Negative cooperativity in H2 relaxin binding to a dimeric relaxin family peptide receptor 1

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Running title: Mechanism of H2 relaxin binding to RXFP1

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Abstract

H2 relaxin, a member of the insulin superfamily, binds to the G-protein-coupled receptor RXFP1 (relaxin family peptide 1), a receptor that belongs to the leucine-rich repeat (LRR)-containing subgroup (LGRs) of class A GPCRs. We recently demonstrated negative cooperativity in INSL3 binding to RXFP2 and showed that this subgroup of GPCRs functions as constitutive dimers. In this work, we investigated whether the binding of H2 relaxin to RXFP1 also shows negative cooperativity, and whether this receptor functions as a dimer using BRET^2. Both binding and dissociation were temperature dependent, and the pH optimum for binding was pH 7.0. Our results showed that RXFP1 is a constitutive dimer with negative cooperativity in ligand binding, that dimerization occurs through the 7TM domain, and that the ectodomain has a stabilizing effect on this interaction. Dimerization and negative cooperativity appear to be general properties of LGRs involved in reproduction as well as other GPCRs.

1. Introduction

Relaxin was first isolated in 1926 by Frederik Hisaw (reviewed in Ziel and Sawin, 2000). After a period of neglect, recent research has given many new insights about the hormones of this peptide family, their receptors and the importance of relaxin family peptide receptors as new drug targets (reviewed in Bathgate *et al.*, 2005b; Ivell *et al.*, 2005; Ivell and Bathgate, 2002; Dschietzig *et al.*, 2006; Van Der Westhuizen *et al.*, 2007).

Relaxin belongs to the insulin/relaxin superfamily of peptides which in humans comprises insulin, IGF-I and IGF-II, relaxins (H1, H2 and H3), and INSL3 – INSL6. H2 relaxin (which we will term relaxin from now on) was traditionally associated with pregnancy. It is synthesized in the corpora lutea of ovaries during pregnancy. Its physiological role appears to be species-specific (see Sherwood, 2004 and Dschietzig *et al.,* 2006, for recent reviews). In nonhuman mammals, relaxin is highest in the days before birth (Hudson *et al.*, 1983). Its major biological effect is to remodel the mammalian reproductive tract to facilitate the birth process (loosening of the pubic symphysis and relaxation of the cervix) (Ivell, 2002; Porter, 1972). Besides, relaxin also promotes the development of the mammary organs, thus enabling normal lactational performance. During pregnancy, relaxin inhibits uterine contractility and promotes the osmoregulatory changes of pregnancy in rats. In males, relaxin has been shown to be expressed in almost all parts of the male reproductive tract, with high levels in testis and vas deferens (Filonzi *et al.*, 2007). In humans, relaxin is at its highest in the first trimester of pregnancy. Its involvement in decidualization and in preterm premature rupture of the fetal membranes has been extensively studied by Bryant-Greenwood's group (see Bryant-Grenwood *et al.,* 2005, for recent review). Relaxin has also a number of nonreproductive actions (see Dschietzig *et al.,* 2006, for recent review).

Relaxin-3 is not involved in reproduction but is believed to be a putative neuropeptide involved in appetite regulation (McGowan *et al.*, 2005). Relaxin-3 is most likely the ancestor to the entire relaxin peptide family (Wilkinson *et al.*, 2005). INSL3, expressed in the testis and ovary, and its receptor RXFP2 were shown to initiate oocyte maturation and to suppress male germ cell apoptosis. The administration of an RXFP2 antagonist resulted in an increased germ cell apoptosis, suggesting that

INSL3 antagonists may have potential as novel contraceptive agents (Del Borgo *et al.*, 2006). It has been proposed that INSL3 plays an important role in spermatogenesis as well as differentiation and maintenance of the male phenotype (reviewed in Ivell and Bathgate, 2002). In females, INSL3 has a role in the regulation of the oestrus cycle and possibly in follicular development, explaining the impaired fertility in INSL3 knockout mice (Nef and Parada, 1999). The functions of H1 relaxin and INSL4 to INSL6 remain unknown (Wilkinson *et al.*, 2005).

Relaxin binds and activates RXFP1, which is assumed to be the cognate receptor of relaxin. It is also able to bind with lower affinity to RXFP2, although the significance of this binding in vivo is unknown (Svendsen *et al.*, 2008). RXFP1 and RXFP2 belong to Type C LGRs (leucine-rich repeat containing G-protein coupled receptor) of the class A subtype of GPCRs (rhodopsin-like family). They are unique because they have N-terminal domains which have homology to the LDLa modules that constitute the ligand binding repeats found in the LDL receptor family (Scott *et al.*, 2006). The ectodomain of RXFP1 and RXFP2 functions as the primary ligand binding domain. Ligand binding leads to the activation of adenyl cyclase and the protein kinase A-dependent pathway in many target tissues (Bathgate *et al.*, 2005a; Hsu *et al.*, 2002).

There is increasing evidence that GPCRs are allosteric proteins (Springael *et al.*, 2007). Allosterism is a property displayed by many oligomeric proteins. The binding of a molecule at one site induces a change in the binding properties of another site of the protein. In the case of negative cooperativity, the receptor sites do not have a fixed affinity, rather, the affinity of the receptors decreases as a function of the occupancy of the receptor population and is usually measured by ligand-accelerated tracer dissociation in an infinite dilution procedure (De Meyts *et al.*, 1973). Negative cooperativity is a mechanism that increases the range of the effective concentrations of the ligands.

After negative cooperativity in ligand binding was demonstrated for the insulin receptor in the early seventies (De Meyts *et al.*, 1973), it was also demonstrated for the β_2-adrenergic (Limbird *et al.*, 1975) and TSH receptors (De Meyts, 1976), later found to be GPCRs. The homodimerization of the β_2-adrenergic receptor and the TSH receptor was subsequently established (Graves *et al.,* 1996; Angers *et*

al., 2000; Latif *et al.* 2001; Rapoport, 2007), and the link between homo- and heterodimerization and the existence of negative cooperativity in LGR receptors including the TSH receptor was recently demonstrated, confirming the earlier TSH receptor observations (Urizar *et al.*, 2005). Similar findings with chemokine receptors suggest that dimerization and negative cooperativity may be the rule rather than the exception among GPCRs (Springael *et al.*, 2005). The dimerization of receptors has been shown to have many physiological roles such as receptor maturation, regulation of ligand binding, G-protein selectivity, or internalization (reviewed in Terrillon and Bouvier, 2004), and therefore it is of importance to investigate this mechanism in any particular receptor/ligand system. The relevance of dimerization and allosterism for the physiological properties of GPCRs, and implications for drug design, has been recently reviewed (see Springael *et al.*, 2007). However, no data were available on the RXFP group of receptors involved in human reproduction until we recently showed that INSL3 binding to RXFP2 shows negative cooperativity, and that RXFP2 forms homodimers, as well as heterodimers with RXFP1 (Svendsen *et al.*, 2008).

