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Comparison of relaxin receptors in rat isolated atria and uterus by use of synthetic and native relaxin analogues

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1 The receptors for relaxin in the rat atria and uterus were investigated and compared by use of a series of synthetic and native relaxin analogues. The assays used were the positive chronotropic and inotropic effects in rat spontaneously beating, isolated right atrium and electrically driven left atrium and the relaxation of K^+ precontracted uterine smooth muscle.

2 Relaxin analogues with an intact A- and B-chain were active in producing powerful chronotropic and inotropic effects in the rat isolated atria at nanomolar concentrations. Single-chain analogues and structural homologues of relaxin such as human insulin and sheep insulin-like growth factor I had no agonist action and did not antagonize the effect of the B29 form of human gene 2 relaxin.

3 Shortening the B-chain carboxyl terminal of human gene 1 (B2–29) relaxin to B2–26 reduced the activity of the peptide and removal of another 2 amino acid residues (B2–24) abolished the activity. This suggests that the B-chain length may be important for determination of the activity of relaxin. More detailed studies are needed to determine the effect of progressive amino acid removal on the structure and the bioactivity of relaxin.

4 Porcine prorelaxin was as active as porcine relaxin on a molar basis, suggesting that the presence of the intact C-peptide did not affect the binding of the prorelaxin to the receptor to produce functional responses.

5 Relaxin caused relaxation of uterine longitudinal and circular smooth muscle precontracted with 40 mM K^+. The pEC_{50} values for human gene 2 and porcine relaxins were lower than those in the atrial assay, but rat relaxin had similar pEC_{50} values in both atrial and uterine assays. Rat relaxin was significantly less potent than either human gene 2 or porcine relaxin in the atrial assay, but in the uterine assay they were equipotent. The results suggest that the relaxin receptor or the signalling pathway in rat atria may differ from that in the uterus.

Keywords: Relaxin; functional receptor; rat atria; rat uterus; signalling pathway

Introduction

Relaxin is a peptide hormone with structural similarities to insulin and insulin-like growth factors (Bedarker *et al*., 1977; Isaacs *et al*., 1978; Eigenbrot *et al*., 1991). It is produced by the ovary during pregnancy and acts on the mammalian reproductive system to ripen the cervix, elongate the pubic symphysis and inhibit uterine contraction (see review by Sherwood, 1994). In addition to its role as a reproductive hormone, relaxin also produces chronotropic and inotropic responses in rat isolated atria (Kakouris *et al*., 1992) and participates in the regulation of blood pressure (St. Louis & Massicotte, 1985; Mumford *et al*., 1989; Parry *et al*., 1990). It also regulates fluid balance (Weisinger *et al*., 1993) and release of neuropeptides such as vasopressin and oxytocin (Summerlee *et al*., 1984; Dayanithi *et al*., 1987; Way & Leng, 1992) by binding to the circumventricular organs (organum vasculosum of the lamina terminalis and the subfornical organ) and the neurosecretory magnocellular hypothalamic nuclei (paraventricular and supraoptic nucleus) of the brain. These non-reproductive functions of relaxin have been supported by the recent discovery of relaxin binding sites in rat atria (Osheroff *et al*., 1992) and brain (Osheroff & Phillips, 1991), and the demonstration that intraventricular injection of relaxin increases neuronal activity in regions of the hypothalamus and lamina terminalis associated with fluid and electrolyte balance and oxytocin secretion (McKinley *et al*., 1997).

Relaxin has been isolated from a variety of species. Although the amino acid sequences vary from as close as 2% (porcine vs minke whale or porpoise) to as diverse as 75% (rat vs skate) (Sherwood, 1994), these relaxins are structurally similar. The relaxin molecule contains two peptide chains, A and B. The A-chain is 20–24 and the B-chain is up to 36 amino acid residues in length. These chains are connected by two disulphide bonds, in addition to an intrachain disulphide bond in the A-chain. The amino acids which are conserved between all species are the cysteine residues for the formation of disulphide bonds, and the adjacent glycines. Two arginines in the n, n+4 positions in the B-chain (B13 and B17 of human gene 2 relaxin, h2Rlx, or B16 and B20 of rat relaxin, see Table 1) are also conserved in all species except for gorilla gene 1 relaxin (Evans *et al*., 1994). Structure-activity studies show that these arginines are essential for binding of the relaxin to the receptor (Büllesbach *et al*., 1992; Rembiesa *et al*., 1993) or to the rabbit anti-porcine polyclonal antibody R6 (Büllesbach *et al*., 1996).

The guinea-pig pubic symphysis palpation (Kroc *et al*., 1959) and mouse interpubic ligament assays (Steinetz *et al*., 1969) have been used to determine the bioactivity of relaxin. In the mouse interpubic ligament assay, the rat native relaxin was less potent than porcine native relaxin (Sherwood, 1979; 1982) or B29 form of h2Rlx, which have similar potencies (Entenmann *et al*., 1983; Ferraiolo *et al*., 1989; Büllesbach & Schwabe, 1991). However, a recent study in which this bioassay was used showed that rat synthetic relaxin was as active as h2Rlx (Büllesbach & Schwabe, 1996). Partial denaturation of the native relaxin during extraction and purification was suggested for the difference in bioactivity between native and synthetic relaxins (Büllesbach & Schwabe,

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Table 1 The amino acid sequence of relaxins and synthetic analogues used in this study

