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Quantitative autoradiographic studies of relaxin binding in rat atria, uterus and cerebral cortex: characterization and effects of oestrogen treatment

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1 The binding characteristics of the relaxin receptor in rat atria, uterus and cortex were studied using a [^{33}P]-labelled human gene 2 relaxin (B33) and quantitative receptor autoradiography.

2 The binding kinetics of [^{33}P]-human gene 2 relaxin (B33) were investigated in slide-mounted rat atrial sections. The binding achieved equilibrium after 60 min incubation at room temperature ($23 \pm 1^{\circ}$C) and dissociated slowly. The association and dissociation rate constants were $4.31 \pm 0.34 \times 10^{8}$ M^{-1} min^{-1} and $1.55 \pm 0.38 \times 10^{-3}$ min^{-1} respectively. Thus, the kinetic dissociation constant was 3.46 ± 0.59 pM.

3 Binding was saturable to a single population of non-interacting sites throughout atria, in uterine myometrium and the 5th layer of cerebral cortex. The binding affinities (pK_D) of [^{33}P]-human gene 2 relaxin (B33) were 8.92 ± 0.09 in atrial myocardium and 8.79 ± 0.04 in cerebral cortex of male rats, and 8.79 ± 0.10 in uterine myometrium. Receptor densities in the cerebral cortex and atria were higher than in uterine myometrium, indicating that relaxin also has important roles in non-reproductive tissues.

4 In male rats, treatment with 17β-oestradiol (20 μg in 0.1 ml sesame oil s.c., 18–24 h) significantly decreased the density of relaxin receptors in atria and cerebral cortex. Identical treatment in female rats had no significant effect in atria and cerebral cortex, but it significantly increased the density of relaxin receptors in uterine myometrium.

5 Relaxin binding was competitively displaced by porcine and rat native relaxins. Porcine native relaxin binds to the relaxin receptor in male rat atria (8.90 ± 0.02), and cerebral cortex (8.90 ± 0.03) and uterine myometrium (8.89 ± 0.03) with affinities not significantly different from human gene 2 (B33) relaxin. Nevertheless, rat relaxin binds to the receptors with affinities (8.35 ± 0.09 in atria, 8.22 ± 0.07 in cerebral cortex and 8.48 ± 0.06 in uterine myometrium) significantly less than human gene 2 (B33) and porcine relaxins.

6 Quantitative receptor autoradiography is the method of choice for measurement of affinities and densities of relaxin receptor in atria, uterine myometrium and cerebral cortex. High densities were found in all these tissues. 17β-Oestradiol treatment produced complex effects where it increased the densities of relaxin receptors in uterus but decreased those in atria and cerebral cortex of the male rats, and had no effect on the atria and cerebral cortex of the female rats.

Keywords: Relaxin receptor; quantitative receptor autoradiography; rat atria; rat uterus; rat brain; binding affinities; receptor densities

Introduction

Relaxin is well established as a hormone of pregnancy and acts on the smooth muscle and connective tissue of the reproductive tract to facilitate parturition (see review by Sherwood, 1994). Relaxin also has direct effects on non-reproductive tissues and produces powerful chronotropic and inotropic responses in rat isolated atria (Kakouris *et al*., 1993), participates in the regulation of blood pressure (Mumford *et al*., 1989; Parry *et al*., 1990) and fluid balance (Weisinger *et al*., 1993), and releases vasopressin and oxytocin (Dayanithi *et al*., 1987; Way & Leng, 1992) by activating the neurosecretory magnocellular hypothalamic nuclei (Osheroff & Phillips, 1991; McKinley *et al*., 1997). These effects may relate to some of the physiological changes observed in females during pregnancy.

The biological actions of relaxin are mediated through specific receptors located on the surface of the target cells. Closely related structural homologues such as insulin and insulin-like growth factors do not cross react with relaxin in either binding or functional assays (Osheroff *et al*., 1990; 1992; Osheroff & Phillips, 1991; Tan *et al*., 1998). Development of a receptor binding assay to characterize these receptors has been difficult. Using a [^{125}I]-labelled porcine relaxin prepared with the modified method of Bolton & Hunter (1973), receptors for relaxin were studied in the homogenate preparations of rat myometrium (Mercado-Simmen *et al*., 1980; 1982a), mouse uterus (Yang *et al*., 1992) and porcine myometrium and cervix (Mercado-Simmen *et al*., 1982b). Incubation of tissue homogenates with radiolabelled relaxin followed by centrifugation to separate bound and free ligand shows high levels of non-specific binding (Yang *et al*., 1992; Garibay-Tupas *et al*., 1995) because relaxin binds to many materials (Greenwood *et al*., 1981). Our initial attempts to characterize the receptor in atria, uterus and cerebral cortex using tissue homogenate followed by filtration were unsuccessful, as specific binding was undetectable in the atria and was less than 50% of the total binding in uterus and cerebral cortex (Figure 1).

