Uptake of relaxin in the uterus and cervix of rats *in vivo*: influence of ovarian steroids and tolerance

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The influence of oestradiol benzoate and progesterone treatment and of tolerance to relaxin on the uptake of ^{125}I -labelled porcine relaxin by reproductive tissues was investigated in anaesthetized female rats. In ovary-intact rats, ^{125}I -labelled relaxin uptake increased with time in reproductive tissues and in tissues concerned with metabolism and excretion. Administration of 50 μg unlabelled porcine relaxin before injection of ^{125}I -labelled relaxin significantly reduced uptake of ^{125}I -labelled relaxin into the uterus and cervix, but had no effect on uptake of ^{125}I -labelled relaxin into other tissues, indicating that specific uptake of relaxin was occurring in the uterus and cervix. In ovariectomized rats, treatment with oestradiol benzoate or oestradiol benzoate plus progesterone for 1 day did not significantly increase uterine or cervical ^{125}I -labelled relaxin uptake compared with corn oil-treated rats, but induced a significant increase in uterine uptake of ^{125}I -labelled relaxin after treatment for 2 days. Induction of tolerance to relaxin by i.v. infusion of a high dose of relaxin significantly reduced uterine and cervical uptake of ^{125}I -labelled relaxin at 3 h after termination of infusion compared with saline-infused rats. By 12 h after termination of infusion, ^{125}I -labelled relaxin uptake in the uterus and cervix was similar in saline-infused rats and in rats given an infusion of relaxin. Infusion of glibenclamide (20 mg kg^{-1}) did not influence uterine or cervical uptake of ^{125}I -labelled relaxin; however, treatment with phentolamine (10 mg kg^{-1}) significantly reduced ^{125}I -labelled relaxin uptake in uterus, bladder and jejunum. This study demonstrates that steroid hormone treatment and tolerance modulate relaxin uptake in reproductive tissues.

Introduction

Relaxin is physiologically important during pregnancy in rats for inhibition of uterine contractions, softening of the cervix and a high incidence of live births (Downing and Sherwood, 1985a–c). Uterine sensitivity to relaxin as an inhibitor of spontaneous uterine contractions in nonpregnant rats *in vivo* is enhanced by oestradiol benzoate and progesterone treatment (Downing and Hollingsworth, 1992a, b). Treatment with a physiological dose of oestradiol benzoate (0.4 $\mu\text{g kg}^{-1} \text{ day}^{-1}$) for 2 days produced a twofold increase and treatment with oestradiol benzoate plus progesterone produced an eightfold increase in uterine sensitivity to relaxin. Oestrogen pretreatment has also been shown to be necessary for relaxin to induce softening of the cervix and maximum effect of relaxin occurs after pretreatment with oestrogen plus progesterone (Kroc *et al.*, 1959; Cullen and Harkness, 1960; Downing and Sherwood, 1985c, 1986). Although there is little information concerning hormonal modulation of specific binding sites for relaxin it has been suggested that oestrogen increases the concentration of binding sites for relaxin in both the uterus and cervix (Mercado-

Simmen *et al.*, 1982). Osheroff *et al.* (1992) showed that relaxin binding in the rat uterus was decreased by 53% following ovariectomy, but was restored to 90% of normal values following oestrogen treatment. The influence of progesterone on relaxin binding has not previously been investigated.

Tolerance to relaxin as an inhibitor of spontaneous uterine contractions develops during prolonged administration (Wigvist, 1959; Downing and Hollingsworth, 1992a). Infusion of 20 μg relaxin $\text{kg}^{-1} \text{ h}^{-1}$ i.v. for 40 h produced an eightfold reduction in uterine sensitivity to relaxin (Downing and Hollingsworth, 1992a). By 24 h after termination of infusion, only partial recovery of uterine sensitivity to relaxin had occurred. Treatment with oestradiol benzoate or oestradiol benzoate plus progesterone did not influence the extent of tolerance development, but in rats treated with oestradiol benzoate plus progesterone, recovery of uterine sensitivity to relaxin after cessation of relaxin infusion was more rapid. Prolonged intravenous infusion of relaxin at a higher rate (50 $\mu\text{g kg}^{-1} \text{ h}^{-1}$) for 40 h produced a 130-fold decrease in uterine sensitivity to relaxin. The mechanism of development of tolerance to relaxin is not known, but may be due to phosphorylation of binding sites, uncoupling of binding sites from second messenger systems or internalization and degradation of binding sites, as observed for other drugs and hormones (Levitzki, 1986; Lefkowitz *et al.*, 1990).