In this study we investigated whether negative cooperativity and dimerization are general properties of the LGR class of GPCRs by investigating whether H2 relaxin binding to RXFP1 displays properties similar to those of INSL3 binding to RXFP2, and whether RXFP1 forms homodimers. Furthermore we have investigated in detail the binding kinetics of H2 relaxin binding to RXFP1. Earlier studies focused solely on the binding affinity while here we have also investigated in detail kinetic properties such as association/dissociation time, temperature-/pH dependence of association and dissociation, and dose response curves for negative cooperativity.

2. Materials and Methods

2.1. Chemicals and reagents

Cell culture reagents, FBS, transfection lipids and antibiotics were purchased from Invitrogen, Copenhagen, DK. Chemicals were purchased from Sigma, Copenhagen, DK. Recombinant H2 relaxin was kindly provided by BAS Medical, and synthetic H3 relaxin and INSL3 were provided by JD Wade.

2.2. Cell culture

HEK293 cells obtained from the European Collection of Animal Cell Cultures (Salisbury, UK) and HEK293T cells stably transfected with RXFP1 (HEK293T-RXFP1) (Bathgate *et al.*, 2006), were cultured in RPMI 1640 with 10% FBS, 2 mM L-glutamine and 100 U/ml of penicillin-streptomycin at 37ºC in a humidified atmosphere of 5% (v/v) CO_2. For the stable transfectants, zeocin (200 µg/ml) was added once a week. We used these cells as there are limited primary cell lines that have enough receptors to be able to adequately carry out binding studies. However, the cells used have also been used for the investigation of the signaling pathways of RXFP1 and RXFP2 (Halls *et al.*, 2005b; Halls *et al.*, 2006), suggesting that the cell system is suitable. Furthermore, these cells are routinely used for expression of both RXFP1, 2, and 3, and the native cells do not bind ^{125}I-ligands or respond to ligands with cAMP production, indicating that these cells have low endogenous levels of these receptors and possible splice forms which could interfere with the assays (Bathgate *et al.*, unpublished data).

2.3. Labeling of H2 relaxin

Recombinant H2 relaxin was iodinated as described before (Palejwala *et al.*, 1998), using a modification of the chloramine-T procedure.

2.4. Receptor binding assays

All experiments were performed three times in duplicate at 15ºC and in buffer with a pH of 7.6 unless otherwise stated. We chose 15ºC for the performance of the experiments instead of the physiological 37ºC because the kinetics might be too fast to measure any difference at physiological temperature. However, the experiments that could be performed at that temperature were done so. Furthermore the receptor/ligand complex might get internalized at 37ºC, complicating the interpretation of the binding data.

2.5. Cell concentration dependence of H2 relaxin binding to HEK293T-RXFP1 cells. HEK293T-RXFP1 cells with a density ranging from 5.0 x 10^4 cells/ml to 1.6 x 10^6 cells/ml were incubated with a

constant concentration of tracer at 15ºC for 3 hours. Duplicate aliquots were centrifuged and the bound activity counted. Two aliquots were not centrifuged but counted as total. This experiment has only been performed once to determine the cell range in which less than 20% of the tracer is binding, in order to minimize ligand depletion.

2.6. Association assays. ^{125}I-H2 relaxin and HEK293T-RXFP1 cells were incubated at 4, 15, 21 or 37ºC for different time intervals. Specific H2 relaxin binding was determined by centrifugation as previously described (Gavin *et al.*, 1973). Two additional aliquots were not centrifuged but counted as total.

2.7. pH dependence of equilibrium binding. ^{125}I-H2 relaxin and HEK293T-RXFP1 cells were incubated in HBB (Hepes Binding Buffer, 100 mM NaCl, 5 mM KCl, 1.3 mM MgSO4, 1 mM EDTA, 10 mM glucose, 15 mM NaOAc, 100 mM HEPES, 1% BSA, pH adjusted with NaOH or HCl, all from Sigma, USA) with pH values ranging from 5 to 10 in the absence or presence of unlabeled H2 relaxin at 15ºC for 3 hours. Duplicate aliquots were centrifuged and the bound activity counted. Two additional aliquots from each pH value were counted as total.
We are aware that it might be better not to use the same buffer for pH values ranging from value 5 to 10, but previous experiments in our laboratory measuring the binding affinity of insulin to the insulin receptor using either the same buffer or different buffers with a pH optimum at the pH values tested showed that in practice the choice of buffer had no influence on the pH curve.

2.8. pH dependence of dissociation. ^{125}I-H2 relaxin and HEK293T-RXFP1 cells were preincubated in HBB pH 7.6 at 15ºC. After two hours of incubation, the cells were resuspended in an equal amount of buffer. Duplicate aliquots were diluted 40 fold in HBB with pH values ranging from 5 to 10. After 7 hours of incubation, the bound activity was counted.

After the experiments the pH value of the buffers were measured and no change could be detected for pH 5 to 8. At pH 9 and 10 the pH value was slightly decreased (data not shown). Furthermore the cell

viability was tested. At pH 5 to 8, approximately 80% of the cells were alive after incubation time, whereas at pH 9 to 10, most cells were dead with a leaky cell membrane, possibly explaining the decrease in binding at these higher pH values.

2.9. Competition assays were done as described before (Gavin *et al.*, 1973). HEK293T-RXFP1 cells were incubated with a constant amount of ^{125}I-H2 relaxin and increasing concentrations of unlabeled ligand (H2 or H3 relaxin, or INSL3) at 15ºC for 3 hours. This was done in buffer with pH 7.0 or 7.6. Duplicate aliquots were centrifuged after incubation and the bound activity counted. Two additional aliquots were not centrifuged but counted as total.

2.10. Dissociation assays were performed as previously described for insulin and INSL3 (De Meyts *et al.*, 1973; Svendsen *et al.*, 2008). ^{125}I-H2 relaxin and HEK293T-RXFP1 cells were preincubated. After two hours of incubation, the cells were resuspended in an equal amount of buffer. Duplicate aliquots were diluted 40 fold in absence and presence of a constant amount of unlabeled H2 relaxin and incubated at 15ºC or 37ºC. After different time intervals, the cells were centrifuged and the bound activity counted. Two additional aliquots were not centrifuged but counted as total. In a second experiment, duplicate aliquots were diluted 40 fold with increasing concentrations of unlabeled ligand (H2 or H3 relaxin, or INSL3) for a constant amount of time. The experiments have been performed in buffer with a pH value of 7.6 for all ligands.

