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Structural requirements for the interaction of sheep insulin-like factor 3 with relaxin receptors in rat atria

Yean Yeow Tan[a], Nicola F. Dawson[b], Andrew R. Kompa[a], Courtney P. Bond[a], Antonia Claasz[b], John D. Wade[b], Geoffrey W. Tregear[b], Roger J. Summers[a,*]

[a] *Department of Pharmacology, Monash University, P.O. Box 13E, Clayton 3800, Victoria, Australia*
[b] *Howard Florey Institute of Experimental Physiology and Medicine, University of Melbourne, Parkville 3010, Victoria, Australia*

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Abstract

Relaxin is a peptide with various reproductive and nonreproductive functions. The site for the peptide–receptor interaction contains two arginines (Arg) and an isoleucine (Ile) or valine (Val) residue in the B-chain with a configuration of -Arg-X-X-X-Arg-X-X-Ile/Val-X-. The sheep insulin-like peptide 3 (INSL3), a structural homologue of relaxin, also contains the *n*, *n*+4 arginines in the B-chain but they are displaced towards the carboxyl terminus by four residues (-X-X-X-X-Arg-X-X-Val-Arg-). Human INSL3 increases the activity of human relaxin in mouse bioassays. Here, we investigated whether sheep synthetic INSL3 affects the relaxin activity in rat atria. INSL3 lacked relaxin-like agonist activity but blocked the activity of relaxin and competed for relaxin binding sites at high concentrations. We also synthesized analogues of INSL3, with amino acid substitutions in the arginine-binding region. Analogues A, D and E, which have the arginines in positions identical to relaxin, showed weak relaxin-like agonist activity. These results suggest that other sites in the relaxin molecule are involved in high-affinity peptide–receptor interaction for the production of the relaxin biological responses.

Keywords: Relaxin; INSL3 (Insulin-like peptide 3); Atria, rat; Chronotropic; Inotropic; Receptor autoradiography

1. Introduction

Relaxin is a structural homologue of insulin (Bedarker et al., 1977; Isaacs et al., 1978; Eigenbrot et al., 1991) but it does not cross-react with insulin (Osheroff et al., 1990; Tan et al., 1998). It is produced principally by the ovary during pregnancy, and acts via its own receptors to soften the cervix and the interpubic ligaments to facilitate birth (reviewed by Sherwood, 1994). It also has positive chronotropic and inotropic effects in the rat heart (Kakouris et al., 1992; Ward et al., 1992; Tan et al., 1998), and acts on relaxin receptors (Osheroff and Phillips, 1991; McKinley et al., 1997) located in the circumventricular organs to modulate blood pressure (Mumford et al., 1989; Parry et al., 1990) and plasma osmolality (Weisinger et al., 1993), as well as receptors in the neurosecretory magnocellular hypothalamic nuclei to release the neuropeptides oxytocin and vasopressin (Summerlee et al., 1984; Dayanithi et al., 1987; Way and Leng, 1992).

Although there is relatively little amino acid sequence homology between relaxins from various species (Sherwood, 1994), the primary active site consists of two arginine residues at positions *n* and *n*+4 and an isoleucine or valine residue within the B-chain α-helix in a configuration of -Arg-X-X-X-Arg-X-X-Ile/Val-X- (for example B13, B17 and B20 in human gene 2 (H2) (B29) relaxin) (Fig. 1). These residues are conserved in almost all relaxins, and studies have shown that substitution of these residues causes a reduction in or an abolition of the peptide activities (Büllesbach and Schwabe, 2000; Büllesbach et al., 1992). The X-ray crystal structure of relaxin shows that the arginine residues are positioned in the first and second loops of the α-helix with their side chains project away from the core molecule into the surrounding water (Büllesbach et al., 1992) (Fig. 2). It has been suggested that these arginines, together with the side chain of isoleucine/valine, form a triangular contact with the receptor-binding sites

* Corresponding author. Tel.: +61-3-9905-1440; fax: +61-3-9905-8192.
E-mail address: roger.summers@med.monash.edu.au (R.J. Summers).