Relaxin	*Amino acid sequence*
Human gene 2 (B33)	ZLYSALANKCCHVGCTKRSLARFC DSWMEEVIKLCGRELVRAQIAICGMSTWS*KRSL*
Human gene 1 (B2–33)	RPYVALFEKCCLIGCTKRSLAKYC KWKDDVIKLCGRELVRAQIAICGMSTWS*KRSL*
Porcine	RMTLSEKCCQVGCIRKDIARLC QSTNDFIKACGRELVRLWVEICGSVSWGRTA
Porcine prorelaxin	LLLWVGLLYSFLRPM SQLPREIP AKLEQPLNPVFELMMKLIEADKTISSPMTEAPP T G LSERQPSLRELQQSASKDSNLNFEEFKKIIL T N E RKKRSHKDLGLNKLELLSKDEAENQR L L Q F RMTLSEKCCQVGCIRKDIARLC SLEEP L GQSTNDFIKACGRELVRLWVEICGSVSWGRTA
Rat	QSGALLSEQCCHIGCTRRSIAKLC RVSEEWMDQVIQVCGRGYARAWIEVCGASVGRLAL
Rat A [C^{1}, S^{11}]-bis-cyclic	CSGALLSEQCSHIGCTRRSIAKLC
Rat A [$S^{11,24}$]	ZSGALLSEQCSHIGCTRRSIAKLS
Rat B (15–25)	GRGYARAWIEV
Human gene 1 B[$S^{11,23}$]	KWKDDVIKLSGRELVRAQIAISGMSTWS

The human gene 2 (B29) and human gene 1 (B2–29) relaxins have 4 residues (*in italics*) less than their respective longer forms. The arginines in the B-chain which are important for binding are underlined.

1996). These bioassays are time consuming to perform and do not generate a graded dose-response relationship so that the activity of various relaxins can be easily quantified and compared. In contrast, the atrial (Kakouris *et al*., 1992) and uterine (St. Louis, 1982) bioassays provide graded dose-response relationships to known concentrations of relaxins and can be normalized to a reference drug known to evoke a maximal response in these tissues.

In the present study the rat isolated atrial bioassay was used to investigate the activity of human (gene 1 and gene 2), rat and porcine relaxins, and synthetic analogues. The activity of these relaxins was also investigated in the uterine smooth muscle relaxation assay to compare and differentiate the functional receptors in atria and uterus, as oestradiol treatment was shown to increase the receptor number in the uterus but had no effect in the atria (Osheroff *et al*., 1992).

Methods

Atrial bioassay

Male Sprague-Dawley rats (200–250 g) were anaesthetized in 80% CO_2 and 20% O_2 before decapitation. The heart was rapidly isolated and the atria dissected in warmed Krebs bicarbonate (KB) (composition in mM: NaCl 118.4, KCl 4.7, $CaCl_2$ 1.9, $NaHCO_3$ 25, $MgSO_4.7H_2O$ 1.2, glucose 11.7 and $NaH_2PO_4.2H_2O$ 1.2) containing 2,3-butanedione monoxime (BDM, 0.03 M), a short acting calcium antagonist to prevent tissue damage. The atria were then mounted separately in 5 ml organ baths containing KB maintained at 37°C and bubbled with 95% O_2/5% CO_2. Right atria were allowed to beat spontaneously while left atria were stimulated at 5 Hz with 2 ms square-wave pulses at 1.5 times the threshold voltage (1–2 V) via punctate electrodes connected to a Grass S6 stimulator. The responses of the tissues were recorded with isometric transducers (Grass FTO3c) connected to a MacLab system.

The tissues were washed several times over a 30 min period to remove the BDM and were exposed briefly to 0.1 μM (−)-isoprenaline to test their viability. After the responses had returned to baseline, a length/force relationship was established in the left atrial preparation by gradually increasing the resting force (tissue lengthening) until further increases did not produce a corresponding increase in force of contraction with tissue stimulation. The resting force was then set at a level producing 50–60% of the maximum contraction (Gille *et al*., 1985). Cumulative concentration-response curves to relaxins were then constructed and the chronotropic and inotropic responses recorded. Only one concentration-response curve was determined on each preparation as the responses did not return to baseline, even after 6 h of washing. At the end of the concentration-response curves, 0.1 μM (−)-isoprenaline was added to the baths to determine the maximum response of the tissues.

The bioactivity of human insulin, sheep insulin-like growth factor type I (IGF-I) and other synthetic single-chain peptides

such as human gene 1 relaxin B chain [$Ser^{11,23}$] (2–29), rat A [Ser^{11}, Ser^{24}] (1–24), rat A [Cys^{1}, Ser^{11}]-bis-cyclic and rat B (15–25) was also investigated. In these experiments, the atria were again first exposed briefly to 0.1 μM (−)-isoprenaline. This was followed by a high concentration (0.1 μM) of the peptides, 3 nM h2Rlx (B29) (80–90% maximum response) and 0.1 μM (−)-isoprenaline.