2.11. Vector construction

Human cDNAs of RXFP1 and RXFP1 TM1-7 (Bathgate *et al.*, 2006) were cloned into pcDNA3.1 (+/-) vectors (Invitrogen, Copenhagen, DK) containing either Rluc or GFP^2 inserts using standard molecular biology methods. The Rluc and GFP^2 inserts were fused in frame to the C-terminal of the receptors. The RXFP1 TM1-7 receptor is a truncated version of RXFP1 containing only the 7TM domain. The C-terminally GFP^2-tagged neurokinin type 1 receptor (NK1R) was generated (Svendsen *et al.*, 2008). All clones were verified by sequencing.

2.12. BRET^2 experiments

BRET^2 measurements were performed as described (Heding, 2004; Vrecl *et al.*, 2004; Vrecl *et al.*, 2006). For BRET^2 saturation experiments, HEK293 cells were cotransfected with a constant amount of vectors coding for Rluc-tagged receptors (1 μg) and increasing amounts of vectors coding for GFP^2-tagged receptors (0.1 to 5 μg) or with a constant amount of RXFP1 Rluc (1 μg) with increasing amounts of NK1R GFP^2 (0.05 to 5 μg; control experiment). The total amount of cDNA for transfection was kept uniform at 6 μg by adding empty vector. For BRET^2 competition assays, HEK293 cells were cotransfected with a constant amount of Rluc- and GFP^2-tagged receptors at a 1:2 cDNA ratio while increasing the amount of untagged receptor. 1 μg of Rluc- and 2 μg of GFP^2-tagged constructs were used and the amount of untagged construct varied from 0.5 to 5 μg. The total amount of cDNA used for transfection was kept uniform at 8 μg by adding empty vector. For the heterodimerization experiments, HEK293 cells were cotransfected with constant amounts of RXFP1 Rluc (1 μg) with increasing amounts (0.25 to 5 μg) of a construct devoid of the N-terminal hormone-binding domain (RXFP1 TM1-7 GFP^2).

Expression levels of Rluc- and GFP^2-tagged constructs for each BRET^2 experiment were monitored by luminescence and fluorescence measurements as described (Vrecl *et al.*, 2004).

3. Results and Discussion

3.1. Cell concentration dependence of H2 relaxin binding to HEK293T-RXFP1 cells

H2 relaxin was labeled with ^{125}I at position Tyr^{A3} as has been described before (Palejwala *et al.*, 1998). The labeling gave two batches (A and B) whereof batch A was oxidized at unknown position, most likely outside the binding cassette. Both batches showed similar affinity and could be used for the experiments (Figure 1). The binding of the tracer was a linear function of the cell concentration. As shown in Figure 1 there is a linear increase in the Bound/Free of ^{125}I-H2 relaxin over a 30-fold increase in cell concentration. The highest concentration of cells was additionally incubated with ^{125}I-H2 relaxin in the presence of 160 nM unlabeled H2 relaxin. The bound radioactivity in this experiment shows the nonspecific binding which was approximately 2% of total binding.

3.2. Temperature dependence of association and dissociation

The binding of ^{125}I-H2 relaxin to RXFP1 is time and temperature dependent, although the curves for 4, 15 and 21ºC are not statistically different ($p>0.05$, t-test). However, all curves are different from the curve at 37ºC ($p<0.05$, t-test) (Figure 2 A). Maximum binding occurs at 15ºC or at temperatures below. Steady state at 15ºC is reached after approximately 3 hours. Like the binding of insulin to the insulin receptor, the binding of H2 relaxin to RXFP1 is only slightly faster at higher temperatures and the maximum binding at 21ºC and 37ºC is less than at lower temperatures and is further diminished as a function of the duration of incubation (Gavin *et al.*, 1973). This suggests that the dissociation is much more temperature dependent than the association, which was indeed the case (Figure 2 B and C). At 15ºC, the dissociation by dilution alone is slow and is accelerated by the presence of unlabeled ligand; the accelerating effect of unlabeled ligand increases with time. At 37ºC, the dissociation of ^{125}I-H2 relaxin is faster and the accelerating effect starts immediately. However, after 1 hour of dissociation, both curves reach an identical horizontal asymptote showing that approximately 5% of the tracer is undissociable, possibly due to internalization. Kern and Bryant-Greenwood have recently shown that RXFP1 is indeed internalized upon ligand stimulation (Kern, A. and Bryant-Greenwood, G.D. The relaxin receptor (LGR7): desensitization and internalization in stably transfected HEK293 cells and primary chorionic cytotrophoblast; ENDO 2008, San Francisco, June 2008, abstract P3-97). Callander *et al.* have also investigated the internalization of both RXFP1 and 2 upon stimulation with ^{125}I-H2 relaxin and ^{125}I-INSL3, respectively (Callander, G.E., Thomas, W.G. and Bathagte, R.A.D., submitted for publication). They found that both receptors are internalized, but the internalization is never greater than 10%, supporting the concept that the undissociable 5% observed in our work may be due to internalization of the receptor. Alternatively, some of the RXFP1 may get sequestered within a so-called membrane microdomain. Latif *et al.* (2007) showed that an oligomeric form of the TSH receptor is preferentially localized in lipid microdomains on the plasma membrane. Further studies will have to be done to investigate if this is the case for RXFP1 and 2 as well.

The temperature dependence of H2 relaxin binding to RXFP1 is similar to that observed for insulin binding to the insulin receptor (De Meyts *et al.*, 1976), but is in contrast to that seen in the binding of INSL3 to RXFP2, where the ligand association gets much faster with increasing temperatures and

where all association curves reach the same level of equilibrium binding, suggesting that both association and dissociation are affected to the same degree by the increase of temperature (Svendsen *et al.*, 2008).

3.3. pH dependence assay

GPCR activity and function is regulated by a variety of mechanisms (reviewed in Ferguson, 2001). These mechanisms can act at the level of GPCR ligand specificity, G-protein activation, and effector regulation. But also GPCR desensitization and endocytosis can act as molecular switches coupling GPCRs to alternative signal transduction pathways. After internalization, the receptor/ligand complex can either be recycled or degraded, dependent on if the ligand sticks to the receptor or dissociates from it. This might depend on the pH of the surrounding environment. Therefore, the investigation of the pH dependence of association and dissociation could give valuable information about receptor activity regulation. Internalization upon ligand binding has been shown for a variety of GPCRs (Ferguson, 2001), among others for the TSH receptor (Singh *et al.*, 2004), the LH receptor (Ghinea *et al.*, 1992), and the FSH receptor (Piketty *et al.*, 2006).