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The mechanism by which relaxin inhibits uterine contractions is not fully known. Relaxin induces a moderate increase in myometrial concentrations of cAMP in the presence of phosphodiesterase inhibitors *in vitro* (Cheah and Sherwood, 1980; Sanborn *et al.*, 1980; Hsu *et al.*, 1985). However, relaxin induces only a very small increase in uterine cAMP concentrations *in vivo* (Downing *et al.*, 1992). Relaxin is antagonized *in vivo* by glibenclamide, which blocks ATP-sensitive potassium channels (Downing and Hollingsworth, 1991), but such antagonism was not observed *in vitro* (Hughes *et al.*, 1992). Phentolamine, which blocks both α -adrenoceptors and ATP-sensitive potassium channels (Plant and Henquin, 1990), also delays the onset of inhibition of uterine contractions *in vivo* by relaxin (Porter *et al.*, 1979).

The aim of this study, therefore, was to determine whether specific uptake of ^{125}I -labelled porcine relaxin, as an indicator of relaxin binding sites, occurs in the reproductive tract in anaesthetized rats and is modulated by pretreatment with oestradiol benzoate and progesterone, by induction of tolerance to relaxin or by treatment with glibenclamide or phentolamine.

Materials and Methods

Experiment A: determination of biological activity of Bolton–Hunter-labelled relaxin

Porcine relaxin was modified by attachment of Bolton–Hunter reagent (3-*p*-hydroxyphenyl propionic acid *N*-hydroxysuccinimide ester) (Sigma Chemical Co., Poole, Dorset) by the method of Cheah and Sherwood (1980). Briefly, 5 mg porcine relaxin was stirred with 1.25 mg Bolton–Hunter reagent for 24 h at 4°C in sodium borate buffer (0.1 mol l⁻¹, pH 8.5), and then dialysed extensively against sodium hydrogen orthophosphate (0.001 mol l⁻¹) and sodium chloride (0.01 mol l⁻¹, pH 11.0) for 4 days to remove small unincorporated molecules. The degree of incorporation of the (3-*p*-hydroxyphenyl) propionate group into relaxin, estimated by the method of Bencze and Schmid (1957), was approximately 2 mol (3-*p*-hydroxyphenyl) propionate into 1 mol hormone. The protein concentration of Bolton–Hunter-labelled porcine relaxin was determined by the method of Lowry *et al.* (1951). Tube gel electrophoresis was performed on relaxin and modified relaxin by the method of Davis (1964), using 7.5% acrylamide, pH 4.3. Relaxin, 50 µg, or modified relaxin was layered onto the gel in 250 µl 5% sucrose. Gels were run at 6 mA per gel for 45 min, fixed and stained in 1% amido black in 7% acetic acid, and destained in 7% acetic acid.

Female rats, 200–250 g, purchased from Charles River (Margate) were anaesthetized with tribromoethanol (240 mg kg⁻¹, i.p.) and bilaterally ovariectomized. A small latex balloon was placed in one uterine horn and the right jugular vein was cannulated (PP50 tubing, internal diameter 0.58 mm, outside diameter 0.96 mm; Portex Ltd, Hythe). All rats were given 5 mg morphine sulfate kg⁻¹ for post-operative analgesia. Twenty-four hours after surgery, continuous recording of pressure cycles produced by uterine contractions commenced, using Elcomatic EM750 or Statham P23Db pressure transducers and Grass preamplifiers and polygraphs (Grass Co., Quincy, MA). Uterine contractions were defined as a rise and fall of at least

5 mm Hg. Contractions were quantified as the integral of the area under the pressure curve produced by contractions using Grass 7P10B integrators (Downing and Hollingsworth, 1992a). Rats were given three or four bolus doses of pig relaxin (2, 5, 20 µg kg⁻¹, *n* = 8 rats) or Bolton–Hunter-labelled pig relaxin (2, 5, 20, 50 µg kg⁻¹, *n* = 8 rats) i.v. in 1 ml saline kg⁻¹. Three hours elapsed between each bolus dose to allow the relaxin to clear. The uterine response to each bolus dose was recorded for 60 min and expressed as percentage inhibition of the integral of uterine contractions recorded for the 60 min immediately before each bolus dose. Dose–response curves were constructed for each animal using a logarithmic transformation. Log ID₅₀ values (log of dose producing 50% inhibition of integral of uterine contractions) were estimated by probit analysis using a computer program (Tallarida and Murray, 1987).