Uterine smooth muscle relaxation assay

Female Sprague-Dawley rats (200–250 g) were injected with 20 μg of β-oestradiol in sesame oil (s.c.) 18–24 h before experimentation and were killed as above. Segments (1.5 cm lengths) of the uterine horns were cut and mounted under 0.5 g force in 5 ml organ baths containing KB to investigate the responses of the longitudinal smooth muscle. To investigate the responses of the circular smooth muscle, spiral strips were cut before mounting. The preparations were allowed to equilibrate for 30 min after which they were exposed twice to modified KB containing 40 mM K^+ to stabilize the tissues. After the third contraction, concentration-response curves to relaxins were constructed. Relaxation responses were recorded via isotonic transducers (Ugo-Basile type 7006) into a MacLab system. Papaverine, a non specific smooth muscle relaxant (50 μM) was added to the baths at the end of experiment to determine the maximum relaxation of the tissues.

Statistics

Values are given as mean ± s.e.mean. The concentration-response data were analysed by use of a sigmoidal dose-response function with a variable slope in the computer programme PRISM (GraphPad Software Inc.) to obtain the pEC_{50} values. Student's unpaired t test was used to compare the activity of relaxins from other species to that of h2Rlx (B29). Probability (P) values of less than 0.05 were regarded as statistically significant.

Materials used

Human gene 2 relaxins (B33 and B29 forms) (Genentech); porcine relaxin and prorelaxin, human gene 2 with glutamine substitution at A1 (Gln), human gene 1 (B2–33, B2–29, B2–26 and B2–24), human gene 1 B [Ser^{11}, Ser^{23}] (2–29), rat native and synthetic, rat A [Ser^{11}, Ser^{24}] (1–24), rat A [Cys^{1}, Ser^{11}]-bis-cyclic and rat B (15–25) relaxins (Table 1) (Howard Florey Institute); sheep IGF-I (GroPep); human insulin (Humulin R) (Eli Lilly); (−)-isoprenaline (+)-bitartrate, papaverine hydrochloride, 2,3-butanedione monoxime and β-oestradiol (1,3,5[10]-oestratriene-3,17β-diol) (Sigma).

Stock peptides (5–10 μM) were prepared in 0.1% aqueous trifluoroacetic acid (TFA) containing 0.5 mg ml^{-1} lyophilized bovine serum albumin (BSA). Further dilutions were made in KB or in modified KB during the experiments. BDM (0.03 M) was prepared in warmed KB and stock solutions of (−)-isoprenaline (10 mM) and papaverine (5.5 mM) were prepared in 10 mM HCl.

Results

Atria bioassay

Human gene 2 relaxin (h2Rlx; B29) caused positive chronotropic and inotropic responses in the rat isolated atria. The pEC_{50} values for chronotropic and inotropic responses were 9.06 ± 0.10 (n = 6) and 9.03 ± 0.07 (n = 6), respectively. The longer form of h2Rlx (B33) was significantly more active than h2Rlx (B29) for the chronotropic response (9.35 ± 0.07, P = 0.04) but not for the inotropic response (9.05 ± 0.08, P = 0.85) (Figure 1a). Substitution of the pyroglutamine (Z) residue at A1 with glutamine (Q) did not significantly change the activity of h2Rlx (B29) (chronotropic: 9.17 ± 0.05; inotropic: 9.06 ± 0.17, P > 0.05, all n = 6) (Figure 1a).

Synthetic human gene 1 relaxin (h1Rlx) was also active in the atrial bioassay. The pEC_{50} values for h1Rlx (B2–29) were 8.74 ± 0.05 (n = 6) for chronotropic and 8.83 ± 0.04 (n = 6) for inotropic responses. Both values were significantly lower than the corresponding values for h2Rlx (B29) (P < 0.05, Figure 1b). The longer B-chain form, h1Rlx (B2–33), was more active for the chronotropic responses (8.97 ± 0.16) but not the inotropic responses (8.67 ± 0.12) (Figure 1b). However, neither value differed significantly from the corresponding values for h1Rlx (B2–29) (P > 0.20, n = 6) (Figure 1b). Shortening the B-chain of h1Rlx caused a progressive loss of bioactivity. Thus h1Rlx (B2–26) has 3 amino acid residues from the carboxyl terminal removed and was significantly less potent than h1Rlx (B2–29) (chronotropic: 8.38; inotropic: 8.36, n = 2, Figure 1b). Further removal of another 2 amino acid residues from the carboxyl terminal (h1Rlx (B2–24)) totally abolished the bioactivity of the peptide (n = 2) (Figure 1b).

Porcine relaxin had bioactivity comparable to that of h2Rlx (B29) with regard to chronotropic (9.12 ± 0.07) and inotropic responses (8.91 ± 0.03, n = 6, P > 0.05) (Figure 2a). The precursor of porcine relaxin, porcine prorelaxin was also active in increasing the atrial rate (9.01 ± 0.06) and force of contraction (8.95 ± 0.06, n = 6). The potency of this precursor peptide did not differ significantly from that of porcine relaxin or h2Rlx (B29) (P > 0.05) (Figure 2a). Rat native and synthetic relaxins were both active in this bioassay. The pEC_{50} values for rat native relaxin (chronotropic, 8.52 ± 0.08; inotropic, 8.60 ± 0.09) did not differ from those obtained with rat synthetic relaxin (chronotropic, 8.60 ± 0.05; inotropic, 8.69 ± 0.09, n = 6, P > 0.05) (Figure 3b). However, these values were significantly less than those obtained for h2Rlx (B29) (chronotropic, P < 0.005; inotropic, P < 0.02) (Figure 2b) or porcine relaxin (chronotropic, P < 0.0005; inotropic, P < 0.04, Figure 2b (see Table 2 for summary). All the active analogues investigated in this study had similar efficacy relative to (−)-isoprenaline.