The association of H2 relaxin binding to RXFP1 is slightly affected by pH with a bell-shaped curve with a pH optimum at about pH 7.0 (Figure 3 A). This indicates that some residues of H2 relaxin or of its cognate receptor must be charged for optimal binding (Waelbroeck, 1982). In this setup, the effect of pH in the range of 5 to 10 was investigated, and thus very strongly basic or acidic groups could not be determined, since it requires a pH range of 0 to 14 to detect all possible active groups. For insulin binding to the insulin receptor, a sharp pH optimum indicates that the ionization constants of the protonated and deprotonated groups are very close as the binding optimum is between pH 7.6 and 8.0 (Waelbroeck, 1982). However, H2 relaxin binding to RXFP1 has a wider pH optimum, suggesting that their pK values are farther apart. The binding optimum of INSL3 binding to RXFP2 was found to be pH 6.0 (Svendsen *et al.*, 2008). The dissociation rate was fastest at pH 5 and pH 10, the most acidic and most basic pH values (Figure 3 B). The slowest dissociation rate in presence of unlabeled ligand was observed around pH 6.5. In addition, the difference between absence and presence of ligand was markedly increased at neutral pH values, and less pronounced at the most basic and acidic pH values

tested, leading to the assumption that the effect of negative cooperativity is markedly decreased at these pH values. The dissociation rate of the insulin-IR complex in the high affinity state increased markedly at pH values lower than 7.8. The dissociation rate of the complex in the low affinity state (in the presence of unlabeled insulin) was increased only at pH values lower than 7.0 (Waelbroeck, 1982). For the binding of INSL3 to RXFP2, the opposite picture was seen. Here, the dissociation rate decreased at lower pH values both in the absence and presence of unlabeled ligand. No major difference between those two curves was seen (Svendsen *et al.*, 2008). The fact that the dissociation is markedly increased at pH values between 5 and 6 suggests that the ligand is dissociating from the receptor upon internalization, as the pH value in the endosome is about 5.5. This enables the receptor to recycle to the cell-membrane, whereas the ligand may be degraded. Further investigations will have to be performed to examine the exact mechanism. We make no quantitative assumptions of the slopes of the curves; it is the pH optimum for binding and dissociation that is important for the ligand/receptor complex stability in the endosome, which may play a role in the modulation of intracellular signaling pathways. It has indeed recently been shown for the insulin receptor that the pathways that get activated upon ligand binding depend on whether the insulin receptor is located on the cell surface or in the endosomal compartment (Jensen *et al.*, 2007).

3.4. Affinity of H2 relaxin/RXFP1 binding

Two K_d values were estimated by computer curve-fitting of competition curves of ^{125}I-H2 relaxin with unlabeled H2 relaxin (Figure 4). The K_d values were 0.33 ± 0.1 and 1.76 ± 2.29 nM respectively (at pH 7.6) using a sequential model and 0.41 ± 0.07 and 3.15 ± 1.85 nM at pH 7.0. Using a one site model, the value was estimated to be 0.68 ± 0.2 nM at pH 7.6 and 0.69 ± 0.02 nM at pH 7.0. The difference between the affinities at pH 7.0 and 7.6 is not statistically significant ($p>0.05$, t-test) (Figure 4 A). This is consistent with previous findings (Halls *et al.*, 2005a; Halls *et al.*, 2005b; Sudo *et al.*, 2003).

The K_d of H3 relaxin towards RXFP1 was much lower than the affinity of H2 relaxin (18.55 ± 3.9 nM) (Figure 4 B), as has been shown before (Sudo *et al.*, 2003). There is no competition with INSL3

at the highest concentration used, also consistent with previous findings (Sudo *et al.*, 2003) (Figure 4 B).

3.5. Negative cooperativity in H2 relaxin/RXFP1 binding

The presence of unlabeled ligand caused an enhancement of radioligand dissociation at both 15 and 37ºC ($p<0.05$) (Figure 2 B and C). The accelerating effect of the unlabeled ligand is observed within minutes at 37ºC and it is slightly slower at 15ºC. The dissociation of both insulin from the insulin receptor and TSH from the TSHR also show an immediate accelerating effect of the unlabeled ligand (De Meyts *et al.*, 1973; De Meyts *et al.*, 1976; Urizar *et al.*, 2005). This contrasts with the dissociation of INSL3 from RXFP2 (Svendsen *et al.*, 2008) and alprenolol from the β_2-adrenergic receptor (Limbird *et al.*, 1975), as well as FSH and LH from their receptors (Urizar *et al.*, 2005) where there is a slower onset of the accelerating effect. The dose-response curve for negative cooperativity shows that the degree of acceleration of dissociation increases as the concentration of the unlabeled ligand gets higher. The dose-response curve is monophasic, instead of bell-shaped (Figure 5) like insulin binding to IR (De Meyts and Whittaker, 2002) or INSL3 binding to RXFP2 (Svendsen *et al.*, 2008), but similar to what has been observed for IGF-I binding to the IGF-I receptor (De Meyts and Whittaker, 2002). The molecular mechanisms of these differences are difficult to establish in the absence of structures of the ligand-receptor complexes. H3 relaxin has a reduced potency for accelerated dissociation in comparison to H2 relaxin, consistent with its higher K_d value. As INSL3 is unable to compete for RXFP1, it also has no potency in the dissociation assay.

3.6. Evidence for RXFP1 homo- and heterodimerization by BRET^2

Homo- and heterodimerization of other members of class A GPCRs has been shown previously (Angers *et al.*, 2000; Latif *et al.*, 2001; Urizar *et al.*, 2005). We recently showed negative cooperativity for INSL3 binding to RXFP2 with a bell-shaped dose response curve for negative cooperativity and constitutive dimerization (Svendsen *et al.*, 2008). These findings suggest that RXFP2 functions as a dimer and that the ligand may bind to the high affinity site in one molecule of the dimer and to the low affinity site in the second molecule of the dimer ("trans" rather than "cis" binding mechanism). To

