33}P]-H2 relaxin (100pM, figure 3, table 3). The affinity of H2 relaxin was significantly increased at RXFP2-BP compared to the RXFP2 receptor ($p<0.05$), while INSL3 and Rhesus monkey relaxin had similar affinities. The pK$_D$ values for RXFP2-BP and the high affinity RXFP2 site were similar. Both H2 relaxin (10-8M) and INSL3 (10^{-8} & 3×10^{-8}M) competed for H2 relaxin binding to RXFP2-TM (figure 4B).

cAMP accumulation studies were also conducted at the RXFP2 receptor (figure 5, table 4). Again the ability of the peptides to produce cAMP accumulation was related to their binding affinity and the rank order of potency was INSL3 > H2 relaxin > Porcine relaxin > Rhesus monkey relaxin >> Rat relaxin (no response). The rank orders of potency at RXFP2 and the RXFP2/1 chimera were the same. Interestingly however, the maximum response to INSL3 was significantly decreased from 13.93% (of forskolin stimulation) at RXFP2 to 11.69% at RXFP2/1 ($p<0.05$). Cells expressing RXFP2-TM produced a normal cAMP response to forskolin (10μM) but there was no detectable response to H2 relaxin or INSL3 (10^{-10}-10^{-6}M).

Discussion

We have examined the pharmacology of the RXFP1 and RXFP2 receptors. At the RXFP1 receptor, the rank order of potency H2 relaxin=Rhesus monkey relaxin>Porcine relaxin>Rat relaxin>>INSL3 was observed for both binding and cAMP signalling. At the RXFP2 receptor, although the rank order of potency INSL3>H2 relaxin>Porcine relaxin=Rhesus monkey relaxin>>Rat relaxin (none) was observed for binding, Porcine relaxin was more potent than Rhesus monkey relaxin for cAMP signalling. Interestingly, interaction at the RXFP2 receptor (or the rat ortholog, data not shown) is a species-specific event not observed for Rat relaxin. This observation may have important implications for the biology of the INSL3-RXFP2 receptor pairing, especially as most of the biology of INSL3 and RXFP2 receptors has been determined in rodents where there is no interaction between relaxin and RXFP2. The current study also demonstrates, for the first time, a primary high-affinity site and a second lower-affinity site at the RXFP2 receptor. Although previous studies provided evidence for two binding sites at the RXFP1 receptor (Sudo et al., 2003) these were not observed here in saturation studies. However, the behaviour of chimeric, ectodomain-only and transmembrane-only receptors in competition binding and cAMP accumulation studies confirmed their presence, suggesting closer similarity in site affinity than at the RXFP2 receptor. The general location of the two sites was also determined. The similar pK_D values for RXFP2-BP and the high affinity RXFP2 receptor site suggested that the high-affinity site was located in the ectodomain. Confirmation was obtained by examination of INSL3 binding characteristics. INSL3 exhibited high-affinity binding at both the RXFP2 and RXFP2/1 receptors, but only poor binding at the RXFP1/2 chimera. This suggested the presence of a high affinity binding site on the ectodomain of the RXFP2 receptor (figure 6A). The low affinity binding of INSL3 at the RXFP1/2 chimera supported a low-affinity RXFP2 receptor site only, as INSL3 binds only weakly to the RXFP1 receptor at high concentrations. This conclusion was supported by experiments with the transmembrane-only constructs that bound H2 relaxin. Binding was competed for by H2 relaxin and INSL3 with pharmacological properties appropriate for each receptor. Thus the low-affinity site appears to be located upon the transmembrane segment of the receptor.

In order to further differentiate between the RXFP2 receptor sites, two concentrations of $[^{33}P]$-H2 relaxin were used, based on the pK_D values for each site. Low concentrations (100pM) should identify primarily the high affinity site, whereas higher concentrations (1nM) should also identify a greater proportion of the low affinity sites. Peptides (H2, Rhesus monkey and Porcine relaxins) exhibited similar or decreased binding in competition with the higher ligand

13

concentration, confirming the presence of an additional site. Interestingly, Rat relaxin appeared to compete weakly at the higher ligand concentration, suggesting some affinity for the low affinity RXFP2 receptor site (no binding at high affinity site). INSL3 had significantly increased binding affinity in the presence of the higher ligand concentration, suggesting that this ligand binds with high affinity to both RXFP2 receptor sites ($p<0.01$).