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A technique originally described for the preparation of [^{32}P]-labelled human γ-interferon (Rashidbaigi *et al*., 1985) has been used successfully to synthesize a biologically active [^{32}P]-labelled human gene 2 relaxin (h2Rlx (B33)) (Osheroff *et al*., 1990). [γ-^{32}P]-ATP specifically monophosphorylates the serine residue in the position B32 of the h2Rlx (B33) without affecting other serine residues in the A- and B-chain, thus producing a radioligand free of contamination from analogues with alternative phosphorylation sites (Osheroff *et al*., 1990). This radioligand was tested for its bioactivity and was found to be similar to unlabelled h2Rlx (B33) in increasing cyclic AMP levels in primary human uterine cell line (Osheroff *et al*., 1990). Receptor autoradiography was then used to demonstrate specific binding sites for relaxin located in the smooth muscle layer of the rat uterus (Osheroff *et al*., 1990; 1992), cervix (Osheroff *et al*., 1990), and for the first time in rat atria (Osheroff *et al*., 1992) as well as in a number of areas of rat brain (Osheroff & Phillips, 1991).

In the present study, we have produced [33p]-labelled h2Rlx (B33) that when combined with phosphorimaging, allows rapid development of images that can be accurately quantitated (Figure 2). The advantage of using [^{33}P] is that it emits low energy radioactive particles which react only with image producing media that lie immediately in their path, thereby giving images of high resolution (Hudson, 1993). Quantitative receptor autoradiography enables the determination of the density of relaxin receptors and the affinity of binding, so allowing the characterization of the receptor in atria, brain and uterus which has mainly involved functional assays. We also examined the role of oestrogen in the control of relaxin receptor expression in both the male and female since oestrogen priming has been shown to increase the response of female reproductive organs to relaxin (reviewed by Sherwood, 1994) and increases binding sites in the smooth muscle of uterus (Osheroff *et al*., 1992). 17β-Oestradiol treatment significantly reduced the relaxin binding sites in male atria and cerebral cortex, had no significant effect in female atria and cerebral cortex, and increased the binding sites in uterine myometrium. The reason for this differential receptor regulation is yet to be determined. Competition

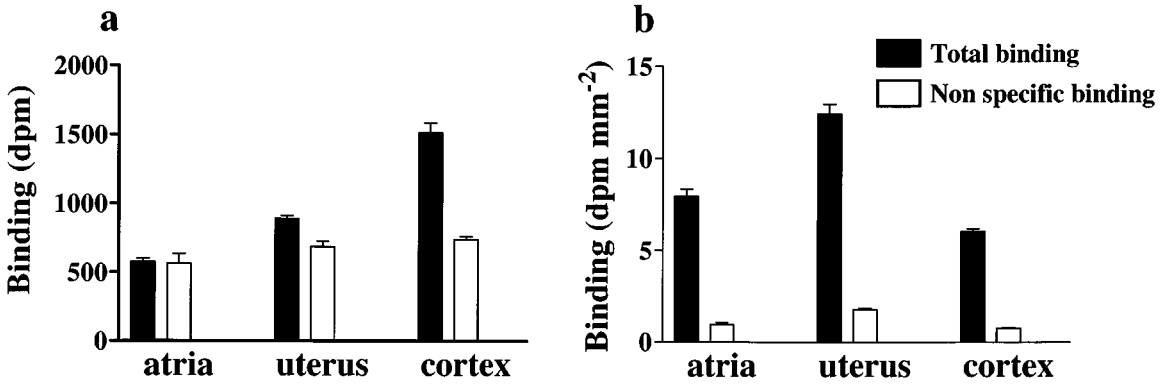

Figure 1 Levels of total and non-specific binding obtained in rat atria, uterus and cerebral cortex using (a) homogenate binding assays and (b) receptor autoradiography. Note that specific binding was not detected in atria and was less than 50% in uterus and cortex with homogenate binding assays. With receptor autoradiography, non-specific binding was reduced to 10–20% of total binding.

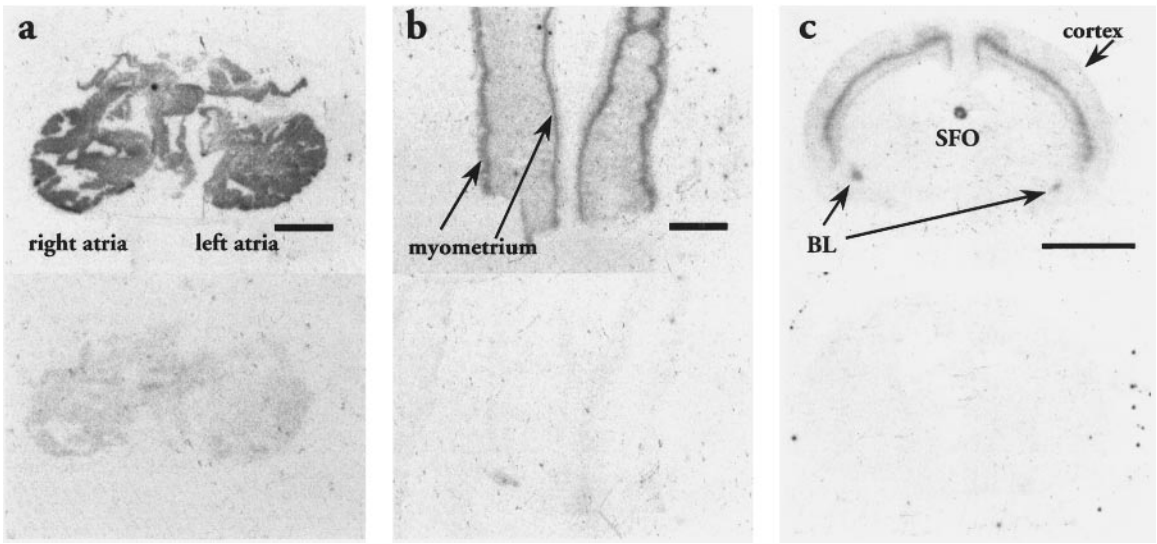

Figure 2 Autoradiographic localization of [^{33}P]-h2Rlx (B33) binding to slide-mounted sections of rat (a) atria with non-specific binding (NSB) in lower panel (bar = 2 mm), (b) uterus with NSB in lower panel (bar = 2 mm) and (c) brain with NSB in lower panel (bar = 5 mm). Note that the specific binding was evenly distributed in atria but was confined to myometrium and specific areas in the brain, such as the 5th layer of cortex, SFO and basolateral amygdaloid nucleus (BL).