Experiment B: time course of relaxin uptake

Porcine relaxin and porcine insulin were radioiodinated by the method of Cheah and Sherwood (1980) using ^{125}I -Bolton–Hunter reagent (New England Nuclear, Dupont de Nemours, GmbH, Dreich). Briefly, 5 µg porcine relaxin in sodium borate buffer (0.2 mol l⁻¹, pH 8.5) was added to 1 mCi ^{125}I -labelled Bolton–Hunter reagent and stirred for 1 h over ice, and then for 3 h at 4°C. The ^{125}I -labelled relaxin was separated from unreacted ^{125}I -Bolton–Hunter reagent and hydrolysed reagent by gel filtration using a 1.4 cm × 25 cm Sephadex G25 (coarse) column (previously equilibrated with phosphate buffer (0.5 mol l⁻¹, pH 8.5) containing 2.5% gelatin then rinsed with 30–40 ml phosphate buffer, 0.5 mol l⁻¹). Fractions (1.5 ml) were collected into tubes containing 0.5 ml phosphate buffer (0.5 mol l⁻¹, pH 8.5) 0.25% gelatin and the radioactivity in 2 µl of each fraction determined. Radiolabelling of insulin was carried out in a similar way. Mean specific activity of ^{125}I -labelled relaxin was 0.07 ± 0.02 mCi µg⁻¹ hormone and specific activity of ^{125}I -insulin was 0.06 mCi µg⁻¹ hormone as estimated by the relative quantities of radioactivity contained in the hormone-bound ^{125}I and unbound ^{125}I peaks eluted from the G-25 Sephadex column.

Female rats were anaesthetized with tribromoethanol (240 mg kg⁻¹, i.p.) and the right jugular vein and carotid artery cannulated (PP50 polyethylene tubing). ^{125}I -labelled porcine relaxin (5 µCi) was given via the jugular cannula in 0.1–0.3 ml phosphate buffer (0.1 mol l⁻¹, pH 8.5) and flushed into the animal with 0.5 ml saline (0.9% w/v NaCl). When the duration of the experiment was greater than 20 min, the dose of 5 µCi ^{125}I -labelled relaxin was given as three separate doses of 1.7 µCi, 20 or 40 min apart. Anaesthesia was maintained throughout with tribromoethanol given i.v. via the jugular cannula. At 20 min (*n* = 5 rats), 60 min (*n* = 5 rats) or 120 min (*n* = 5 rats) after the first injection of ^{125}I -labelled relaxin, blood (1 ml) was taken via the carotid cannula and placed in a vial for determination of radioactivity. The animals were immediately killed by an overdose of tribromoethanol. Reproductive (ovaries, uterus, cervix and vagina) and other tissues (bladder, jejunum, ileum, pancreas, liver, spleen, kidney, lung, diaphragm, ventricle of heart) were rapidly dissected out and samples of up to 500 mg were placed in pre-weighed vials. The vials were reweighed to determine wet weight of the tissues. The radioactivity in the blood and tissue samples was determined and the radioactivity

(c.p.m.) obtained per 100 mg of tissue was divided by the radioactivity (c.p.m.) obtained per 100 μ l of blood. A ratio of counts significantly greater than 1.0 indicates uptake of 125 I-labelled relaxin.

The following three experiments were performed to determine the specificity of uptake of 125 I-labelled relaxin. One group of rats was given 50 μ g unlabelled porcine relaxin i.v. just before the first injection of 125 I-labelled relaxin ($n = 5$) and blood and tissue samples were taken 60 min after the first injection of 125 I-labelled relaxin (60 min was selected to ensure that sufficient unlabelled relaxin remained in the circulation to compete with 125 I-labelled relaxin). A second group of rats was given 5 μ Ci unconjugated 125 I-Bolton–Hunter reagent i.v. and blood and tissue samples were taken at 20 min ($n = 5$), 60 min ($n = 5$) or 120 min ($n = 5$) after the first injection of 125 I-Bolton–Hunter reagent. Some of the rats were given 50 μ g unlabelled relaxin i.v. just before the first injection of 125 I-Bolton–Hunter reagent and blood and tissue samples were taken 60 min after the first injection of 125 I-Bolton–Hunter reagent ($n = 5$). A third group of rats was given 5 μ Ci 125 I-labelled porcine insulin ($n = 5$) or 50 μ g unlabelled relaxin just before the first injection of 125 I-labelled insulin ($n = 5$) and blood and tissue samples were taken 60 min after the first injection of 125 I-labelled insulin.