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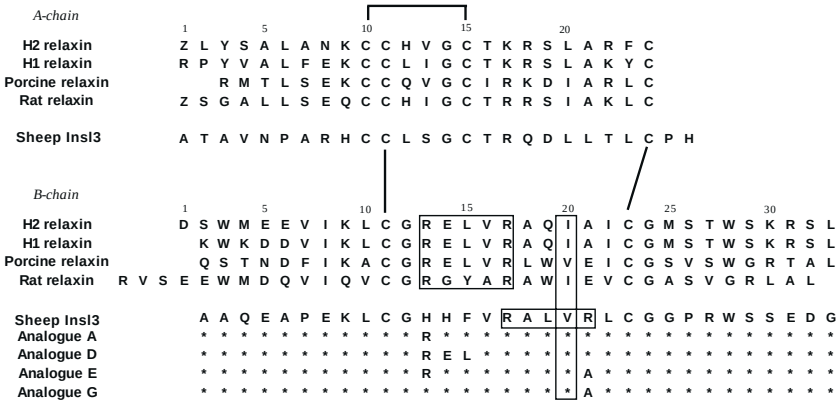

Fig. 1. The amino acid sequence of human gene 2 (H2), human gene 1 (h1), porcine and rat relaxins, sheep insulin-like factor 3 (INSL3) and synthetic analogues (A, D, E and G). The regions in the relaxin B-chain which are involved in the peptide–receptor interaction are boxed. The amino acid residues in these regions include two arginines and an isoleucine or valine with the configuration of -R-X-X-X-R-X-X-I/V-X-. In sheep INSL3, the *n*, *n*+4 arginines are further down towards the C-terminus by four residues (also boxed), having a configuration of -X-X-X-X-R-X-X-V-R-.

(Büllesbach and Schwabe, 2000). The true dynamic of the peptide–receptor interaction is yet to be established as the relaxin receptors have only recently been identified as members of the orphan leucine-rich repeat-containing G protein-coupled receptor (LGR) family, LGR7 and LGR8 (Hsu et al., 2002).

A relaxin-like gene sequence has been found in the sheep genome but it is incapable of producing RNA that can be translated to a functional peptide (Roche et al., 1993). Nevertheless, a peptide structurally similar to relaxin and insulin is expressed in the sheep testicular Leydig cells and in female reproductive tissues (Roche et al., 1996). This peptide, described as Leydig cell insulin-like peptide (Ley I-L), relaxin-like factor (RLF) or insulin-like factor 3 (INSL3), is also expressed in cows (Bathgate et al., 1996)

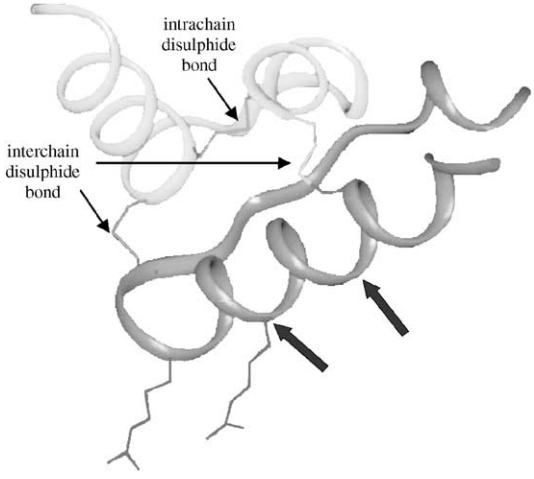

Fig. 2. The molecular structure of relaxin as determined by X-ray crystallography. The A-chain consists of two α-helices (top left), while the B-chain has only one α-helix. In the B-chain, the arginine residues important for receptor binding reside in the first and second loops of the α-helix. The broad filled arrows point to the second and third loops of the α-helix where the arginine residues for INSL3 may reside.

and other mammals including humans (Burkhardt et al., 1994; Tashima et al., 1995; Ivell et al., 1997), pigs (Adham et al., 1993) and rodents (Pusch et al., 1996; Zimmermann et al., 1997; Balvers et al., 1998; Spiess et al., 1999). The structural configuration of native INSL3 is not known as it has yet to be isolated from a biological source.