studies were also performed to determine the binding affinities of porcine and rat native relaxins in these tissues, which allows comparison with the relative potencies of the peptides in functional studies (Tan *et al*., 1998). [^{33}P]-h2Rlx (B33) and porcine relaxin bind to rat atrial, uterine and cerebral cortical sections with similar affinities, but rat relaxin binds with significantly lower affinities to these tissues. This observation is in contrast to previous findings which demonstrated that h2Rlx (B33), porcine and rat relaxins had similar potency in relaxing K^+-precontracted uterine myometrium (Tan *et al*., 1998).

Methods

Tissue preparation for receptor autoradiography

Sprague-Dawley rats (male and intact female, 200–250 g) were randomly separated into control and hormone treatment groups. The rats in the treatment group were treated for 18–24 h with a single injection of 20 μg 17β-oestradiol in sesame oil (0.1 ml, s.c.). The heart and brain from male and female rats, and uterus from female rats were then isolated after anaesthetizing the animals with 80% CO_2 in 20% O_2 and decapitation. Atria and uterus were dissected and trimmed in chilled 1:1 Krebs phosphate (KP):0.32 M sucrose solution (composition in mM: NaCl, 119; KCl, 4.8; $MgSO_4.7H_2O$, 1.2; $CaCl_2$, 1.24 and $NaH_2PO_4.2H_2O$, 10.0, pH 7.2). The brain was washed briefly in KP:sucrose to remove blood. The tissues were then frozen in isopentane precooled with liquid nitrogen and were either stored at -70°C or mounted in OCT compound (Miles Inc. U.S.A.) for sectioning in a cryostat at -23°C (Jung Frigocut 2800E, Leica). Sections (10 μm) were collected on precleaned microscope slides coated with gelatin/chromic potassium sulphate (Young & Kuhar, 1979). Every tenth brain section was stained with 0.5% cresyl violet to locate the organum vasculosum of the lamina terminalis (OVLT) and subfornical organ (SFO).

Labelling of human gene 2 (B33) relaxin

H2Rlx (B33) was labelled with [^{33}P] using the catalytic subunit of cyclic AMP-dependent protein kinase (cAMPdPK) according to the procedure of Osheroff *et al*. (1990) with modifications. Briefly, 4 μg of h2Rlx (B33) relaxin was mixed with a reaction mixture of 20 mM Tris-HCl, 100 mM NaCl, 20 mM $MgCl_2$, pH 7.5, 10 μCi of [γ-^{33}P]-ATP (specific activity 2000 Ci mmol^{-1}, DuPont), 10 μg of lyophilized BSA in 0.1% trifluoroacetic acid (TFA, 10 mg ml^{-1}) and 400 units of cAMPdPK from bovine heart (Promega), and incubated in a water bath at 37°C for 1 h. At the end of the incubation period, the mixture was chilled on ice to stop the reaction and was loaded onto a Sep-Pak C_{18} cartridge (Waters) precoated with BSA (1 mg ml^{-1} in 0.1% TFA) and equilibrated with 1 mM ATP in 0.1% TFA. The cartridge was then washed with 1 mM ATP in 0.1% TFA followed by 10% acetonitrile in 0.1% TFA. [^{33}P]-Labelled and unlabelled relaxins were then eluted with 80% acetonitrile in 0.1% TFA. Fractions (10 μl) were analysed for radioactivity by liquid scintillation spectrometry.

Separation of [^{33}P]-labelled and unlabelled relaxins was then performed using ion exchange high performance liquid chromatography (Waters). The radioactive eluate from Sep-Pak C_{18} was injected onto a Poly CAT A column (Poly LC, U.S.A.) equilibrated with 25 mM KH_2PO_4, pH 7 in 25% acetonitrile (v v^{-1}) (buffer A). The column was then eluted with a linear gradient of 0–0.5 M KCl in buffer A over 50 min at 1 ml min^{-1}. Fractions were collected at 1 min intervals and aliquots (10 μl) were counted for radioactivity. The fraction with peak radioactivity was then stored at 4°C after adding 1 mg of lyophilized BSA and used within 7–10 days.