Experiment C: influence of oestradiol benzoate and progesterone on uptake of relaxin

Rats were anaesthetized with tribromoethanol (240 mg kg^{-1} , i.p.) and subjected to bilateral ovariectomy via flank laparotomy. Rats were given 5 mg morphine sulfate kg^{-1} s.c. postoperatively for analgesia. The animals were then treated with one of the following: corn oil (1 ml kg^{-1}) s.c. daily for 1 ($n = 5$ rats) or 2 days ($n = 5$ rats); oestradiol benzoate (0.4 $\mu\text{g kg}^{-1}$ in 1 ml corn oil kg^{-1}) s.c. daily for 1 ($n = 5$ rats) or 2 days ($n = 5$ rats); oestradiol benzoate (40 $\mu\text{g kg}^{-1}$ in 1 ml corn oil kg^{-1}) s.c. daily for 2 days ($n = 9$ rats); oestradiol benzoate (0.4 $\mu\text{g kg}^{-1}$) plus progesterone (4 mg kg^{-1}) s.c. in 1 ml corn oil kg^{-1} daily for 1 ($n = 5$ rats) or 2 days ($n = 5$ rats).

Uptake by reproductive and other tissues of $3 \times 1.7 \mu\text{Ci}$ 125 I-labelled relaxin given i.v. under tribromoethanol anaesthesia was then determined as described for Expt B for 120 min. Rats treated with 40 μg oestradiol benzoate kg^{-1} received either $3 \times 1.7 \mu\text{Ci}$ 125 I-labelled relaxin i.v. alone ($n = 5$) or 50 μg unlabelled relaxin just before the first injection of 125 I-labelled relaxin ($n = 4$ rats).

Experiment D: influence of tolerance on relaxin uptake

Rats were anaesthetized with tribromoethanol (240 mg kg^{-1} , i.p.) subjected to bilateral ovariectomy and the right jugular vein cannulated; the cannula tubing passed subcutaneously to the back of the neck, where it was exteriorized and protected by a long metal spring which allowed the animals largely unrestricted movement within the cages. The rats were given 5 mg morphine sulfate kg^{-1} s.c. postoperatively for analgesia. The rats were then given one of the following: saline infusion i.v. at 0.15 ml h^{-1} for 40 h ($n = 15$ rats); relaxin infusion i.v. at 20 $\mu\text{g kg}^{-1} \text{h}^{-1}$ ($n = 11$ rats) or 50 $\mu\text{g kg}^{-1} \text{h}^{-1}$ ($n = 20$ rats) in 0.15 ml saline h^{-1} for 40 h. These rates of infusion of

relaxin have previously been shown to produce eightfold and 130-fold reductions in uterine sensitivity to relaxin as an inhibitor of spontaneous contractions, respectively (Downing and Hollingsworth, 1992a).

At 3 or 12 h after termination of infusion, uptake by reproductive and other tissues of 125 I-labelled relaxin ($3 \times 1.7 \mu\text{Ci}$, i.v.) was then determined over 120 min under tribromoethanol anaesthesia as described for Expt B. Three hours after termination of infusion was selected as the earliest time to test uptake of 125 I-labelled relaxin to allow relaxin in the infusate to clear. Studies have shown that relaxin has a half-life of approximately 20 min in plasma (Sherwood *et al.*, 1984). Four saline-infused rats and nine rats given an infusion of relaxin at 50 $\mu\text{g kg}^{-1} \text{h}^{-1}$ received 50 μg or 100 μg unlabelled relaxin i.v. just before the first injection of 125 I-labelled relaxin.