Like relaxin, INSL3 also has two arginine residues in the B-chain α-helix with the *n*, *n*+4 configuration, but they are offset towards the carboxyl terminus by precisely four residues (Roche et al., 1996). By means of circular dichroism spectroscopy, we showed that sheep synthetic INSL3 is structurally similar to relaxin (Dawson et al., 1999), suggesting that the side chain of the arginine residues in INSL3 would project out from the second and the third loops of the B-chain α-helix instead of the first and the second loops (Fig. 2). Sheep INSL3 also has a valine residue in the position corresponding to the B20 of H2 (B29) relaxin. The shift of the arginine residues, therefore, produced a different configuration (-X-X-X-X-Arg-X-X-Val-Arg-) compared to relaxin (Fig. 1). Theoretically, this configuration would interrupt the interaction of INSL3 with relaxin receptor but a study has shown that human synthetic INSL3 lacks relaxin-like bioactivity but it enhances the activity of H2 relaxin in elongating mouse pubic symphysis and competes with relaxin for binding sites in mouse brain and uterine homogenates at high concentrations (Büllesbach and Schwabe, 1995).

In this study, we used an in vitro rat atrial bioassay (Tan et al., 1998) and quantitative receptor autoradiography with [^{33}P]-labelled H2 (B33) relaxin (Tan et al., 1999) to investigate the interaction of sheep synthetic INSL3 with rat atrial relaxin receptors. Using the sheep INSL3 as a template, we then synthesized analogues with amino acid substitutions in the B-chain α-helix (Fig. 1) and tested their biological and binding activities to determine regions of the peptide molecule that are important for interaction with the relaxin receptor. We found that although the correct configuration and position of the *n* and *n*+4 arginines in the B-chain is

essential for relaxin-like agonist activity, other region(s) must also be involved in determining the affinity of the relaxin peptide–receptor interactions.

2. Materials and methods

2.1. Materials

Recombinant human gene 2 relaxins (B33 and B29) (Genentech and Connetics, San Francisco, USA), cyclic AMP-dependent protein kinase (Promega), [γ-^{33}P]ATP (DuPont), HEPES (BDH), phenylmethylsulphonylfluoride (Sigma), other salts and organic solvents used were of analytical quality.

2.2. Peptide syntheses and characterization

The procedures for the synthesis of sheep INSL3 and analogues—the synthesis of A- and B-chains separately followed by chain combination—have been published elsewhere (Dawson et al., 1999). Overall, seven analogues of sheep INSL3 with amino acid changes in the B-chain α-helix were designed, and four analogues were successfully synthesized. Analogue A has the histidine residue in B13 (numbered according to the H2 (B29) relaxin, although it is the 12th residue in sheep INSL3; see Fig. 1) replaced by arginine, producing an extra -Arg-X-X-X-Arg- cassette in the same position as that in relaxin. Analogue D has further substitutions of glutamic acid for histidine in B14 and leucine for phenylalanine in B15, making the sequence in that region of INSL3 identical to H2 (B29) relaxin. Analogue E is the derivative of analogue A in which the arginine in B21 is replaced by alanine, while analogue G has a single amino acid substitution, alanine for arginine in B21 (see Fig. 1 for summary). All peptides were purified with reverse phase high-performance liquid chromatography (HPLC), and the identities confirmed with mass spectrometry, amino acid analysis and circular dichroism spectroscopy (Dawson et al., 1999; Claasz et al., 2001). Peptides were dissolved and diluted in 0.1% aqueous trifluoroacetic acid (aq. TFA) (0.1% v/v in distilled water) before being used.

2.3. Rat atrial bioassay

Right and left atria from male Sprague–Dawley rats (200–250 g) were isolated and mounted in organ baths as previously described (Tan et al., 1998). The chronotropic (expressed as beats per minute, bpm) and inotropic (expressed as milli-Newton, mN) responses of the tissues were recorded with isometric transducers (Grass FTO3c) connected to a MacLab system.

The tissues were first exposed briefly to 0.1 μM (−)-isoprenaline to test their viability. The resting force of the tissues was then set at a level producing 50–60% of the maximum contraction when the corresponding left atria were stretched (Gille et al., 1985; Tan et al., 1998). After the tissues had stabilized, they were exposed to 1 μM sheep INSL3 or the analogues (A, D, E or G) for 20 min, or until the responses achieved plateau if the peptides were causing positive chronotropic and inotropic responses. This was followed by 1 nM (effective concentration to produce 50% of maximum response = EC_{50}, Tan et al., 1998) and 10 nM of H2 (B29) relaxin added cumulatively to test whether the presence of INSL3 or analogues had synergistic or inhibitory effects on the relaxin response in the atria. At the end of the experiment, 0.1 μM (−)-isoprenaline was added to the baths to determine the maximum response of the tissues.