The behaviour of INSL3 at the RXFP2 receptor warranted closer examination. Although INSL3 had the highest binding affinity ($p<0.01$ versus all peptides tested), H2 relaxin produced the greatest maximum cAMP response ($p<0.05$). Thus, INSL3 behaved as a partial agonist. Although this is a novel conclusion, other published studies contain similar examples of this phenomenon. For the RXFP1 and RXFP2 receptors, a study of cAMP accumulation to the INSL4 peptide (Lin et al., 2004), also examined H2 relaxin and INSL3 and showed a greater cAMP response to the former. Similar results were obtained with other signalling assays in our laboratories (data not shown), again suggesting that INSL3 behaves as a partial agonist. INSL3 also behaved as a partial agonist at the RXFP1/2 chimera. Although the maximum cAMP response was small, INSL3 had quite high potency.

Differences in the INSL3 response were also noted for the two RXFP2 receptor sites in cAMP accumulation assays. These are associated with the RXFP2 receptor structure in the chimeras. As INSL3 does not bind to the RXFP1 receptor at the concentrations tested, signalling at the RXFP1/2 chimera can only occur through the available RXFP2 receptor site, the low affinity site on the transmembrane domain. The INSL3 cAMP response at the RXFP1/2 chimera is therefore relatively small when compared to the RXFP2 receptor. At the RXFP2/1 chimera, cAMP accumulation was decreased compared to the RXFP2 receptor, consistent with INSL3 binding solely to the high-affinity site on the ectodomain. Thus, binding to both RXFP2 receptor sites is required for optimal cAMP signal transduction. Similarly, Rat relaxin exhibited decreased responses at the RXFP1/2 chimera compared to the RXFP1 receptor, and no response at the RXFP2/1 chimera. Therefore, the high-affinity binding site couples to cAMP signalling with high efficiency, while the low-affinity site couples with lower efficiency.

The ectodomain has the major influence upon receptor behaviour, consistent with this area containing the primary, high-affinity binding site. As chimeric receptors show altered behaviour compared to RXFP1 and RXFP2 receptors, the transmembrane region appears to have a small role in optimising ligand binding and signal transduction. The

experiments with the transmembrane-only constructs showed that although able to bind ligand this domain alone was unable to cause signal transduction that clearly requires an intact ectodomain. Interaction between the two receptor domains was clearly illustrated by Rat relaxin binding to the RXFP1 receptor. Since this relaxin does not bind to the RXFP2 receptor, affinity and efficacy changes were due only to variations in RXFP1 receptor site availability. Rat relaxin had decreased affinity at the RXFP1/2 chimera compared to the RXFP1 receptor. This suggested that although the ectodomain plays the primary role in Rat relaxin binding, the transmembrane region of the RXFP1 receptor has an influence upon the strength of ligand interaction. This was reiterated by cAMP accumulation responses: Rat relaxin had decreased potency at the RXFP1/2 chimera compared to the RXFP1 receptor ($p<0.001$). The combined influence of the two receptor domains upon the strength of ligand binding has been previously demonstrated for the H3 relaxin-RXFP1 receptor interaction (Sudo et al., 2003). The influence of the transmembrane site was further reiterated using ectodomain-only receptor constructs. Whether the relative affinity of the ectodomain site increased or decreased in the presence of the transmembrane site was dependent upon the peptide involved. At the RXFP1 receptor, H2 relaxin had increased affinity ($p<0.05$), H3 relaxin affinity remained unchanged and INSL3 had decreased affinity ($p<0.05$) compared to RXFP1-BP. Similar trends occurred between the RXFP2 receptor and RXFP2-BP. Most interestingly, INSL3 was able to bind well to RXFP1-BP, however binding was abolished in the presence of the transmembrane region (RXFP1 and RXFP1/2 receptors).

The varying affinity of peptides has suggested that ligand structure influences the degree of receptor binding and activation. Despite low amino-acid sequence conservation between relaxin peptides, some features remain invariant (figure 7). Most importantly, the motif Arg-XXX-Arg-XX-Ile/Val located within the B-chain (Schwabe et al., 1976; Bullesbach and Schwabe, 2000), is required for relaxin bioactivity and high affinity RXFP1 receptor binding. The most potent relaxins (Porcine and H2) have identical features surrounding this motif (Cys-B11—Arg-B17) (Bullesbach and Schwabe, 1991) and, together with Rhesus monkey relaxin, have identical structure between the two arginines (Arg-B13—Arg-B17). These peptides also exhibited the highest binding affinity and signalling efficacy at the RXFP1 receptor. However, Rat relaxin and INSL3, both of which had lower-affinity at the RXFP1 receptor, have different residues between the binding arginines. In the case of INSL3, the RXFP1 receptor binding motif is reduced to Arg-XXX-Arg and displaced toward the C-terminus by four residues (Büllesbach and Schwabe, 1995). Consequently, INSL3 only had low affinity for the RXFP1 receptor, reiterating the importance of binding motif

location. Thus although the arginines in the binding motif are essential for activity (Bullesbach and Schwabe, 1991),

additional residues surrounding this area may play important roles in the strength of ligand-receptor interactions.