Experiment E: influence of glibenclamide and phentolamine on relaxin uptake

Rats were anaesthetized with tribromoethanol (240 mg kg^{-1} , i.p.) and subjected to bilateral ovariectomy. The animals were given 5 mg morphine sulfate kg^{-1} s.c. postoperatively for analgesia. Twenty-four hours after surgery, the animals were again anaesthetized with tribromoethanol and the right jugular vein and carotid artery cannulated. Groups of animals were then treated with either an infusion of glibenclamide (20 mg kg^{-1} , $n = 5$ rats) or vehicle (0.2 mol sodium hydroxide l^{-1} in 4% w/v glucose, 5 ml kg^{-1} , $n = 5$ rats) i.v. over 5 min. This dose of glibenclamide produces more than 19-fold antagonism of relaxin as an inhibitor of spontaneous contractions *in vivo* (Downing and Hollingsworth, 1991). Other groups were given an injection of phentolamine (10 mg kg^{-1} , $n = 5$ rats) or saline (1 ml kg^{-1} , $n = 5$ rats) i.v. This dose of phentolamine delays the onset of inhibition of uterine contractions by relaxin (Porter *et al.*, 1979). Fifteen minutes after infusion or injection, the rats were given 125 I-labelled relaxin ($3 \times 1.7 \mu\text{Ci}$) i.v. and blood and tissue samples were taken at 60 min after the first injection of 125 I-labelled relaxin as described for Expt B. The period of 60 min was selected as it has been found that the effectiveness of glibenclamide as an antagonist of relaxin declines markedly after this time in rats (Downing and Hollingsworth, 1991).

Reagents

Porcine relaxin was isolated and purified from pregnant sow ovaries by the method of Sherwood and O'Byrne (1974). Relaxin potency was bioassayed *in vitro* by inhibition of contractions of electrically stimulated uterus from oestrogen pretreated rats (100 μg oestradiol benzoate kg^{-1} s.c.) (Bradshaw *et al.*, 1981), using highly purified porcine relaxin, kindly donated by O.D. Sherwood (University of Illinois, Urbana, IL) as standard. Our relaxin preparation was equipotent with highly purified relaxin. Porcine insulin, oestradiol benzoate, progesterone and phentolamine were obtained from Sigma Chemical Co. (Poole, Dorset). Glibenclamide was supplied by Hoechst Ltd (Hounslow). Tribromoethanol was obtained from Fluka Chemicals (Glossop) and morphine sulfate from Evans Ltd (Dunstable). All other reagents were of Analar grade.

Fig. 1. Dose-response curves for inhibition of uterine contractions by porcine relaxin (○, eight rats) and Bolton-Hunter-labelled porcine relaxin (●, eight rats). Values are means $\pm$ SEM. Ordinate: inhibition of uterine contractions over 60 min as a % of integral of 60 min preceding i.v. bolus dose. Abscissa: dose on a log scale.

Statistical analysis

Results are presented as means $\pm$ SEM. Values were compared statistically by analysis of variance followed by Student's *t* test.

Results

Experiment A: determination of biological activity of Bolton-Hunter-labelled relaxin

There were no significant differences in log ID₅₀ values (porcine relaxin, $0.92 \pm 0.12 \mu\text{g kg}^{-1}$; Bolton-Hunter relaxin, $1.19 \pm 0.14 \mu\text{g kg}^{-1}$) or slopes of the regression lines for the dose-response curves (porcine relaxin, $45.8 \pm 10.9\% \mu\text{g}^{-1} \text{kg}^{-1}$; Bolton-Hunter relaxin, $34.1 \pm 6.0\% \mu\text{g}^{-1} \text{kg}^{-1}$) (Fig. 1). There was, therefore, no significant change in biological activity of relaxin as an inhibitor of uterine contractions owing to attachment of Bolton-Hunter reagent to porcine relaxin. Gel electrophoresis of relaxin and modified relaxin produced protein bands that were clearly separated suggesting that biological activity of modified relaxin was not due to residual unmodified relaxin in the preparation.