2.4. Quantitative receptor autoradiography

H2 (B33) relaxin was labelled with [γ-^{33}P]ATP using the catalytic subunit of cyclic AMP-dependent protein kinase and purified by ion exchange HPLC (Tan et al., 1999). Slide-mounted, 10-μm thick atrial sections of male Sprague–Dawley rats (200–250 g) were prepared. The sections were first preincubated in 100 μl of 25 mM HEPES, 300 mM KCl, pH 7.2, containing 1 mM of protease inhibitor phenylmethylsulphonylfluoride for 30 min in a moisture chamber at room temperature (23 ± 1 °C). After removing the preincubation buffer, the sections were covered in 100 μl of HEPES/KCl with bovine serum albumin (1 mg/ml) containing approximately 100 pM of [^{33}P]H2 (B33) relaxin for 90 min in the absence or presence of increasing concentrations of sheep INSL3 or analogues (0.1–10 μM). Nonspecific binding was defined with 1 μM of H2 (B29) relaxin. The slides were then rinsed briefly and washed (2 × 10 min) in HEPES/KCl, followed by a brief rinse in distilled water and were blown dry with cooled dehumidified air. The slides were apposed to phosphorimaging plates for 2–3 days, which were then scanned with Phosphorimager SI™ (Molecular Dynamics, USA) and the images analysed using the computer program ImageQuant™ (Molecular Dynamics). Binding data were fitted using the single site competition function in PRISM™ (GraphPad, San Diego, USA) with the minimum fixed at zero, and the binding affinities of INSL3 and analogues to the rat atrial relaxin receptors calculated using the built-in Cheng and Prusoff (1973) equation.

2.5. Statistics

The raw data from the rat atrial bioassay are presented as means ± S.E.M. from experiments performed in *n* number of animals. These data were then normalized as a percentage of the maximum response produced by 0.1 μM (−)-isoprenaline before statistical analyses. Student's unpaired *t*-test or analysis of variance (ANOVA) were used to compare the activity of sheep INSL3 and analogues in producing positive chronotropic and inotropic effects, their effects on relaxin bioactivity in atria and their binding

affinities to rat atrial sections. Probability (P) values of less than 0.05 were regarded as statistically significant. Data analysed with ANOVA that showed statistical significance were further analysed with Bonferroni's post test in PRISM.

3. Results

3.1. Effects of sheep INSL3 and analogues on the rat atria and the chronotropic and inotropic responses produced by relaxin

Rat isolated atria were incubated in sheep INSL3 or the peptide analogues (all at 1 μM) for approximately 20 min, followed by H2 (B29) relaxin (cumulative 1 and 10 nM or 10 nM only), and (−)-isoprenaline (0.1 μM) to determine the maximum response of each preparation. INSL3 failed to increase the beating rate (chronotropic) of the isolated right atrial preparations or the force of contraction (inotropic) of the isolated left atrial preparations. The addition of H2 (B29) relaxin evoked changes in chronotropic response from baseline of 106 ± 11 bpm in control, 82 ± 12 bpm in INSL3 (1 μM) treated at 1 nM, and 145 ± 11 bpm in control, 127 ± 13 bpm in INSL3 treated at 10 nM; and changes in inotropic response from baseline of 1.40 ± 0.45 mN in control, 1.56 ± 0.56 mN in INSL3 treated at 1 nM, and 2.78 ± 0.32 mN in control, 3.36 ± 0.74 mN in INSL3 treated at 10 nM (n = 5). Statistical analyses (two-way ANOVA) on these data showed that the presence of INSL3 did not significantly affect the responses produced by H2 (B29) relaxin. When the results in the individual preparations were expressed as a percentage of the maximum response produced by 0.1 μM (−)-isoprenaline to normalize the differences between tissue responsiveness, the reductions in chronotropic response in the presence of INSL3 were statistically significant (P = 0.02), while the changes in inotropic response remained not significant (P = 0.51, two-way ANOVA; Fig. 3). However, further statistical test (Bonferroni's post test) performed to compare the chronotropic responses produced by 1 nM of relaxin in control and INSL3 treated atrial preparations or those produced by 10 nM of relaxin did not show any statistical significance (P>0.05).