The synthetic single-chain peptides, h1Rlx-B [S^{11}, S^{23}] (2–29), rat A [S^{11}, S^{24}] (1–24), rat A [C^{1}, S^{11}]-bis-cyclic and rat B (15–25) as well as human insulin and sheep IGF-I were all inactive in this bioassay at 0.1 μM (all n = 2). They also did not appear to antagonize the responses produced by 3 nM of h2Rlx (B29) (chronotropic > 60% and inotropic > 55% of 0.1 μM (−)-isoprenaline), or 0.1 μM (−)-isoprenaline (both chronotropic and inotropic responses were similar to pre-peptide levels).

Uterine bioassay

In this bioassay, the h2Rlx (B29) relaxed the K^+ (40 mM) precontracted uterine myometrium from β-oestradiol treated rats. The pEC_{50} values obtained in longitudinal or circular smooth muscle from either the left or right horn of the uterus were not significantly different (P > 0.05, n = 5, Table 3) but the values were significantly lower than those for the atrial bioassay (P < 0.05, Table 2). The activity of the longer form of h2Rlx (B33) (n = 2), porcine relaxin (n = 4) and prorelaxin (n = 2) were not significantly different from that of the h2Rlx (B29) (Table 3, Figure 3a and b). However, the porcine relaxin

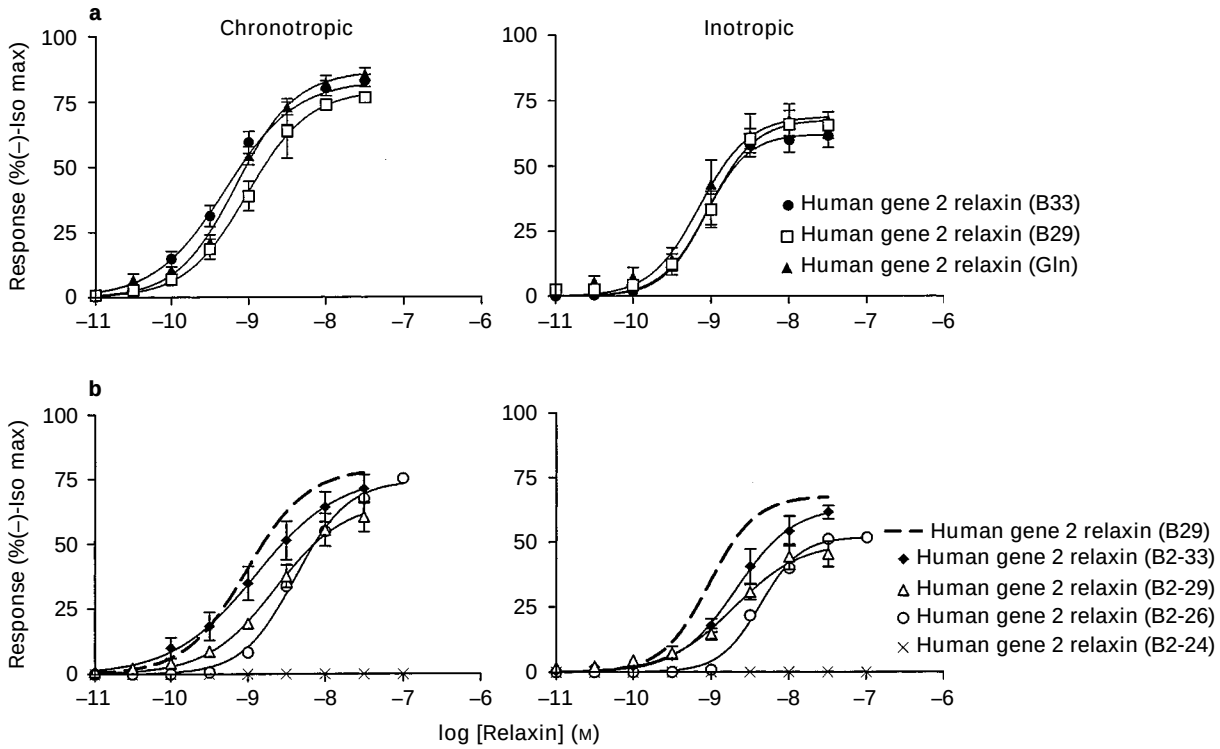

Figure 1 Effects of human relaxins in producing chronotropic (left) and inotropic (right) responses in rat isolated atria. Shown here are mean cumulative concentration-response curves expressed as a percentage of the (−)-isoprenaline maximum for (a) human gene 2 relaxins comprising the B33, B29 and Gln forms (all $n=6$) and (b) human gene 1 relaxins comprising the B2−33, B2-29, B2-26 and B2−24 in comparison to human gene 2 (B29) relaxin.