verify the possible dimerization of RXFP1 we performed BRET^2 experiments. Saturation curves were obtained by cotransfecting HEK293 cells with a constant amount of vectors coding for Rluc-tagged receptors and increasing amounts of vectors coding for GFP^2-tagged receptors both with and without stimulation by the ligand. The saturation curve for the RXFP1-RXFP1 homodimer showed a clear signal with a maximum level of approximately 130 mBU (Figure 6 A) with no change of the signal upon ligand stimulation. The role of the large ectodomain in the LGRs dimerization has been controversial (Fan and Hendrickson, 2005; Liang *et al.*, 2003; Moyle *et al.*, 2005). Recent research on the TSH receptor however has suggested that the 7TM domain is sufficient for dimerization and that the ectodomain plays only a minor role (Urizar *et al.*, 2005). The maximum level for the BRET^2 signal of the truncated form of the receptor was 70 mBU (Figure 6 B) versus 130 mBU for the full receptor, confirming the stabilizing function of the ectodomain. The heterodimerization curve (Figure 6 C) between the holoreceptor and RXFP1 construct devoid of the ectodomain (RXFP1 TM1-7) showed a saturation curve with a maximum level of 80 mBU, suggesting that both ectodomains are needed for stabilization. However, a change in the orientation could also be the cause for the lower signal. While this paper was being readied for publication, Kern *et al.* (2008) published a thorough paper also showing BRET data supporting the constitutive homodimerization of RXFP1, in agreement with our results. In addition, we here demonstrate also heterodimerization between the holoreceptor and a truncated form of the receptor, consisting of only the 7 TM domain, thereby determining the role of the ectodomain. Kern *et al.* (2008) did not study the kinetic properties of the ligand-receptor interaction.

The specificity of BRET experiments has been challenged (James *et al.*, 2006) and therefore, proper control experiments have to be done using this method for the detection of protein interactions. The BRET^2 signal necessarily increases with increasing abundance of GFP^2 associated to the plasma membrane. However, this curve would be linear and would only show a saturation level with all Rluc constructs close to GFP^2 constructs. We used neurokinin type 1 receptor as a negative control (Figure 7 A). The curve showed a very weak signal within the GFP/Rluc ratio at which the RXFP1 pair reached saturation. Furthermore, when constant amounts of RXFP1 Rluc and –GFP^2 were cotransfected with increasing amounts of untagged receptor (Figure 7 B), the BRET^2 signal decreased.

The WT receptor competes for the dimerization between the RXFP1 Rluc and RXFP1 GFP^2 and this is further evidence for the specificity of the interaction.

As was the case for INSL3 binding to RXFP2 (Svendsen *et al.*, 2008), the data suggests that RXFP1 is a constitutive dimer and that dimerization occurs through the 7TM domain. Although BRET^2 experiments alone may not be sufficient for the proposition that the receptor functions as a dimer, the combination of BRET^2 experiments with the binding studies strongly supports this concept together with previous findings on the binding mechanisms of RXFP2 and other GPCRs.

3.7. Mechanistic and physiological implications of our findings

It is difficult to provide a precise mechanistic interpretation of our observation that RXFP1 and 2 exhibit negative cooperativity, since no structure of the ligand-receptor complexes are available. Clearly, the fact that they exist as dimers provides a structural basis for allosteric site-site interactions. This appears to be a general property of GPCRs (Springael *et al.*, 2007) as well as several receptor tyrosine kinases (De Meyts, 2008). It took over three decades to unravel the structural basis of negative cooperativity at the insulin receptor (De Meyts and Whittaker, 2002; McKern *et al*., 2006; Ward *et al*., 2008), including a recent structure-based mathematical model (Kiselyov and De Meyts, submitted for publication). The recently published structure of the β_2-adrenergic receptor, which shows a crystal lattice of dimers (Cherezov *et al.,* 2007), hopefully ushers an era where more structures of ligand-GPCR complexes will become available.

It is interesting that most of the LGRs involved in human reproduction: RXFP1 and 2 (our work) as well as the gonadotrophin receptors (Urizar *et al.*, 2005) show negative cooperativity in the context of a receptor dimer. That dimerization is physiologically relevant was shown *in vitro* for both the LH (Lee *et al.,* 2002) and the TSH receptor (Urizar *et al.,* 2005) by complementation, by cotransfecting cells with two non-functional mutant receptors. That the dimerization is also relevant in the physiology of reproduction was recently elegantly demonstrated for the first time *in vivo* by Huhtaniemi's group (Rivero-Müller *et al*., 2008) who was able to rescue the LH receptor knockout

phenotype by complementation by co-expressing two non-functional receptor mutants in the knockout mice.

Negative cooperativity due to an accelerated dissociation at higher ligand concentrations provides a mechanism whereby the ligand residence time on the receptor becomes shorter as the ambiant ligand concentration increases. It has been shown that variable ligand residence times can allow selective activation of different signaling pathways, some of which may require sustained signaling from the receptor (Shymko *et al.*, 1997) and therefore may become extinguished if the residence time is shortened. Further work is needed to investigate the impact of negative cooperativity on RXFP signaling.

The observation that RXFP1 and 2 form heterodimers (Svendsen *et al.*, 2008) is also of potential physiological relevance, especially since both receptors appear to be widely co-expressed on various cell types of the male reproductive tract including late germ cells (Filonzi *et al.*, 2007). Heterodimerization may extend the pharmacological properties of GPCRs (Springael *et al.*, 2007). The recent implication of heterodimers between serotonin and glutamate receptors in schizophrenia underlines the potential importance of this mechanism (González-Maeso *et al.*, 2008). Agnati *et al.* (2005) have proposed that GPCRs form functional clusters or "mosaics" in which crosstalk between different kinds of GPCRs occur. It would be of interest to test if RXFPs have the ability to form heterodimers with other members of the LGR class, since it would raise the intriguing possibility of cross-regulation between relaxin and gonadotrophins.

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Figure legends

Figure 1: A) Association of ^{125}I-H2 relaxin (■ tracer A from Novo Nordisk; ▲ tracer B from Novo Nordisk; ● non-specific binding) to HEK293T-RXFP1 cells. The Bound/Free tracers were plotted as a function of the cell number. Only one experiment was performed.

Figure 2: A) Association of ^{125}I-H2 relaxin at 4 (■), 15 (▼), 21 (●) and 37ºC (▲). Bound/Total ^{125}I-ligand is plotted as a function of time. B) Dissociation of H2 relaxin from RXFP1 at 15ºC and C) at 37ºC with (closed symbols) and without (open symbols) addition of 167 nM unlabeled ligand. Bound tracer at time t divided by bound tracer at time 0 was plotted as a function of time.

Figure 3: pH dependence of steady state binding and dissociation.
A) Cell-associated radioactivity after 3 hours of incubation time was plotted as the logarithm of Bound/Free ligand as a function of pH. B) The logarithm of bound tracer after 7 hours/Bound tracer at time 0 was plotted as a function of the pH value. The curve with the open symbols shows dissociation in dilution only; the curve with closed symbols shows dissociation plus unlabeled hormone.