In terms of the RXFP2 receptor, a distinct motif (Gly-Gly-Pro-Arg-Trp) was identified at the INSL3 B-chain C-

terminal (Büllesbach and Schwabe, 1999) which was involved in INSL3 binding. The tryptophan residue is essential

for receptor binding and the proline residue is likely important for maintaining its correct conformation (Büllesbach

and Schwabe, 2004). The tryptophan residue is conserved in most relaxin peptides except Rat relaxin and may

explain the lack of activity of Rat relaxin on the RXFP2 receptor. However, this tryptophan alone is not enough for

receptor binding as H3 relaxin (Sudo et al. 2003) and INSL6 (Bogatcheva et al., 2003) that contain this residue in the

correct position do not bind to the RXFP2 receptor. Furthermore Rhesus monkey relaxin, which does bind to the

RXFP2 receptor, albeit with lower affinity, also does not contain this tryptophan residue. The relaxin motif may also

play a role in high affinity RXFP2 receptor binding – peptides that bound with high affinity (INSL3, H2, Porcine and

Rhesus monkey relaxins) contained Arg-Ala/Glu-Leu-Val-Arg of the RXFP1 binding motif. Rat relaxin and H3

relaxin did not, and did not bind to the RXFP2 receptor, suggesting that relaxin-RXFP2 receptor interactions are

species-specific. Studies utilising peptide chimeras may isolate the important structural areas in receptor-peptide

interactions.

Results from this study, and previous work (Sudo et al., 2003), provide evidence for a two-stage binding model. In

this model (figure 6B), the peptide initially binds to the high-affinity LRR site to form a structure important for

receptor-ligand interaction. This binding leads to disruption of the constraint upon the transmembrane region, such

that the peptide is able to interact with the low-affinity site. Thus the primary high-affinity binding site directs the

receptor response, but subsequent ligand association with the lower-affinity site is required for full receptor

activation and stabilisation. Studies with the transmembrane-only receptors showed that although they bind

radioligand the ectodomain is necessary for the cAMP response.

This study has identified two binding sites on the RXFP2 receptor and has confirmed the presence of analogous sites

on RXFP1. The high-affinity binding site for both RXFP1 and RXFP2 receptors is located on the ectodomain,

likely within the LRR region. This site has primary influence over receptor binding and signal transduction. The

lower-affinity site is contained within the transmembrane region. Binding and signalling differences between relaxin family peptides suggest an influence of amino-acid sequence surrounding the binding motif, or the presence of an additional binding motif responsible for interaction with the low-affinity site. Further, we have provided evidence for a two-stage model of receptor activation.

Acknowledgements

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Figure Legends

Figure 1. Saturation binding data expressed as Scatchard plots for RXFP2 (A) and RXFP2-BP (B). Each graph is a representative plot. Experiments were performed 5-7 times in duplicate/triplicate.

Figure 2. Competition between H2 relaxin (■), Rat relaxin (○) and INSL3 (●) for [^{33}P]-H2 relaxin binding at RXFP1 (A), RXFP2 (B), RXFP1/2 (C), RXFP2/1 (D) and RXFP2 (1nM labelled-H2 relaxin) (E). Peptides competed against either 100pM [^{33}P]-H2 relaxin (A-D) or 1nM [^{33}P]-H2 relaxin (E). Symbols represent means, and vertical bars standard errors of the mean of 4-5 different experiments.

Figure 3. Competition binding (24-well plate format) between H2 relaxin (■), H3 relaxin (□), INSL3 (●) and Rhesus monkey relaxin (◇) for [^{33}P]-H2 relaxin binding at RXFP1 (A), RXFP2 (B), RXFP1-BP (C) and RXFP2-BP (D). Peptides competed against 100pM [^{33}P]-H2 relaxin. Symbols represent means, and vertical bars standard errors of the mean of 3-4 different experiments.