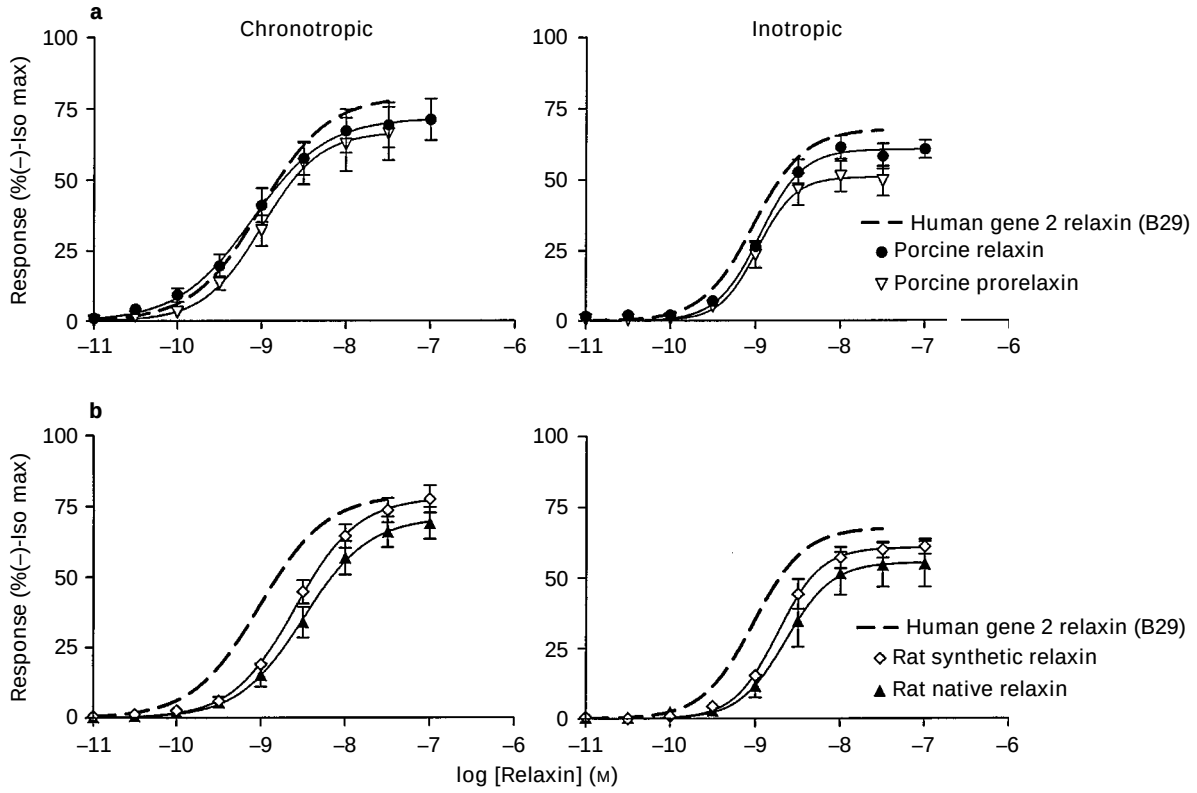

Figure 2 Effects of porcine and rat relaxins in producing chronotropic (left) and inotropic (right) responses in rat isolated atria. Shown here are mean cumulative concentration-response curves expressed as a percentage of the (−)-isoprenaline maximum for (a) porcine relaxin and prorelaxin in comparison to human gene 2 (B29) relaxin and (b) rat synthetic and native relaxins in comparison to human gene 2 (B29) relaxin (all $n=6$).