Binding kinetics of [^{33}P]-h2Rlx (B33)

The association and dissociation rate constant of the [^{33}P]-h2Rlx (B33) were investigated at room temperature (23 ± 1°C) and at 37°C using the atrial sections from untreated male rats. The slide-mounted sections were first covered in 100 μl of 25 mM HEPES, 300 mM KCl, pH 7.2, 1 mM of phenylmethylsulphonylfluoride (PMSF) for 30 min in a moisture chamber. After removing the preincubation buffer, the slides were incubated in 100 μl of HEPES/KCl/BSA (1 mg ml^{-1}) containing approximately 100 pM of [^{33}P]-h2Rlx (B33) for 5, 10, 20, 30, 60 or 120 min. The slides were rinsed briefly and washed (2×10 min) in HEPES/KCl, followed by a brief rinse in distilled water and were then blown dry with cooled dehumidified air. They were then apposed for 2 days to phosphorimaging plates which were then scanned with Phosphorimager SI (Molecular Dynamics, U.S.A.). The images were analysed using the computer program ImageQuant (Molecular Dynamics, U.S.A.) and the intensities, expressed arbitrarily as phosphorimager units (PU) were obtained. These values were converted to d.p.m. mm^{-2} using standards made with [γ-^{33}P]-ATP in rat ventricle paste, frozen and cut in a cryostat (10 μm), and either mounted onto a microscope slide or the radioactivity directly counted by liquid scintillation spectrometry (Figure 3). The standards were

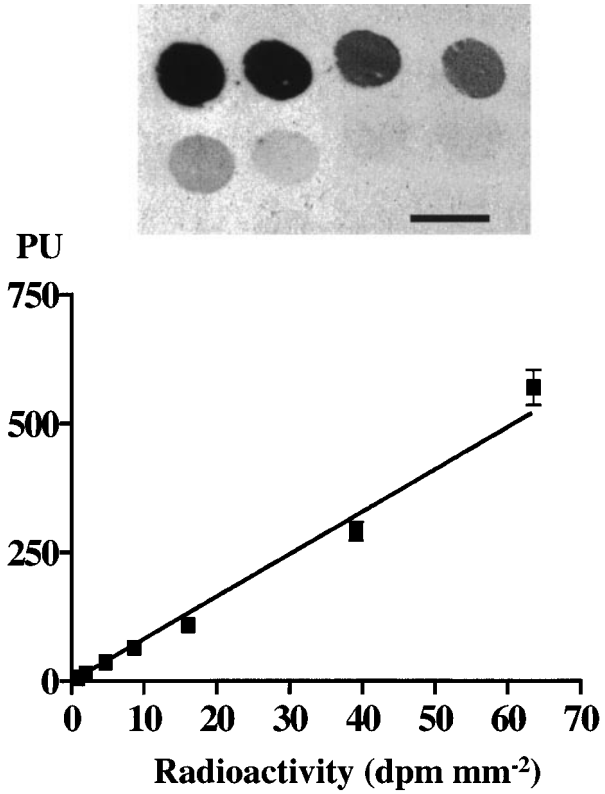

Figure 3 Phosphorimager images of [^{33}P] standards prepared as described in Methods (bar = 5 mm). The relationship between intensity (expressed as phosphorimager units (PU)) produced by different levels of radioactivity (expressed as d.p.m. mm^{-2}) is shown in the lower panel. Note that the curve is linear over the range of radioactivity used in this study and does not approach saturation.

apposed to the phosphorimaging plate together with the labelled tissue sections. After subtraction of non-specific binding which was defined by the presence of 1 μM of h2Rlx (B29), the data were fitted using the one phase exponential association function in the computer program PRISM (GraphPad Inc., San Diego, U.S.A.).

For dissociation experiments, after 30 min preincubation in PMSF buffer and 60 min incubation with 100 pM of [^{33}P]-h2Rlx (B33), the slides were washed in 30 ml HEPES/KCl/BSA without [^{33}P]-h2Rlx (B33) for 10, 20, 30, 60, 120 or 240 min. This was followed by the washing and image processing steps as described for association studies. Data obtained were analysed using the single phase exponential decay function in PRISM.

Saturation experiments

Slide-mounted sections of atria, brain and uterus from control and treated male and female rats were preincubated at room temperature in PMSF buffer. They were then incubated with 100 pM of [^{33}P]-h2Rlx (B33) for 90 min in the absence or presence of increasing concentrations of unlabelled h2Rlx (B33) (100 pM to 3 nM). Non-specific binding was defined with 1 μM of h2Rlx (B29) relaxin. The slides were then processed and images analysed. The amount of protein in each tissue section was determined by Coomassie brilliant blue (1.5% w v^{-1}) staining (Miller *et al.*, 1988; Summers, 1993). Binding data were first converted to d.p.m. mm^{-2} using radioactive standards (Figure 3). They were then further transformed according to the proportion of radiolabelled and total concentrations of h2Rlx (B33) present in each incubation solution, and corrected for the amount of protein. The transformed binding data were analysed using the one site binding function in PRISM to obtain estimates of binding affinity (pK_D) and the maximum density of binding sites (B_{max}).

Competition experiments with porcine and rat native relaxins

The experimental protocol was similar to that described above for saturation experiments but higher final concentrations were used (up to 50 nM). Binding data were analysed using the single site competition function in PRISM and the binding affinities (pK_i) of porcine and rat relaxins were calculated using the built-in Cheng & Prusoff equation (Cheng & Prusoff, 1973).

Statistics

Values are given as mean $\pm$ s.e.mean from experiment performed in n number of animals. Student's unpaired t-test was used to compare the binding affinities and the number of binding sites in male and female rats, the effects of hormone treatment and the binding affinities of porcine and rat relaxins with those of h2Rlx (B33). Probability (P) values of less than 0.05 were regarded as statistically significant.