Figure 4: Competition assays.
A) Homologous competition assay with labeled and unlabeled H2 relaxin at pH 7.0 (▲) and 7.6 (■). B) Homologous competition assay with labeled and unlabeled H2 relaxin (■). Heterologous competition assay with labeled H2 relaxin and unlabeled H3 relaxin (▼) or INSL3 (▲). All curves are plotted as Bound/Total labeled H2 relaxin as a function of the logarithm of the concentration of unlabeled ligand.

Figure 5: Dose response curves for negative cooperativity.
A constant amount of labeled H2 relaxin is dissociated from RXFP1 as a function of increasing concentrations of unlabeled H2 relaxin (■). The same experiment was repeated with the same constant

amount of labeled H2 relaxin and increasing concentrations of unlabeled H3 relaxin (▼) or INSL3 (▲).

Figure 6: A) $BRET^2$ saturation curve for RXFP1. B) Saturation curve for the RXFP1 construct devoid of the ectodomain (RXFP1 TM1-7). C) Saturation curve for the heterodimerization between RXFP1 and RXFP1 TM1-7. All curves are obtained by stimulating the cells with 1.6 nM H2 relaxin (▼), 10 nM H2 relaxin (▲), or without stimulation (■).

Figure 7: A) $BRET^2$ control experiment for RXFP1. The unstimulated curve (■) from Figure 6 A is compared with the result obtained by transfecting the cells with RXFP1 Rluc together with increasing amounts of NK1R GFP^2 (Δ). B) RXFP1-RXFP1 pair and increasing amounts of WT RXFP1. $BRET_0$ is the $BRET^2$ signal obtained in the absence of competitor.

Figure 1

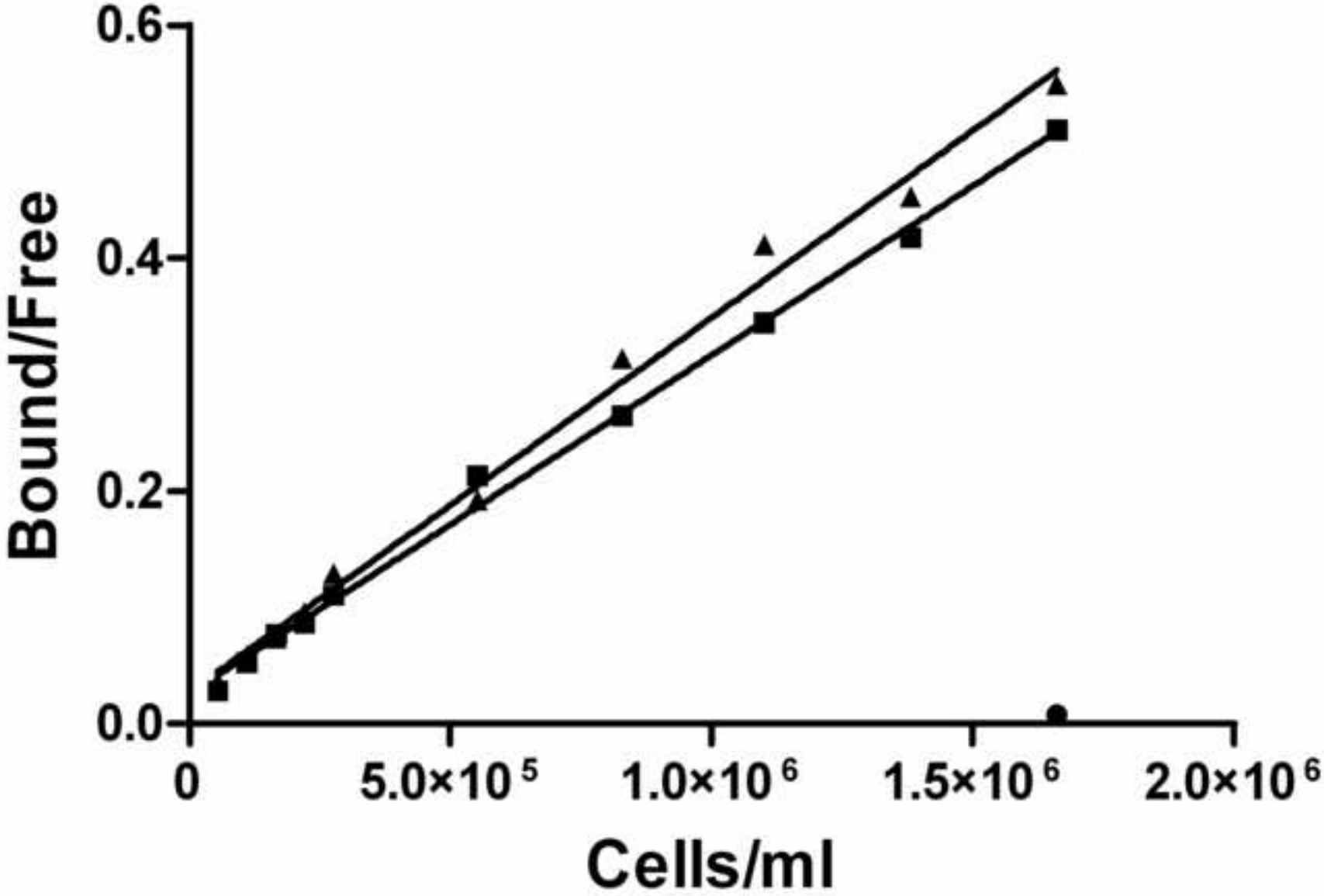

Figure 2

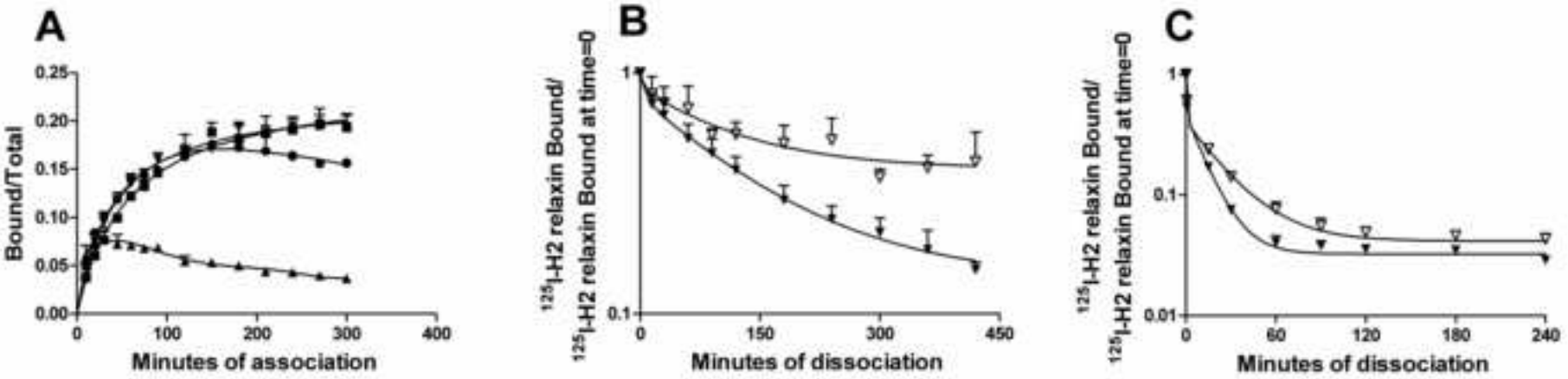
A
Bound/Total
0.25
0.20
0.15
0.10
0.05
0.00
0
100
200
300
400
Minutes of association
B
^{125}I-H2 relaxin Bound/
^{125}I-H2 relaxin Bound at time=0
1
0.1
0
150
300
450
Minutes of dissociation
C
^{125}I-H2 relaxin Bound/
^{125}I-H2 relaxin Bound at time=0
1
0.1
0.01
0
60
120
180
240
Minutes of dissociation