Experiment B: time course of uptake of ¹²⁵I-labelled relaxin

Values of ratios of ¹²⁵I content in tissues compared with ¹²⁵I content of blood (relative uptake) at various times after injection of ¹²⁵I-labelled relaxin are shown (Table 1) and ratios at various times after injection of unconjugated ¹²⁵I-Bolton-Hunter reagent or ¹²⁵I-labelled insulin, with or without prior injection of 50 μg unlabelled relaxin are shown (Table 2). Relative uptake of ¹²⁵I-labelled relaxin in reproductive tissues had a ratio of greater than 1.0 at 20 min and increased significantly (two- to three-fold) with time. Tissues concerned with

metabolism or excretion also showed increased uptake of ¹²⁵I-labelled relaxin with time (bladder, jejunum, pancreas, liver, kidney, lung). Other organs containing muscle (diaphragm, ileum) or spleen showed no consistent uptake of ¹²⁵I-labelled relaxin. Samples of ventricles of the heart showed no consistent uptake of relaxin. Injection of 50 μg unlabelled relaxin just before injection of ¹²⁵I-labelled relaxin resulted in significant reduction in uptake of ¹²⁵I-labelled relaxin in uterus and cervix indicating that the enhanced ¹²⁵I content of these tissues was due to specific uptake of relaxin (Table 1). ¹²⁵I-labelled relaxin uptake in other tissues was unaffected by prior injection of unlabelled relaxin. In reproductive and nonreproductive tissues, there was no consistent uptake of label after injection of ¹²⁵I-Bolton-Hunter reagent or ¹²⁵I-labelled insulin whether or not this had been preceded by treatment with unlabelled relaxin (Table 2).

Experiment C: influence of oestradiol benzoate and progesterone on uptake of relaxin

Reproductive tissues. Treatment of ovariectomized rats with 0.4 μg oestradiol benzoate kg^{-1} for 1 or 2 days did not significantly change uterine wet weight (corn oil treatment for 1 day, $315 \pm 32 \text{ mg}$; corn oil treatment for 2 days, $289 \pm 15 \text{ mg}$; 0.4 μg oestradiol benzoate kg^{-1} for 1 day, $278 \pm 18 \text{ mg}$; 0.4 μg oestradiol benzoate kg^{-1} for 2 days, $279 \pm 23 \text{ mg}$). Similarly, cervical wet weight was not altered by treatment for 1 or 2 days with 0.4 μg oestradiol benzoate kg^{-1} ($65 \pm 6 \text{ mg}$, $65 \pm 4 \text{ mg}$, respectively) or 0.4 μg oestradiol benzoate kg^{-1} plus 4 mg progesterone kg^{-1} ($93 \pm 5 \text{ mg}$, $62 \pm 3 \text{ mg}$, respectively) compared with corn oil treatment ($90 \pm 7 \text{ mg}$, $73 \pm 5 \text{ mg}$, respectively). Uterine uptake of ¹²⁵I-labelled relaxin was significantly increased by treatment with 0.4 μg oestradiol benzoate kg^{-1} for 2 days ($P < 0.01$, Fig. 2a, b) compared with corn oil treatment. Treatment with 0.4 μg oestradiol benzoate kg^{-1} plus 4 mg progesterone kg^{-1} for 1 or 2 days again did not significantly increase uterine wet weight ($329 \pm 8 \text{ mg}$, $305 \pm 15 \text{ mg}$, respectively), but significantly increased uterine uptake of ¹²⁵I-labelled relaxin after treatment for 2 days ($P < 0.02$ compared with corn oil-treated rats, Fig. 2a). Treatment with 40 μg oestradiol benzoate kg^{-1} for 2 days produced a significant increase in uterine wet weight ($510 \pm 30 \text{ mg}$, $P < 0.001$) and significantly increased uterine uptake of ¹²⁵I-labelled relaxin compared with corn oil-treated rats ($P < 0.001$) and rats treated with the lower dose of oestradiol benzoate with ($P < 0.01$) or without ($P < 0.01$) progesterone for 2 days.

Unlike results observed for the uterus, treatment with 0.4 μg oestradiol benzoate kg^{-1} or 0.4 μg oestradiol benzoate kg^{-1} plus 4 mg progesterone kg^{-1} for 2 days did not significantly increase cervical uptake of ¹²⁵I-labelled relaxin. Treatment of rats with 40 μg oestradiol benzoate kg^{-1} for 2 days produced significant increases in both cervical wet weight ($122 \pm 7 \text{ mg}$, $P < 0.001$) and ¹²⁵I-labelled relaxin uptake compared with corn oil-treated rats ($P < 0.01$) and rats treated with the lower dose of oestradiol benzoate with ($P < 0.02$) or without ($P < 0.05$) progesterone (Fig. 2b). The marked increase in uptake of ¹²⁵I-labelled relaxin observed in uteri and cervix from rats treated with 40 μg oestradiol benzoate kg^{-1} for 2 days was abolished by prior injection of 50 μg unlabelled relaxin ($P < 0.001$, Fig. 2a, b).