Materials

Recombinant human gene 2 relaxins (B33 and B29 forms) (Genentech); porcine and rat native relaxin (Howard Florey Institute); cyclic AMP-dependent protein kinase (Promega); [γ-^{33}P]-ATP (DuPont); N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES) and cresyl violet (BDH); acetonitrile (EM Science); phenylmethylsulphonylfluoride (PMSF), bovine serum albumin (BSA), adenosine 5′-triphosphate (ATP), 17β-oestradiol (1,3,5[10]-oestratriene-3,17β-diol) and Coomassie brilliant blue (Sigma)

Results

Binding kinetics

Binding of [^{33}P]-h2Rlx (B33) was measured at each time point in quadruplicate sections. Since the binding in sections of right and left atrium did not differ significantly (Figure 2a), these results were pooled. The binding in atrial sections achieved equilibrium after 60 min of incubation at 23°C. The association rate constant (k_1) was $4.31 \pm 0.34 \times 10^8$ M^{-1} min^{-1} ($n = 6$, Figure 4a). Dissociation of the bound radioligand occurred at a very slow rate. After 240 min of washing, more than 50% of the radioligand still remained bound to the receptors (Figure 4b). The dissociation rate constant (k_{-1}) was $1.55 \pm 0.38 \times 10^{-3}$ min^{-1} ($n = 6$). The kinetic dissociation constant (K_D) was therefore 3.46 ± 0.59 pM.The association and dissociation rate constants and the kinetic K_D were all higher at 37°C. Due to evaporation of the binding buffer after prolonged incubation which increased non-specific binding and resulted in deterioration of the tissue sections, all subsequent saturation and competition studies were conducted at 23°C.

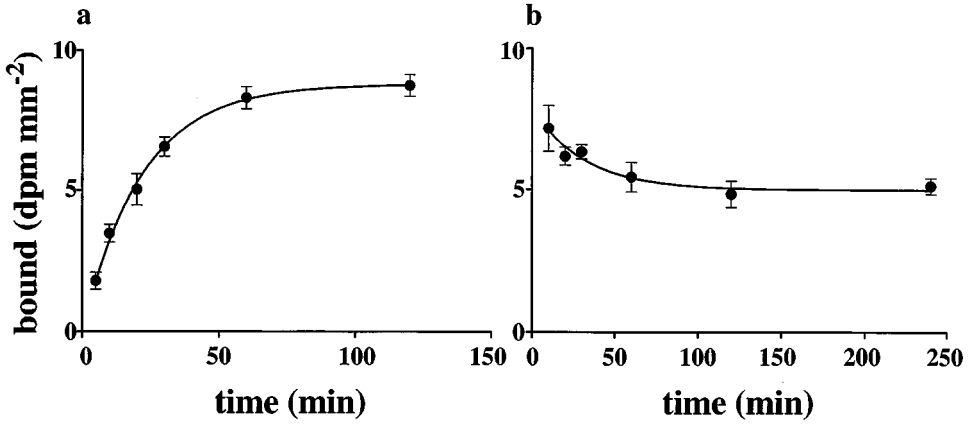

Figure 4 Binding kinetics of [^{33}P]-h2Rlx (B33) to slide mounted sections of rat atria at 23 $\pm$ 1°C. Shown here are mean relationships for (a) association and (b) dissociation of radioligand to sections of atrial myocardium. Note that the binding (a) reaches equilibrium within 60 min and then (b) dissociates slowly on infinite dilution.

Saturation experiments

Binding of [^{33}P]-h2Rlx (B33) to atrial sections and the effect of 17β-oestradiol treatment Saturation isotherms (Figure 5) showed that the binding of [^{33}P]-h2Rlx (B33) was saturable to a single population of non-interacting sites in atria. Binding affinities (pK_D) of the radioligand for atrial sections from male rats (8.92 ± 0.09, $n=6$) was not significantly different from that for atria from female rats (9.01 ± 0.10, $n=6$, $P=0.55$). Treatment with 17β-oestradiol did not have a significant effect on the binding affinities of the radioligand to atrial sections from male rats (9.16 ± 0.12, $n=6$, $P=0.14$) or female rats (8.87 ± 0.07, $n=6$, $P=0.29$). Receptor densities determined in male rat atria (5.91 ± 0.83 fmol mg protein^{-1}, $n=6$) were not significantly different from those in atria from females (4.72 ± 0.89 fmol mg protein^{-1}, $n=6$, $P=0.35$). However, oestradiol treatment significantly decreased the receptor number in the male (3.47 ± 0.39 fmol mg protein^{-1}, $n=6$, $P=0.02$) but had no significant effect in the females (3.22 ± 0.30 fmol mg protein^{-1}, $n=6$, $P=0.14$) (Figure 6).

Binding of [^{33}P]-h2Rlx (B33) to uterine sections and the effect of 17β-oestradiol treatment Binding of the radioligand to the uterine sections was also saturable to a single non-interacting site but the binding sites were confined to myometrium (Figure 2b). The binding affinity (pK_D) in non-treated female rats (8.79 ± 0.10, $n=6$) was not significantly different from that obtained in atria from the same group of rats ($P=0.16$). Oestradiol treatment did not significantly alter the binding affinities of the radioligand to relaxin receptors (8.81 ± 0.05, $n=6$, $P=0.88$) but significantly increased the number of binding sites in the uterine smooth muscle from 2.91 ± 0.42 fmol mg protein^{-1} in untreated to 4.64 ± 0.37 fmol mg protein^{-1} in oestradiol treated rats ($n=6$, $P=0.01$) (Figure 6).