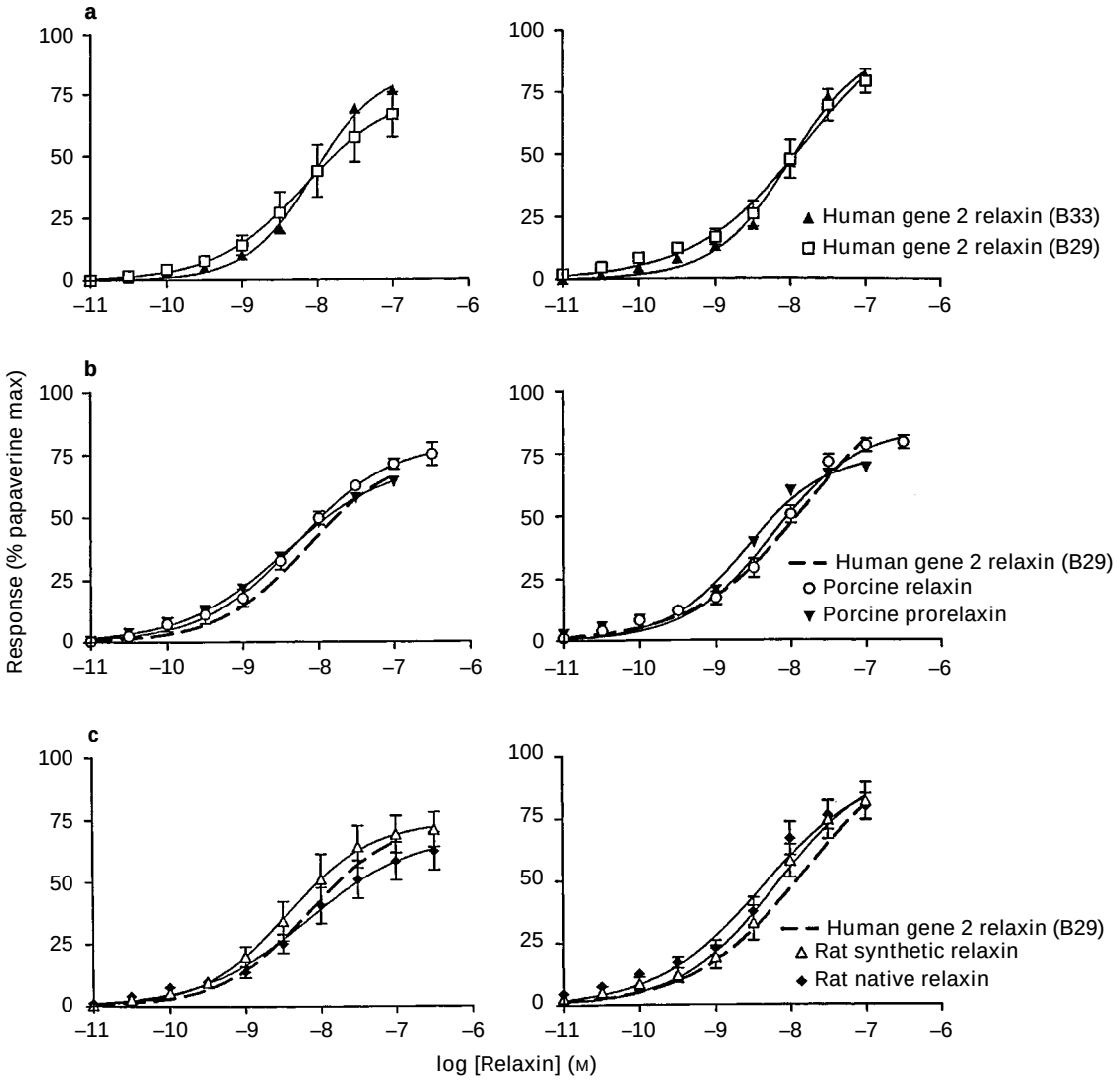

Figure 3 Effects of human, porcine and rat relaxins in relaxing isolated uterine left horn longitudinal (left), and circular (right) smooth muscle from β-oestradiol pretreated rats. Shown here are mean cumulative concentration-response curves expressed as a percentage of the papaverine maximum for (a) B33 and B29 forms of human gene 2 relaxin, (b) porcine relaxin and prorelaxin in comparison to human gene 2 (B29) relaxin and (c) rat synthetic and native relaxins in comparison to human gene 2 (B29) relaxin.

Table 2 Comparison of the maximum response, pEC_{50} value and efficacy of relaxins and synthetic analogues in producing chronotropic and inotropic responses in rat isolated atria

	δ *max. response*		pEC_{50}		*Efficacy*[#]	
	Chrono (b.p.m.)	*Ino* (g)	*Chrono*	*Ino*	*Chrono*	*Ino*
h2Rlx (B29)	130±7	0.21±0.03	9.06±0.10	9.03±0.07	0.77±0.01	0.66±0.05
h2Rlx (B33)	133±9	0.25±0.04	9.35±0.07	9.05±0.08	0.83±0.02	0.62±0.05
h2Rlx (Gln)	136±10	0.31±0.02	9.17±0.05	9.06±0.17	0.85±0.02	0.68±0.03
hlRlx (B2–29)	100±10	0.19±0.02	8.74±0.05*	8.83±0.04*	0.60±0.06	0.45±0.05
hlRlx (B2–33)	107±17	0.31±0.03	8.97±0.16	8.67±0.12	0.71±0.06	0.61±0.03
Porcine native	113±16	0.25±0.05	9.12±0.07	8.91±0.03	0.71±0.07	0.61±0.03
Porcine proRlx	91±7	0.21±0.03	9.01±0.06	8.95±0.06	0.66±0.09	0.50±0.05
Rat synthetic	123±13	0.24±0.02	8.60±0.05‡	8.69±0.09†	0.78±0.05	0.61±0.03
Rat native	117±17	0.23±0.05	8.52±0.08‡	8.60±0.09†	0.69±0.06	0.55±0.08
Rat A [S^{11}, S^{24}] (1–24)	nb	nb	nb	nb	nb	nb
Rat A [C^{1}, S^{11}]-bis-cyclic	nb	nb	nb	nb	nb	nb
Rat B (15–25)	nb	nb	nb	nb	nb	nb
hlRlx B [$S^{11,23}$] (2–29)	nb	nb	nb	nb	nb	nb
Human insulin	nb	nb	nb	nb	nb	nb
Sheep IGF-1	nb	nb	nb	nb	nb	nb

Values given are means±s.e.mean; all $n=6$. nb=no bioactivity or no antagonist activity, $n=2$. *$P<0.05$ c.f. h2Rlx (B29). †$P<0.05$ c.f. h2Rlx (B29) or porcine relaxin. ‡$P<0.05$ c.f. h2Rlx (B29) or porcine relaxin. [#]Relative to maximum response produced by 0.1 μM (−)-isoprenaline.

and prorelaxin followed the pattern seen with the h2Rlx, where the pEC_{50} values in the uterine assay were significantly less than those in the atrial bioassay ($P<0.05$, Table 2). Rat native ($n=5$) and synthetic relaxins ($n=4$) were active in this assay and did not significantly differ in their activity (Table 3, Figure 3c). In contrast to the atrial bioassay where rat relaxins were less potent than their human counterparts, rat relaxins relaxed uterine myometrium with pEC_{50} values that were not significantly different from the human gene 2 relaxins or the porcine relaxins (Table 3).