Figure 4. Competition binding between H2 relaxin or INSL3 for H2 relaxin binding (5nM) to the receptor transmembrane domains RXFP1-TM (A) and RXFP2-TM (B) transiently expressed in HEK293 cells. Histograms represent means, and vertical bars standard errors of the mean for 2-3 different experiments conducted in duplicate.

Figure 5. Ligand-stimulated cAMP accumulation responses mediated through RXFP1 (A), RXFP2 (B), RXFP1/2 (C) and RXFP2/1 (D). Ligands expressed graphically are H2 relaxin (■), Rat relaxin (○) and INSL3 (●). cAMP accumulation is expressed as a percentage of the forskolin (10μM) response for each receptor. Symbols represent means, and vertical bars standard errors of the mean of 4-5 experiments.

Figure 6. Proposed model of RXFP1/RXFP2 receptor binding and activation. A) Proposed locations of the two binding sites found on both RXFP1 and RXFP2. The primary high-affinity site is located on the ectodomain of the receptor (dotted-line box), likely within the leucine-rich repeat region. The lower-affinity binding site is

24

located within the transmembrane region (solid-line box). B) Initially the receptors are thought to reside in an

inactive conformation (1). Relaxin/INSL3 can then bind to both the primary high affinity ectodomain site and

the lower affinity transmembrane binding site, changing the conformation of the receptor (2).

Figure 7. Amino acid sequences of the relaxin family peptides used in this study: H2 relaxin, H3 relaxin,

Porcine relaxin, Rhesus monkey relaxin, Rat relaxin and INSL3. The RXFP1 binding motif is shaded, and the

RXFP2-INSL3 binding motif is circled and indicated in italics. Residues in bold are conserved within the

binding motif regions. Boxed cysteine residues are those involved in disulphide bond formation, dotted lines

between boxed residues indicate the disulphide bonds themselves. **W** indicates the tryptophan implicated to be

important for RXFP2 activity.

Table 1. Receptor binding site affinity values for all receptor constructs utilised. The table classifies the

receptor construct and lists the relevant pK_D value for $[^{33}P]$-H2 relaxin binding. pK_D values are expressed as

means ± the standard error of the mean of 5-7 different experiments. *** denotes $p<0.001$ versus high affinity

RXFP2 site (unpaired t-test). ^ denotes $p<0.05$ versus the low affinity RXFP2 site (unpaired t-test).

	Receptor Construct	pK_D
Native Receptors	RXFP1	$9.26\pm0.22^{\wedge}$
	RXFP2 (high affinity site)	$9.24\pm0.30^{\wedge}$
	RXFP2 (low affinity site)	$8.35\pm0.10^{***}$
Chimeric Receptors	RXFP1/2	$9.57\pm0.18^{\wedge}$
	RXFP2/1	$9.33\pm0.12^{\wedge}$
Ectodomain-only Constructs	RXFP1-BP	$9.78\pm0.20^{\wedge}$
	RXFP2-BP	$9.28\pm0.36^{\wedge}$

Table 2. Competition binding data showing pK$_i$ values for each tested relaxin family peptide at RXFP1, RXFP2 (100pM and 1nM labelled-H2 relaxin), RXFP1/2 and RXFP2/1. Values are expressed as means ± the standard error of the mean of four experiments. * denotes $p<0.05$, ** $p<0.01$, *** $p<0.001$ versus related RXFP1 value (unpaired t-test), ^ denotes $p<0.05$, ^^ $p<0.01$, ^^^ $p<0.001$ versus related RXFP2 value (unpaired t-test), n/a denotes no affinity.

		H2 relaxin	Porcine relaxin	Rhesus monkey relaxin	Rat relaxin	INSL3
RXFP1	100pM	9.35±0.04	8.56±0.12	9.39±0.02	7.27±0.18	n/a
RXFP2	100pM	8.30±0.15***	7.87±0.60	7.81±0.15***	n/a***	9.68±0.13*
	1nM	8.34±0.11	8.47±0.44	7.71±0.40	n/a	9.19±0.16^^
RXFP1/2	100pM	9.09±0.09**	8.54±0.17	9.09±0.02***	7.67±0.11*	8.57±0.57**
RXFP2/1	100pM	8.96±0.05^^^	8.89±0.24^	7.93±0.17	n/a	9.91±0.04^

Table 3. Competition binding data (24-well plate method) showing pK$_i$ values for each tested peptide at

RXFP1, RXFP2, RXFP1-BP and RXFP2-BP. Values are expressed as means ± the standard error of the mean

of 3-4 experiments. * denotes *p*<0.05 versus RXFP1 (unpaired t-test), u/t denotes untested.