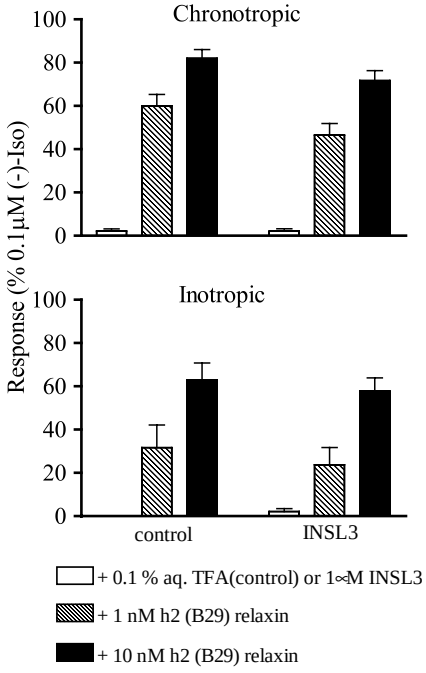

Fig. 3. Effects of INSL3 and H2 (B29) relaxin on chronotropic and inotropic responses in rat isolated atria. Shown here are mean responses of five experiments expressed as a percentage of responses to 0.1 μM (−)-isoprenaline. INSL3 at 1 μM did not have relaxin-like positive chronotropic and inotropic activities, but its presence exhibited a trend in blocking the activities of H2 (B29) relaxin.

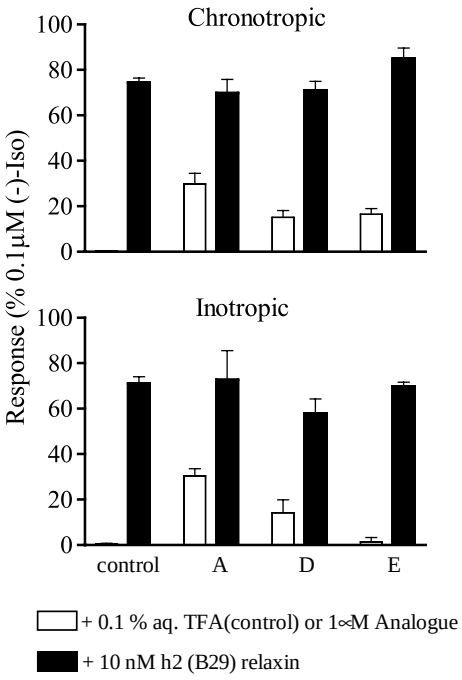

Fig. 4. Effects of analogues A, D and E, and H2 (B29) relaxin on chronotropic and inotropic responses in rat isolated atria. Shown here are mean responses of four to five experiments expressed as a percentage of responses to 0.1 μM (−)-isoprenaline. All peptide analogues (A, D and E) have relaxin arginine-binding cassette in correct position, and at 1 μM they produced relaxin-like positive chronotropic responses. Analogues A and D also produced positive inotropic responses. These responses were weaker compared to those produced by 10 nM H2 (B29) relaxin. The presence of analogues A, D or E did not affect the activity of H2 (B29) relaxin.

Analogue A (1 μM), which has a single amino acid substitution in the position B13 (arginine for histidine) and now has an arginine-binding cassette in the position comparable to relaxin, produced positive chronotropic (48 ± 7 bpm) and inotropic (1.45 ± 0.33 mN, n = 4) responses (Fig. 4). However, these responses were weak compared to the chronotropic (120 ± 8 bpm) and inotropic (2.67 ± 0.23 mN, n = 12) responses produced by 10 nM of H2 (B29) relaxin. When 10 nM of H2 (B29) relaxin was added to the atrial preparations that had been preincubated in analogue A, the positive chronotropic (115 ± 12 bpm)

and inotropic (3.20 ± 0.46 mN) responses produced were not significantly different from those in controls, even after the data were normalized (P>0.70, t-test; Fig. 4).