Discussion

The present study demonstrates that the rat isolated atrial and uterine assays are sensitive, reproducible graded assays suitable for investigation of the bioactivity of relaxins from a variety of species and of a number of synthetic analogues. The results showed that all the relaxins having an intact A- and B-chain were active, while the synthetic single-chain analogues were all inactive and in addition did not act as antagonists since responses to h2Rlx (B29) were unaffected. The structural homologues of relaxin, human insulin and sheep insulin-like growth factor I had no agonist action and did not block the effect of h2Rlx (B29) in the atrial assay. This, together with data from an earlier study with α-, β-adrenoceptor and 5-HT_2 receptor antagonists (Kakouris *et al*., 1993), suggest that the relaxins produce the chronotropic and inotropic responses through a unique receptor. The existence of such a receptor for relaxins was also shown in a binding study where angiotensin II, atrial natriuretic peptide, insulin and insulin-like growth factor I did not compete with radiolabelled relaxin for relaxin receptors in rat atria (Osheroff *et al*., 1992).

The h1Rlx (B2–29) was synthesized based on the amino acid sequence deduced from molecular studies (Hudson *et al*., 1983) and it has yet to be established whether this peptide is produced *in vivo*. The peptide was active in the atrial assay with a potency slightly less than that for h2Rlx (B29). It has also been examined in porcine and human isolated uterine myometrium. H1Rlx (B2–29) at a single concentration (0.13 μg ml^{-1} or 22 nM) was as active as h2Rlx (B29) in inhibiting the spontaneous contraction of the porcine myometrium but both had minimal activity in human myometrium (MacLennan *et al*., 1995), leading to the suggestion that the receptors in the human myometrium may differ from those in the porcine myometrium. However, in this study concentration-response curves were not constructed and hence pEC_{50} values were not available to allow a direct comparison of the activity of the h1Rlx (B2–29) and h2Rlx (B29).

The effect of B-chain shortening, by removing amino acid residues from the carboxyl terminal of the B-chain of the h1Rlx (B2–29), on the bioactivity of the peptide was also investigated in the atrial assay. The h1Rlx (B2–26) was less potent than h1Rlx (B2–29) in producing chronotropic and inotropic responses, with the concentration-response curves being shifted to the right. Removal of a further 2 amino acid residues rendered the peptide (h1Rlx (B2–24)) inactive. The progressive reduction of B-chain length may disrupt the molecular structure of the relaxins, especially with the h1Rlx (B2–24) where the disulphide bond between the cysteines B23 and A24 may be affected. Earlier studies with porcine relaxin also indicated that the length of the carboxyl terminus of the B-chain was important for biological activity (Tregear *et al*., 1982; 1983; Frieden *et al*., 1988). More detailed studies will be needed to confirm this possibility.

The activity of native porcine relaxin did not differ from that of the h2Rlx (B29) in the atrial and uterine bioassays. This was in agreement with data from studies in the mouse interpubic ligament assay (Ferraiolo *et al*., 1989; Büllesbach *et al*., 1992). However, h2Rlx (B29) and porcine relaxins behaved differently in different tissues and were more potent in the rat atria than in the uterus. The precursor peptide prorelaxin, which has not been studied before, also possessed the full activity of the porcine relaxin on a molar basis. The n, n+4 arginines, which are known to interact with relaxin receptors (Büllesbach *et al*., 1992) do not appear to be sterically hindered by the presence of the C-peptide in prorelaxin (Sherwood, 1994). If this is correct, we could predict that all prorelaxins should be as active as their respective mature relaxins.

The rat relaxins, both synthetic and native, were significantly less potent than h2Rlx (B29) and porcine relaxins in the atrial bioassay but equally potent in the uterine assay. In the mouse interpubic ligament assay, rat relaxin was less potent than porcine relaxins (Sherwood, 1979; 1982), but the present study showed for the first time that the difference in the activity of relaxins may be tissue specific. Native rat relaxin is known to be less potent than the synthetic peptide in the mouse assay, possibly due to denaturation of the native peptide during the harsh extraction process (Büllesbach & Schwabe, 1996). This difference was not observed here where the chemical integrity of the synthetic and native peptides was determined (Wade *et al*., 1997). The differences in the activity of h2Rlx (B29), porcine and rat relaxins in rat atria and uterus might suggest that the receptors which mediate the chronotropic and inotropic responses in the rat atria differ from those mediating the relaxation responses in the rat uterus. However, this is not supported by receptor autoradiographic studies with ^{32}P-labelled h2Rlx (B33) which have shown similar binding affinities in both tissues (Osheroff *et al*., 1992).

Another explanation for the anomalies observed for the h2Rlx (B29), porcine and rat relaxins may lie in the 3 amino acid residues which sit between the n, n+4 arginines in the B-

Table 3 Comparison of the pEC_{50} and efficacy of relaxins and synthetic analogues in relaxing the longitudinal (L) and circular (C) smooth muscle of rat isolated left and right uterine horn

	pEC$_{50}$				*Efficacy*$^{\#}$			
	Left L	*Left C*	*Right L*	*Right C*	*Left L*	*Left C*	*Right L*	*Right C*
h2Rlx (B29)	8.30±0.12	8.26±0.11	8.18±0.04	8.32±0.14	0.66±0.09	0.79±0.05	0.63±0.06	0.77±0.06
h2Rlx (B33)	8.17	8.17	8.12	8.17	0.76	0.81	0.68	0.77
Porcine native	8.44±0.08	8.33±0.09	8.19±0.19	8.39±0.12	0.72±0.03	0.80±0.02	0.70±0.05	0.84±0.02
Porcine proRlx	8.64	8.70	8.78	8.76	0.64	0.69	0.67	0.79
Rat synthetic	8.47±0.12	8.42±0.11	8.50±0.08	8.50±0.11	0.71±0.07	0.82±0.07	0.70±0.08	0.79±0.05
Rat native	8.35±0.10	8.62±0.07	8.33±0.08	8.68±0.04	0.63±0.08	0.79±0.05	0.67±0.07	0.82±0.03