Binding of [^{33}P]-h2Rlx (B33) to brain sections and the effect of 17β-oestradiol treatment Binding to the cerebral cortex was confined to the 5th layer (Figure 2c; Swanson, 1992) and was saturable to a single population of sites. The binding affinity (pK_D) for [^{33}P]-h2Rlx (B33) in the male (8.79 ± 0.04, $n=6$) was not significantly different from that in female rats (8.85 ± 0.03, $n=6$, $P=0.25$). However, the density of binding sites in the male (11.81 ± 0.60 fmol mg protein^{-1}, $n=6$) was significantly higher than in the female (8.23 ± 0.69 fmol mg protein^{-1}, $n=6$, $P=0.003$). The effects of oestradiol treatment on relaxin receptor density in the cerebral cortex of male and female rats were similar to that obtained in atrial myocardium. In male rats, binding affinities in cerebral cortex were not altered by the hormone treatment (8.90 ± 0.05, $n=6$, $P=0.15$) but a significant reduction in the number of binding sites (8.70 ± 0.84 fmol mg protein^{-1}, $n=6$, $P=0.01$) was observed; while in the female rats, both the binding affinities (8.86 ± 0.05, $n=6$, $P=0.95$) and the number of binding sites (9.71 ± 0.46 fmol mg protein^{-1}, $n=6$, $P=0.11$) in cerebral cortex were not significantly changed by the hormone treatment (Figure 7). The binding affinities of [^{33}P]-h2Rlx (B33) for OVLT (8.84 ± 0.11, $n=6$) and SFO (8.86 ± 0.06, $n=6$; Figure 2c) in untreated male rats were also determined. These values were not significantly different from those in atria and cortex, but the small area covered by these tissues prevents accurate quantitation of the number of binding sites.

Competition studies

Data obtained from competition studies with porcine and rat native relaxins were best fitted by the one site competition

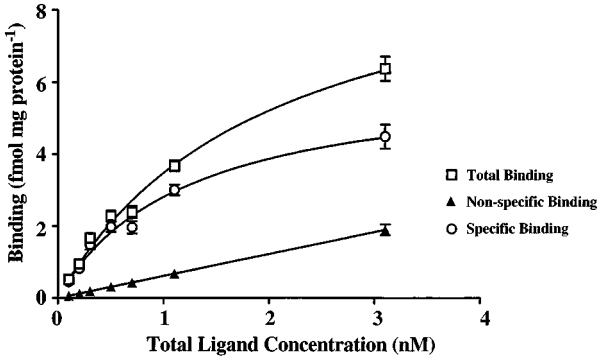

Figure 5 A representative saturation isotherm of [^{33}P]-h2Rlx (B33) binding to male rat atria ($n=6$). Binding was saturable to a single population of non-interacting sites.

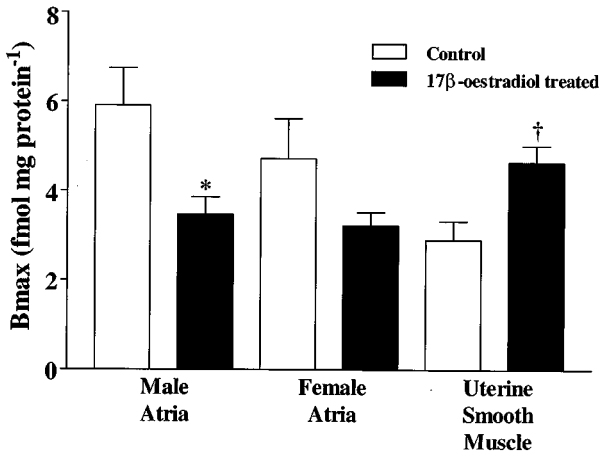

Figure 6 The number (B_{max}) of relaxin receptors, expressed as fmol mg protein^{-1}, determined in either control or 17β-oestradiol treated male and female rat atria and uterus. The hormone treatment significantly reduced the number of receptors in the male atria ($n=6$, $*P=0.02$) but have no affect on female atria. In uterine smooth muscle, the hormone treatment significantly increased the number of binding sites ($n=6$, $\dagger P=0.01$).

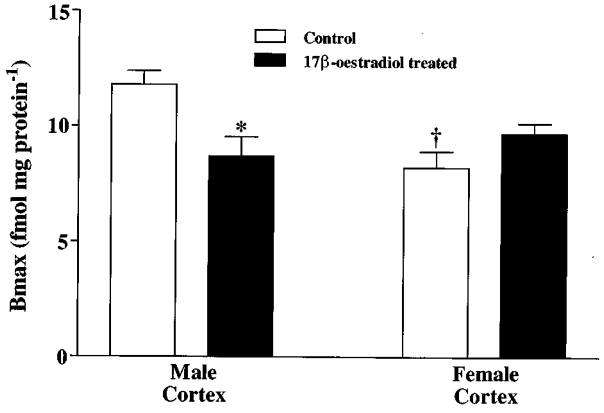

Figure 7 The number (B_{max}) of relaxin receptors, expressed in fmol mg protein^{-1}, determined in either control or 17β-oestradiol treated male and female rat cortex. The number of receptors in the male cortex was significantly more than the female cortex ($n=6$, $\dagger P=0.003$). The hormone treatment significantly reduced the number of receptors in the male cortex ($n=6$, $*P=0.01$) but had no significant effect on the receptor number in the female cortex ($n=6$, $P=0.11$).