Analogue D, with amino acid substitutions at positions B13, 14 and 15 and has arginine-binding cassette identical to relaxin, also produced positive chronotropic (29 ± 7 bpm) and inotropic (0.62 ± 0.27 mN, n = 5) responses at 1 μM (Fig. 4). The chronotropic (P = 0.03, t-test), but not the inotropic (P = 0.05, t-test) responses were significantly less than those produced by analogue A at the same concentration. The presence of analogue D did not affect the chronotropic (131 ± 19 bpm) or inotropic (2.54 ± 0.23 mN) responses of the isolated atria to 10 nM of H2 (B29) relaxin (P>0.20, t-test; Fig. 4). Analogue E, a derivative of analogue A with an additional amino acid substitution at B21 (alanine for arginine), at 1 μM alone produced a weak positive chronotropic response (25 ± 3 bpm, n = 5) but did not evoke a positive inotropic response. The presence of this analogue did not affect the responses produced by 10 nM of H2 (B29) relaxin (Fig. 4). Analogue G, with only one amino acid substitution at B21 (alanine for arginine) and no arginine-binding cassette, had no relaxin-like agonist activity nor did it affect the chronotropic and inotropic effects of 1 and 10 nM of H2 (B29) relaxin (n = 5).

3.2. Competition by sheep INSL3 and analogues for the relaxin-binding sites in rat atria labelled with [^{33}P]H2 (B33) relaxin

[^{33}P]H2 (B33) relaxin binds with high affinity to relaxin receptors in rat atrial sections (pK_d = 8.92 ± 0.09, K_d = 1.2 nM; Fig. 5; Tan et al., 1999). Sheep INSL3, however, competed for these binding sites only at high concentrations. At 1 μM, it occupied approximately 3–4% of the specific relaxin-binding sites and some 27% at 10 μM. The binding affinity (pK_i) of INSL3 was estimated at 4.69 ± 0.11 (K_i = 20 μM, n = 6), or approximately 4.2 orders of magnitude less than relaxin. Analogues A and D, which showed weak relaxin-like agonist activities both competed for the relaxin-binding sites with higher affinities than INSL3. The competition curves for these analogues showed that at 10 μM, more than 50% of the [^{33}P]H2 (B33) relaxin-binding was displaced. The pK_i value for analogue A was 5.15 ± 0.03 (K_i = 7.08 μM, n = 6), while that for analogue D was 5.29 ± 0.09 (K_i = 5.13 μM, n = 6). These values were significantly higher than that for INSL3 (analogue A: P = 0.0021; analogue D: P = 0.0016; t-test; Fig. 5, top panel).

Analogue E, which produced weak positive chronotropic activity also competed for the sites labelled by [^{33}P]H2 (B33) relaxin but with an affinity (pK_i = 4.80 ± 0.09, K_i = 16 μM, n = 7) lower than analogues A or D. The competition curve for analogue E showed that its binding activity was not significantly different from that of INSL3 (P = 0.48, t-test). Analogue G did not have any agonist or antagonist activity in the atrial bioassay but had very weak binding activity at the atrial relaxin-binding sites. The pK_i value estimated for analogue G was 4.60 ± 0.17 (K_i = 25 μM, n = 6), not significantly different from that for INSL3 (P = 0.66, t-test; Fig. 5, bottom panel).