Values given are mean±s.e.mean, $n=2-5$. $^{\#}$Relative to maximum response produced by 50 μM papaverine.

chain. These arginines are conserved in all relaxins studied and their replacement reduces or abolishes the activity of the peptides (Büllesbach *et al*., 1992). Incorporation of these important amino acid residues into the human insulin molecule produces a *bona fide* relaxin with all the bioactivity of a relaxin while maintaining 10% of the original insulin activity (Büllesbach *et al*., 1996). H2Rlx (B29) and porcine relaxins have identical amino acid residues (Glu-Leu-Val) at this position in contrast to rat relaxin where the residues are Gly-Tyr-Ala. This difference may change the spatial disposition of the arginines and affect the interaction with the receptors, thus determining the binding ability of the peptides. Whale relaxins have the Glu-Leu-Val sequence and are equipotent with the porcine relaxin (Schwabe *et al*., 1989), whereas skate (Asp-Leu-Ile) (Büllesbach *et al*., 1987), shark (Glu-Phe-Ile) (Reinig *et al*., 1981; Büllesbach *et al*., 1986), dog (Asp-Tyr-Val) (Stewart *et al*., 1992) and horse (Glu-Leu-Ala) (Stewart & Papkoff, 1986) relaxins are all less potent than porcine relaxin. Experiments in which the Glu-Leu-Val combination in h2Rlx (B29) and porcine relaxins are exchanged with Gly-Tyr-Ala to form chimeric relaxins or *vice versa* with rat relaxin would be of great interest to study this possibility. In a recent study, a rat relaxin analogue with a Asp-Leu-Val sequence was a better ligand than rat relaxin in a receptor binding assay but was less potent in the mouse interpubic ligament assay (Büllesbach & Schwabe, 1996), indicating the importance of these residues.

The signalling mechanisms utilized by relaxin in the atria and uterus are still the subject of debate. The protein kinase A (PKA) inhibitors PKI (5–22) (Han *et al*., 1994) and 5–24 amide (Piedras-Rentería *et al*., 1997a;b) inhibit the responses of cardiac cells to relaxin. In the uterus, the PKA inhibitor H-8 inhibited the responses of myometrial cells to relaxin (Anwer *et al*., 1990; Meera *et al*., 1995). Nevertheless, adenosine 3′:5′-cyclic monophosphate (cyclic AMP) may not be the sole second messenger involved because the time course for its generation appears to follow rather than precede the mechanical responses (Cheah & Sherwood, 1980; Judson *et al*., 1980; Sanborn *et al*., 1980; Osa *et al*., 1991). Furthermore, low concentrations of relaxin failed to elevate cyclic AMP levels in the rat isolated left atria although maximum inotropic responses were observed (Summers *et al*., 1995). In an *in vivo* study in rat, relaxin induced only small increases in myometrial cyclic AMP in comparison to salbutamol, a β_2-adrenoceptor agonist (Downing *et al*., 1992). In human granulosa-lutein cells, relaxin released Ca^{2+} from intracellular stores possibly via activation of IP_3-PKC (Mayerhofer *et al*., 1995). These observations indicate that both IP_3 and cyclic AMP may be utilized in signal transduction. Opening of ATP-sensitive K^+ channels with consequent closing of voltage-operated Ca^{2+} channels (Hamilton *et al*., 1986; Noack *et al*., 1992) has also been suggested as a mechanism for myometrial relaxation. However, glibenclamide, an inhibitor of ATP-sensitive K^+ channels (Quast & Cook, 1988) reduced the responses of rat uterus to relaxin *in vivo* (Downing & Hollingsworth, 1991) but had no effect *in vitro* (Hughes *et al*., 1992). In addition to these possibilities, it should also be borne in mind that relaxin is a structural homologue of insulin. Therefore, the relaxin receptor may well be a tyrosine kinase and share the intracellular signalling cascades of the insulin receptor. This may explain the multiplicity of effects observed and the poor temporal correlation between second messenger changes and the response to relaxin.

In conclusion, this study used the rat isolated atrial and uterine bioassays to provide a quantitative analysis of the bioactivity of a variety of relaxins. The results showed that rat relaxin was less potent than h2Rlx (B29) and porcine relaxin in the atrial assay, but was equipotent in the uterine assay, suggesting that the coupling mechanisms or the second messengers involved in producing the responses were different in the rat atria and uterus. The presence of both A- and B-chains with intact disulphide bonds and the length of the carboxyl terminus of B-chain were important to preserve the bioactivity of the relaxin. Porcine prorelaxin was shown, for the first time, to be as active as the mature porcine relaxin in both atrial and uterine bioassays, indicating that the presence of the C-peptide may not affect the binding of the peptide to the receptor. The ease of setting up the atrial and uterine bioassays to obtain quantitative results makes these assays more preferable to the guinea-pig pubic symphysis palpation and mouse ligament assays for the investigation of structure-activity relationships of the relaxin peptide.

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