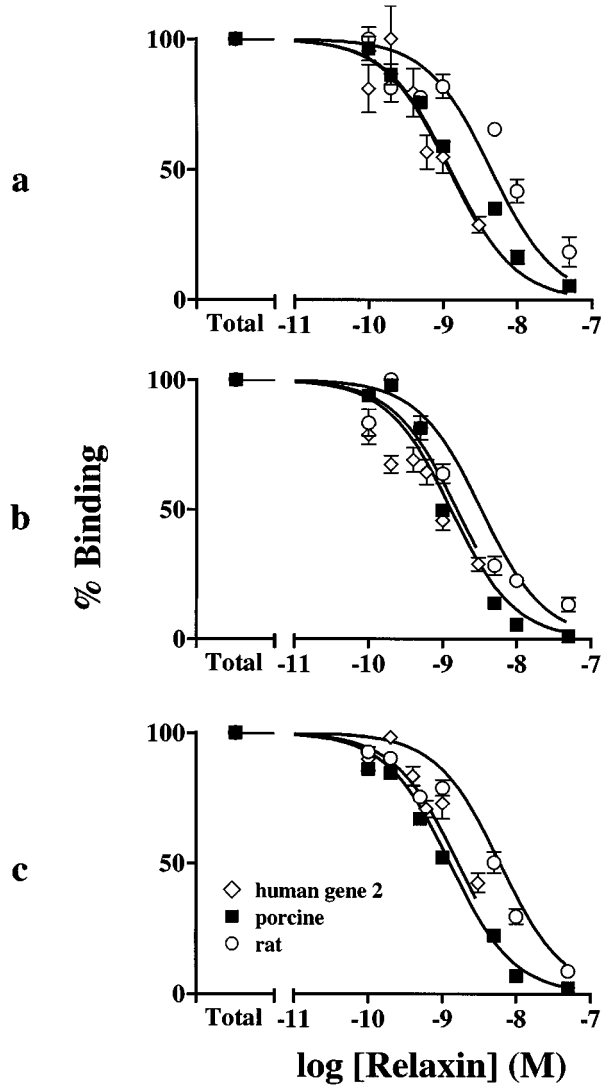

Figure 8 Competition of h2Rlx (B33), porcine and rat relaxin for binding sites in slide-mounted sections of rat (a) atrial myocardium, (b) uterine smooth muscle and (c) cerebral cortex (all $n=6$). Note that the competition curve for rat relaxin was significantly shifted to the right of h2Rlx (B33) and porcine relaxin indicating rat relaxin competed for the binding sites with less affinity than h2Rlx (B33) and porcine relaxin.

function in PRISM. Native porcine relaxin competed for the binding sites with affinities (pK_i) similar to those of h2Rlx (B33) in uterine myometrium from oestradiol treated female rats (8.89 ± 0.03, $n=6$) and in atria (8.90 ± 0.02, $n=6$) and cerebral cortex (8.90 ± 0.03, $n=6$) from untreated male rats ($P>0.05$). Rat native relaxin competed for the binding sites in all tissues ($n=6$) with affinities significantly less than either h2Rlx (B33) or porcine native relaxin. The pK_i values were 8.48 ± 0.06 in uterine myometrium from oestradiol treated female rats, 8.35 ± 0.09 in atria and 8.22 ± 0.07 in cerebral cortex of untreated male rats ($P<0.02$) (Figure 8).

Discussion

Quantitative receptor autoradiography was used to characterize the binding of [^{33}P]-h2Rlx (B33) to rat atria, uterus and cortex. This method was superior to the homogenate binding technique, with levels of non-specific binding of 10–20% of the total binding compared to >50% for homogenate binding (Figure 1).

Binding of [^{33}P]-h2Rlx (B33) to relaxin receptors in the rat atria, uterus and brain was saturable to a single site. This confirms earlier studies using [^{32}P]-h2Rlx (B33) (Osheroff & Phillips, 1991; Osheroff *et al*., 1992) or [^{125}I]-labelled porcine relaxin (Cheah & Sherwood, 1980; Yang *et al*., 1992) but only one study quantitatively measured the density of binding sites (Yang *et al*., 1992). One published study showed that saturation of [^{125}I]-porcine relaxin binding was biphasic in rat uterus (Mercado-Simmen *et al*., 1980). The apparent presence of several binding sites may have been due to the use of a radioligand containing multiple iodinated analogues exhibiting different affinities for the receptors. In the present study, the [^{33}P]-h2Rlx (B33) was eluted from salt gradient HPLC in a single peak.