Figure 3

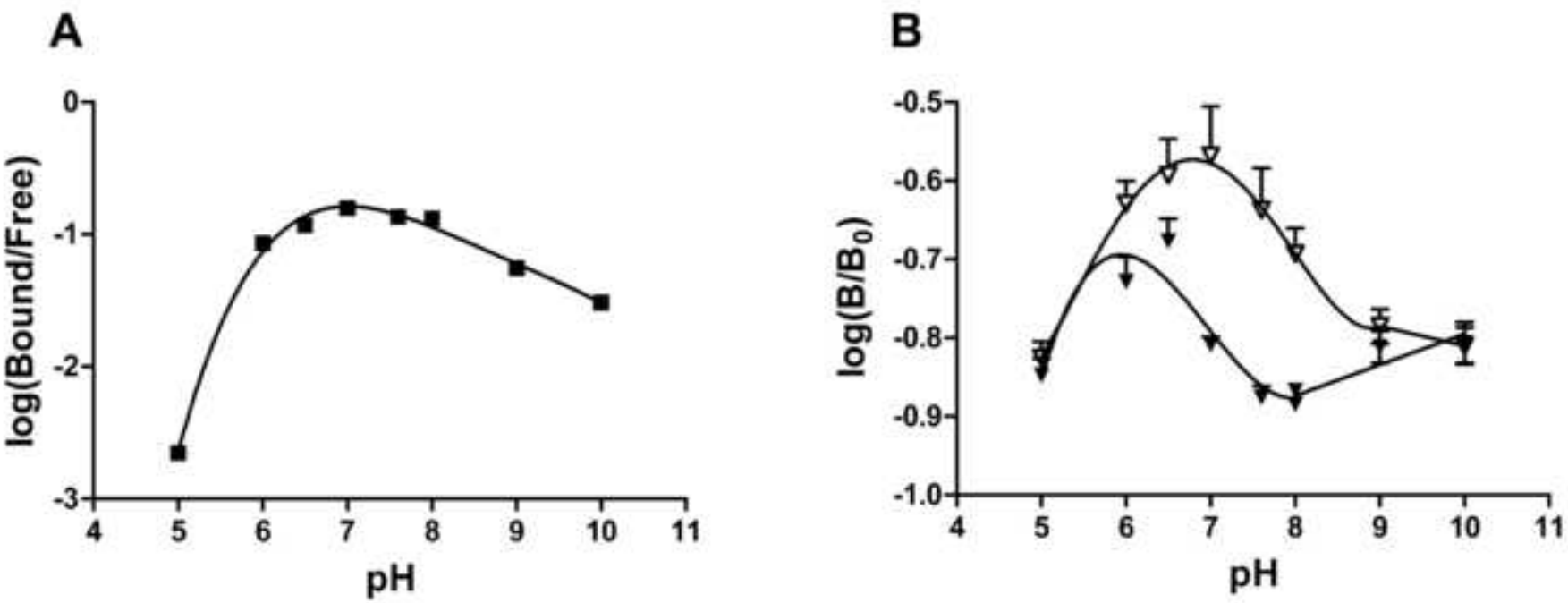

Figure 4

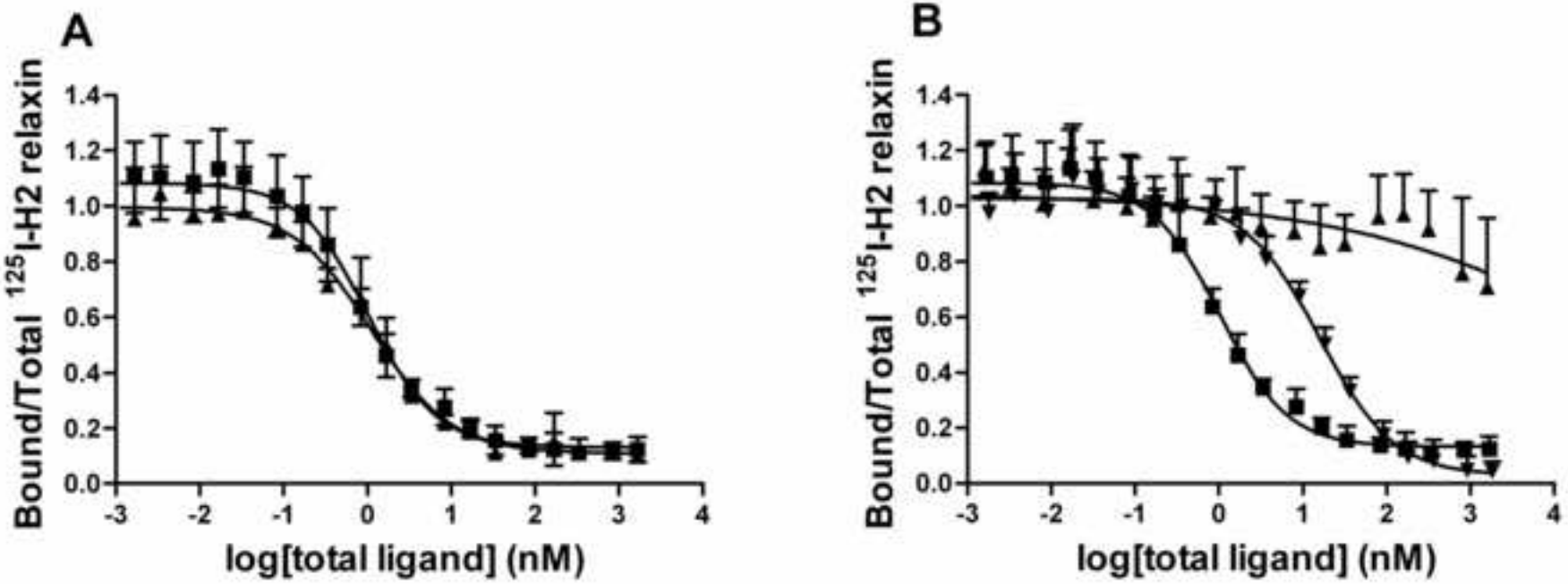

Figure 5

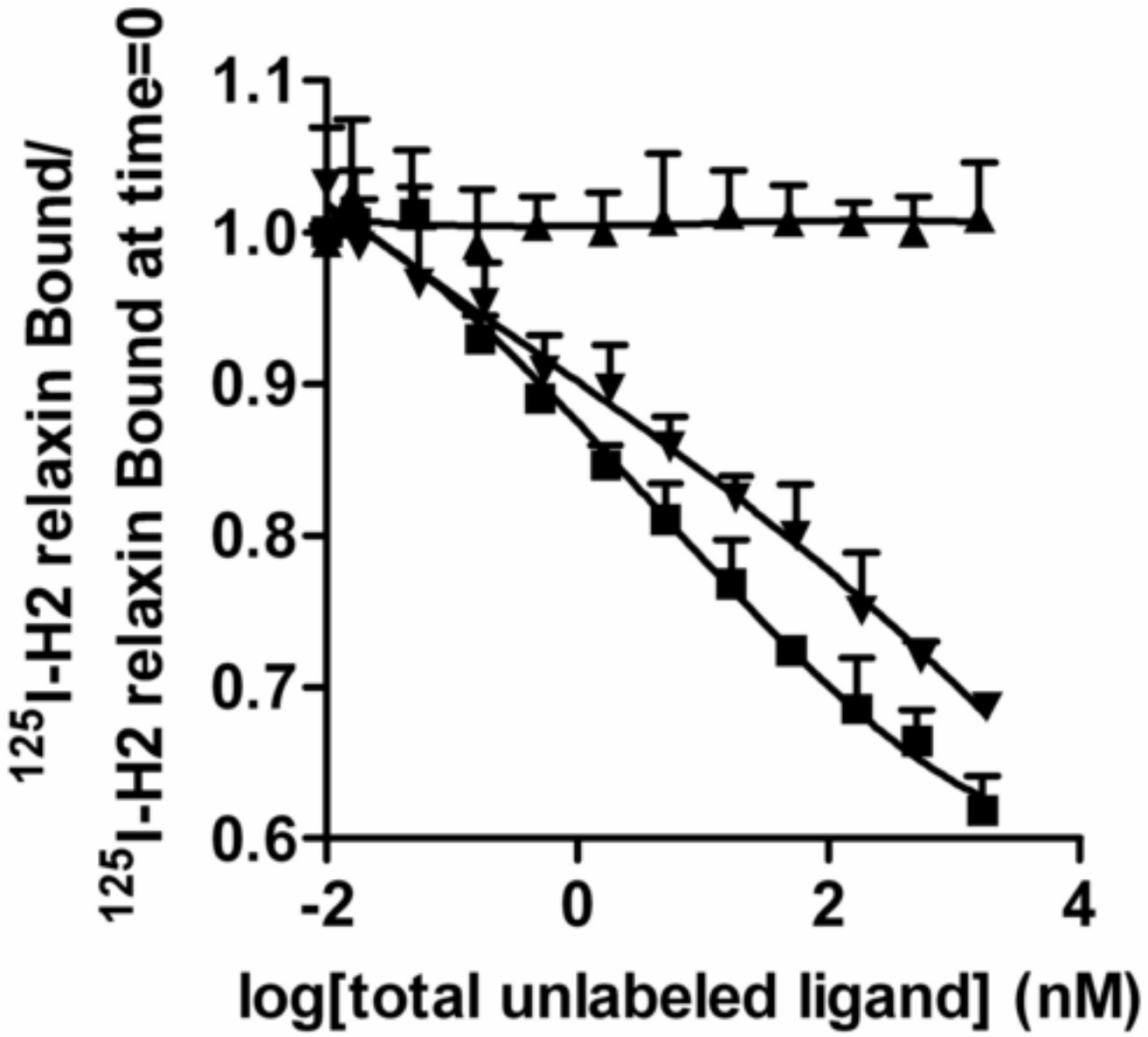
^{125}I-H2 relaxin Bound/