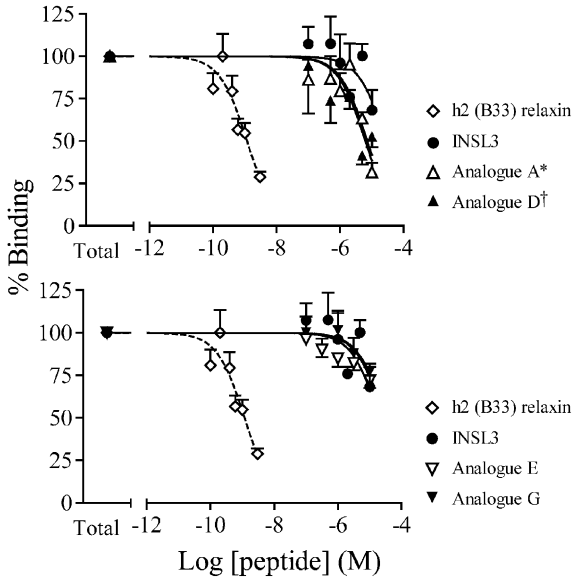

Fig. 5. Competition by sheep INSL3 and synthetic analogues for relaxin-binding sites in slide-mounted sections of rat atrial myocardium labelled with [^{33}P]H2 (B33) relaxin. The competition curves for analogues A and D (top panel) were significantly to the left of INSL3 (*P = 0.0021 for analogue A vs. INSL3, and $^{\dagger}P$ = 0.0016 for analogue D vs. INSL3; t-test, n = 6), indicating that both analogues A and D competed for the relaxin-binding sites with higher affinities than INSL3. The competition curves for analogues E and G (bottom panel) were superimposed with the competition curve for INSL3, indicating that they had similar affinity for relaxin-binding sites in rat atria (P>0.40, t-test, n = 6–7). The binding affinities for INSL3 and all analogues were weak when compared to H2 (B33) relaxin (broken curve).

4. Discussion

The rat atrial bioassay and quantitative receptor autoradiography have been used to study potential relaxin-like activity of sheep INSL3 and analogues. Although INSL3 lacked relaxin-like activity in rat isolated atria, it appeared to reduce the atrial responses to H2 (B29) relaxin. In the receptor autoradiographic studies, the results suggest that INSL3 binds to relaxin receptors at high concentrations (>1 μM) and may act as an antagonist. This conclusion differs with earlier studies investigating the activity of human synthetic INSL3 in mice, which when injected into ovariectomized oestrogen-primed mice (up to 20 μg per mouse) lacked activity on its own but synergised the effect of H2 relaxin on pubic ligament elongation (Büllesbach and Schwabe, 1995). The anomaly may be explained by the difference in species used in both studies, or that the mouse assay is an in vivo assay and relaxin may bind to plasma protein thus reducing the level of free relaxin in circulation in controls. Co-administration of human INSL3, which may compete for the plasma protein binding, could lead to an

increase in the bioavailability of relaxin and thus the responses. In accord with this view, the in vitro competition studies performed in tissue homogenates of mouse uterus using [^{125}I]porcine relaxin and unlabelled human INSL3 showed that human INSL3 competed for the relaxin-binding sites in uterus with low affinity (IC_{50} = 100 ng μl^{-1} or 16 μM, Büllesbach and Schwabe, 1995), a value similar to that obtained in our study (K_i = 20 μM).

The single amino acid substitution in analogue A creates two *n*, *n* + 4 arginine-binding cassettes in the B-chain α-helix. The first *n*, *n* + 4 arginine residues are in the precise disposition relative to the two cysteines for interchain disulphide bonds as in the relaxin B-chain. Analogue A had significant weak chronotropic and inotropic activities in the rat isolated atria but did not affect H2 (B29) relaxin responses. In binding studies, it competed with [^{33}P]H2 (B33) relaxin for atrial relaxin receptors with a significantly higher affinity than INSL3. Analogue A is, therefore, a weak agonist rather than a partial agonist at relaxin receptors as a partial agonist would be expected to reduce the activity of the full agonist. The results also confirm that the structure of INSL3 must resemble relaxin to allow a single amino acid substitution to alter the pharmacological profile of INSL3 to resemble relaxin. This is supported by the published circular dichroism spectroscopy data (Büllesbach and Schwabe, 1995; Dawson et al., 1999).

Despite having the essential binding cassette in the right position, the activities of analogue A were still far weaker than H2 (B29) relaxin, raising the question of the structural features that determine the potency of relaxins and related peptides. Previously, we showed that rat relaxin had lower functional (Tan et al., 1998) and binding (Tan et al., 1999) activities than human or porcine relaxins in rat atria. We suggested that the difference in amino acid residues within the arginine-binding cassette, where human and porcine relaxins have identical residues (-Arg-Glu-Leu-Val-Arg-) in contrast to rat relaxin (-Arg-Gly-Tyr-Ala-Arg-), may be involved. Analogue D, with a binding cassette identical to that of human and porcine relaxins was therefore synthesized and tested to determine whether this analogue would resume full relaxin-like activity comparable to H2 (B29) relaxin. Although analogue D was active in the atrial bioassay, it was less effective than analogue A and did not have a binding affinity significantly different from analogue A. A recently published study showed that the amino acid residues in B14 and B16, that is -Glu- and -Val- for H2 relaxin and porcine relaxin, and -Gly- and -Ala- for rat relaxin, are not important for activity (Büllesbach and Schwabe, 2000).