	H2 relaxin	**H3 relaxin**	**INSL3**	**Rhesus monkey relaxin**
RXFP1	10.18±0.23	8.01±0.19	5.38±0.83	u/t
RXFP2	9.21±0.13	u/t	9.69±0.20	7.86±0.20
RXFP1-BP	9.53±0.25[*]	8.28±0.17	7.67±0.22[*]	u/t
RXFP2-BP	9.46±0.12	u/t	9.69±0.19	7.48±0.10

Table 4. cAMP accumulation data including pEC_{50}, Max (%F) (maximum values expressed as a percentage of 10μM forskolin stimulation) and Max (pmol) (total cAMP accumulation expressed as pmol produced per well) for each relaxin family peptide tested at RXFP1, RXFP2, RXFP1/2 and RXFP2/1. Values are expressed as means ± the standard error of the mean of four experiments. * denotes $p<0.05$, ** $p<0.01$ and *** $p<0.001$ versus the related RXFP1 value (unpaired t-test), ^ denotes $p<0.05$, ^^ $p<0.01$ and ^^^ $p<0.001$ versus the related RXFP2 value (unpaired t-test), n/r denotes no response.

		H2 relaxin	Porcine relaxin	Rhesus monkey relaxin	Rat relaxin	INSL3
RXFP1	**pEC_{50}**	9.57±0.57	9.09±0.18	9.76±0.38	7.70±0.10	n/r
	Max (%F)	19.52±1.93	17.14±3.84	17.67±4.07	18.34±3.99	0.04±0.06
	Max (pmol)	23.35±4.22	29.64±8.32	30.78±7.74	31.55±8.92	0.04±0.07
RXFP2	**pEC_{50}**	7.72±0.22***	8.05±0.29***	5.40±0.73***	n/r	8.16±0.27***
	Max (%F)	15.68±2.03*	13.58±5.17	10.28±2.96	n/r	15.58±1.48***
	Max (pmol)	19.23±5.35	26.16±11.42	12.73±3.74*	0.19±0.01**	13.93±3.36***
RXFP1/2	**pEC_{50}**	9.20±0.53	8.84±0.17	8.91±0.24**	6.58±0.16***	8.50±0.89***
	Max (%F)	17.11±3.29	18.34±2.56	16.98±2.89	19.41±3.55	3.49±1.57*
	Max (pmol)	15.54±3.89	17.39±2.24	15.13±3.23*	17.87±3.05	2.20±0.29***
RXFP2/1	**pEC_{50}**	8.19±0.15^	8.03±0.11	6.31±0.50	n/r	9.01±0.54^
	Max (%F)	14.60±4.05	15.22±3.13	12.72±4.87	0.17±0.04^^^	11.69±1.65^
	Max (pmol)	15.29±2.72	16.85±0.66	11.24±3.34	1.33±1.19	11.50±2.24

Fig 1

Fig 2

Fig 3

Fig 5

Fig 6

A)

N-terminus

Primary, High Affinity
Binding Site

Low Affinity
Binding Site

C-terminus

B)