The dissociation constants determined from the kinetic studies in atria were significantly less than those determined in the saturation studies. Although values from kinetic and saturation studies should ideally be similar, dissociation constants determined in kinetic studies are often higher than those in saturation studies (Molenaar *et al*., 1987). This may be explained by pseudo-irreversible binding characteristics or that relaxin binds to non-receptor sites and remains in close proximity to the receptors thereby allowing reassociation when the receptors become unoccupied. These mechanisms would prolong the apparent dissociation rate and lower the kinetic dissociation constant. The first hypothesis was confirmed in dissociation studies, where more than 50% of labelled relaxin still remained in the tissue after 4 h of washing, and in functional studies where the chronotropic and inotropic responses to relaxin persisted even after 6 h of washing (Tan *et al*., 1998). The second hypothesis has been described in studies on interactions of β_2-agonists with β_2-adrenoceptors (see review by Anderson *et al*., 1994 and Liggett & Green, 1997) but yet to be verified for the relaxin receptor.

The cortex and atria had higher densities of relaxin receptors than the uterine smooth muscle even though the function of relaxin in these tissues is less well defined. The receptor density described here was comparable to a recent homogenate binding study in mouse uterine and brain membranes using [^{125}I]-labelled porcine relaxin (Yang *et al*., 1992). Attempts were made to characterize the relaxin binding sites in the OVLT and SFO but the areas were too small to be accurately quantitated. Published studies have suggested that relaxin receptors may not be evenly distributed in these areas. Central administration of relaxin in conscious rats produces Fos-like immunoreactivity (Fos-LI) in neurons scattered around the most rostral, dorsal and lateral parts of SFO but not in its central caudal zone; while in the OVLT, the neurons exhibiting Fos-LI were situated in its dorsal pole and a few neurons in the central vascular zone and at the lateral edges (McKinley *et al*., 1997). Assuming that only these neurons expressed relaxin receptors, it would require higher resolution receptor autoradiography for proper characterization. The number of neurons with Fos-LI in the caudal part of the SFO was also shown to be significantly less than the rostral part (Heine *et al*., 1997). This may prevent accurate estimation of receptor density since experiments were performed on serially cut sections.

Oestradiol treatment was shown to increase the relaxin binding sites in the uterus but had no effect in both male and female rat atria in a qualitative study (Osheroff *et al*., 1992). Here we measured the affinity and density of binding sites, and confirmed the increased density of binding sites in the myometrium. We also observed a significant reduction of

binding sites in the male atria and cerebral cortex, with no significant change in the female atria and cerebral cortex after the hormone treatment. Oestradiol has been widely used to prepare ovariectomized female animals for testing relaxin bioactivity (Sherwood, 1994). It induces oestrus and promotes growth of the reproductive organs, which could be an explanation for the increase of binding sites in the uterine smooth muscle. Oestradiol treatment may also have additional effect of releasing endogenous relaxin (Goldsmith *et al*., 1982; Lippert *et al*., 1996) which will then bind to and reduce the binding sites available to the radioligand. The tissues studied here were capable of producing relaxin since mRNA for relaxin has been detected in the rat cerebral cortex (Osheroff & Ho, 1993) and heart (Gunnersen *et al*., 1995), and a study has shown that the primary cultures of male rat atrial cardiocytes can release stored relaxin (Taylor & Clark, 1994). The oestradiol treatment produced a significant affect in male rat tissues but not in the female. This may reflect the presence of different levels of circulating relaxin, which is currently below detection in the males.

Binding affinities of porcine native relaxin were similar to those of h2Rlx (B33) in atria, uterus and cortex but rat native relaxin had significantly less affinities in those tissues. The reduced binding activity of rat relaxin was reflected in functional studies where it was less potent in producing positive chronotropic and inotropic effects (Tan *et al*., 1998), and also in the mouse pubic ligament elongation assays (Sherwood, 1979; 1982). Nevertheless, rat relaxin was equipotent with h2Rlx (B33) and porcine relaxin in uterine smooth muscle relaxation assays (Tan *et al*., 1998), differences not observed in binding studies. The mechanism for these anomalies may relate to the control of expression of receptors in the atria and uterus. The expression of relaxin receptors in uterus is under oestrogen control (Osheroff *et al*., 1992), therefore it may be possible to test this hypothesis by examination of binding and functional characteristics for different levels of receptor expression. The amino acid residues located between two arginines in the α-helix of the relaxin B-chain may also have an important role in the difference observed for h2Rlx (B33), porcine and rat relaxins. The arginines are known to participate in the binding of the relaxin to the receptors (Büllesbach *et al*., 1992) and substitution of the amino acid residues in this region has been known to alter the binding and function of the peptides (Büllesbach *et al*., 1992; 1996; Büllesbach & Schwabe, 1996). Since the amino acid residues in this region for h2Rlx (B33) and porcine relaxin are identical (Glu-Leu-Val) but different from rat relaxin (Gly-Tyr-Ala), this may differentiate the binding ability of relaxins to the receptors. The nature of the receptor for relaxin is still under investigation. Studies have indicated that it is similar in size to but distinct from insulin receptor (Büllesbach *et al*., 1995; Osheroff & King, 1995), and receptor activation causes an increase in tyrosine phosphorylation of an intracellular protein (Palejwala *et al*., 1998).

In conclusion, the present study showed that rat relaxin bound to the receptors with affinities lower than h2Rlx (B33) or porcine relaxin in all tissues investigated. These observations agreed with functional studies performed with rat isolated atria but not with those with rat uterus. High densities of relaxin receptors were detected in the atria and cortex, confirming the functional roles of relaxin in non-reproductive tissues. Acute administration of 17β-oestradiol into female rats increased relaxin receptors in the uterus but had no effect in atria and cortex. This treatment also reduced the number of receptors in the male rat atria and cortex.

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