Both analogues A and D have an additional arginine in the third loop of the α-helix that may interfere with binding. Analogue E, a derivative of analogue A but with the arginine residue in the third loop substituted by an alanine, was synthesized to investigate whether the weak activity shown by analogues A and D is due to that additional arginine residue. Analogue E had biological activity weaker than either A or D and a binding affinity for the relaxin receptor comparable to that of INSL3. This result was unexpected because analogue E has all the essential amino acid residues, the two arginines and a valine, in the same disposition as relaxin but it still lacks relaxin-like activity. Analogue G, which was synthesized as a control had no relaxin-like activity. Clearly, the position of arginine-binding cassette is important for relaxin-like activity but it is also apparent that other regions beyond this core of the relaxin molecule have a marked influence on peptide potency.

Recent reports indicate that INSL3 is a novel peptide with distinct expression patterns, receptor distributions and physiological functions unrelated to those of relaxin. The ovary of ruminants expresses relatively high levels of INSL3 (Bathgate et al., 1996; Roche et al., 1996) and the pattern of expression during pregnancy in cows (Bathgate et al., 1996) resembles that of relaxin in pigs or rats (Sherwood, 1994). INSL3 also plays a significant developmental role in males and there is an up-regulation of INSL3 expression during puberty in the mouse testis (Pusch et al., 1996; Zimmermann et al., 1997). In humans suffering from testicular Leydig cell hyperplasia and adenoma, the expression of INSL3 is down-regulated with the gonads remaining in a prepubertal state (Klonisch et al., 1999). Serum concentrations of INSL3 in postpubertal human males are one order of magnitude higher than in females or children (Büllesbach et al., 1999) and in rats, the serum concentrations increase in males only, starting at day 10 after parturition and continuing until adult levels are reached on day 39 after parturition (Boockfor et al., 2001). By use of a [^{125}I]-labelled human INSL3, it has also been demonstrated that INSL3 may have its own novel high-affinity receptors (Büllesbach and Schwabe, 1995; Büllesbach and Schwabe, 1999).

INSL3 gene knockout males are sterile and lack mature sperm due to the failure of development of the male gubernaculum, leading to impaired testicular descent (cryptorchidism) and retention of the testis in the abdomen, an environment not suitable for spermatogenesis (Nef and Parada, 1999; Zimmermann et al., 1999). Female homozygous mutants have impaired fertility associated with deregulation of the oestrus cycle but are not sterile (Nef and Parada, 1999). These results again suggest an important role for INSL3 in the male, although in human the mutations and polymorphism in the INSL3 gene may not be a major cause of cryptorchidism (Marin et al., 2001a,b; Lim et al., 2001; Koskimies et al., 2000; Tomboc et al., 2000; Krausz et al., 2000). The characteristics of INSL3 gene knockout mice are also observed in mice with a disruption of a G protein-coupled receptor encoded by the GREAT gene (Overbeek et al., 2001; Gorlov et al., 2002). Screening of the human genome showed that LGR8 is the human ortholog of the mouse GREAT G protein-coupled receptor, indicating INSL3 may be a ligand for the LGR8. Recent studies have confirmed that LGR8 is indeed the receptor for INSL3 (Kumagai et al., 2002). While INSL3 does not have affinity for the LGR7, a closely related receptor which shares

approximately 60% sequence identity with LGR8, relaxin, however, has high affinity for LGR7 but low affinity for LGR8 (Hsu et al., 2002). Therefore, it would be of interest to investigate the expression of LGR7 and LGR8 in rat atria and the interaction between these receptors to produce the chronotropic and inotropic responses.

In summary, the present study confirmed the importance of the n, $n+4$ arginine-binding cassette in the B-chain of relaxin for relaxin-like agonist activity but only if it is in the correct position and configuration. Studies with INSL3 analogues indicate that other as yet unknown region(s) may be important in relaxin peptide–receptor interactions which affect the affinity of peptide for the receptor.

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