N-terminus

Relaxin/INSL3

N-terminus

Relaxin/INSL3

C-terminus

C-terminus

1. Inactive Conformation

2. Relaxin Binding to Two
Receptor Sites

Fig 7

B Chain

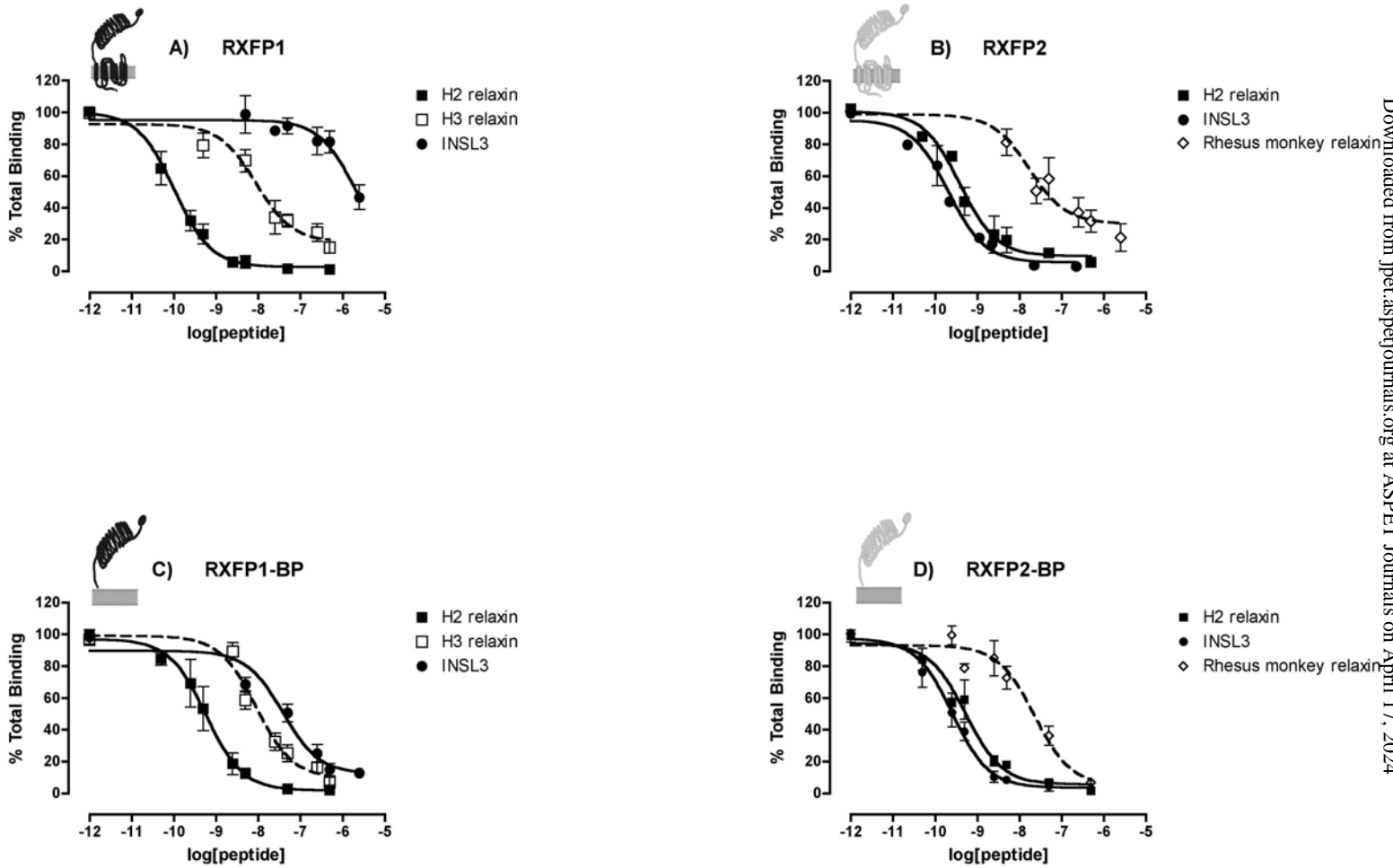|	
H2 relaxin	D S W M E E V I K L **C** G **R** E L V **R** A Q **I** A I **C** G M S T **W** S K R S L
H3 relaxin	R A A P Y G V R L **C** G **R** E F I **R** A V **I** F T **C** G G S R **W**
Porcine relaxin	Q S T N D F I K A **C** G **R** E L V **R** L W V E I **C** G S V S **W** G R T A L
Rhesus monkey relaxin	V A A K W M D D V I K A **C** G **R** E L V **R** A Q **I** A I **C** G K S T L G
Rat relaxin	R V S E E W M D Q V I Q V **C** G **R** G Y A **R** A W **I** E V **C** G A S V G R L A L
INSL3	L G P A P T P E M R E K L **C** G H H F V **R** A L V **R** V **C** *G G P R **W*** S T E A

A Chain

H2 relaxin	Z L Y S A L A N K **C C** H V G **C** T K R S L A R F **C**
H3 relaxin	D V L A G L S S S **C C** K W G **C** S K S E I S S L **C**
Porcine relaxin	R M T L S E K **C C** Q V G **C** I R K D I A R L **C**
Rhesus monkey relaxin	Z L Y M T L S N K **C C** H I G **C** T K K S L A K F **C**
Rat relaxin	Q S G A L L S E Q **C C** H I G **C** T R R S I A K L **C**
INSL3	A A A T N P A R Y **C C** L S G **C** T Q Q D L L T L **C** P Y