^{125}I-H2 relaxin Bound at time=0
1.1
1.0
0.9
0.8
0.7
0.6
-2
0
2
4
log[total unlabeled ligand] (nM)

Figure 6

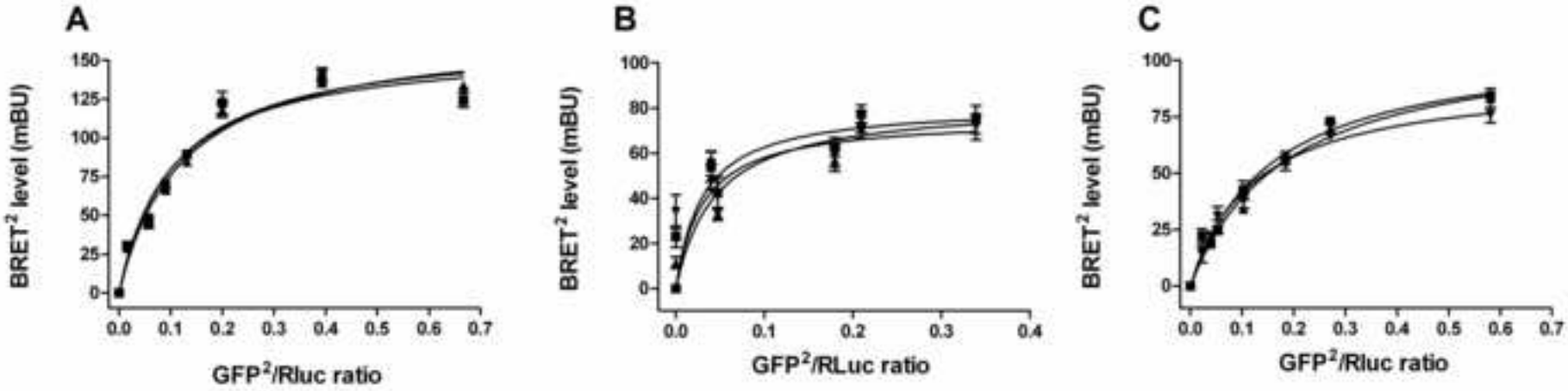

Figure 7