Table 1. Ratios of ^{125}I content in tissues compared with ^{125}I content of blood at 20, 60 or 120 min after injection of 5 μCi ^{125}I -relaxin in intact rats or at 60 min after injection of 50 μg unlabelled relaxin and 5 μCi ^{125}I -labelled relaxin

Treatment Time for uptake (min)	5 μCi ^{125}I -relaxin			50 μg unlabelled relaxin + 5 μCi ^{125}I -labelled relaxin
	20	60	120	60
Tissues				
Ovaries	0.92 ± 0.02^g	1.05 ± 0.08^d	$1.33 \pm 0.09^{d,g}$	0.88 ± 0.04
Uterus	1.50 ± 0.13^a	$2.33 \pm 0.33^{a,e,h}$	4.00 ± 0.20^b	1.06 ± 0.06^e
Cervix	1.93 ± 0.22^b	$3.13 \pm 0.48^{b,f,i}$	5.85 ± 0.24^i	1.29 ± 0.15^f
Vagina	2.07 ± 0.46^c	4.79 ± 1.47	5.73 ± 1.28^c	2.71 ± 0.74
Bladder	1.30 ± 0.06^k	4.51 ± 1.40^k	4.26 ± 0.74	3.66 ± 0.92
Jejunum	$1.10 \pm 0.10^{m,p}$	3.69 ± 0.35^m	4.71 ± 1.06^p	3.41 ± 0.37
Pancreas	$2.29 \pm 0.05^{q,t}$	2.70 ± 0.07^q	2.85 ± 0.11^t	2.85 ± 0.06
Liver	$2.29 \pm 0.06^{r,u}$	2.76 ± 0.09^r	2.81 ± 0.07^u	2.97 ± 0.07
Kidney	25.15 ± 0.26	24.54 ± 2.03^n	33.95 ± 2.92^n	29.99 ± 2.19
Lung	1.43 ± 0.05^s	1.90 ± 0.12^s	1.99 ± 0.13	1.78 ± 0.04
Diaphragm	0.43 ± 0.01	0.46 ± 0.03	0.44 ± 0.02	0.44 ± 0.01
Heart	0.55 ± 0.03	0.51 ± 0.04	0.45 ± 0.03	0.45 ± 0.02
Ileum	0.54 ± 0.02	2.84 ± 1.36	1.00 ± 0.38	1.66 ± 0.90
Spleen	0.93 ± 0.03	1.08 ± 0.10	1.21 ± 0.13	1.17 ± 0.08
<i>n</i>	5	5	5	5

Values are means $\pm$ SEM. Values with the same superscripts are significantly different: a, b, c, k, $P < 0.05$; d, m, n, $P < 0.02$; e, f, p, q, r, s, $P < 0.01$; g, $P < 0.002$; h, i, t, u, $P < 0.001$ (analysis of variance followed by Student's *t* test).

Table 2. Ratios of ^{125}I content in tissues compared with ^{125}I content of blood at 20, 60 or 120 min after injection of 5 μCi ^{125}I -labelled Bolton–Hunter reagent or ^{125}I -labelled insulin in intact rats

Treatment Time for uptake (min)	5 μCi ^{125}I -Bolton–Hunter reagent			50 μg unlabelled relaxin + 5 μCi ^{125}I -Bolton–Hunter reagent	5 μCi ^{125}I -labelled insulin	50 μg unlabelled relaxin + 5 μCi ^{125}I -labelled insulin
	20	60	120	60	60	60
Tissues						
Ovaries	1.12 ± 0.24	0.54 ± 0.04	0.50 ± 0.01	0.70 ± 0.05	0.85 ± 0.18	0.66 ± 0.03
Uterus	0.77 ± 0.09	0.55 ± 0.06	0.75 ± 0.04	0.68 ± 0.02	0.81 ± 0.06	0.91 ± 0.07
Cervix	1.58 ± 0.45	0.58 ± 0.04	0.65 ± 0.04	0.89 ± 0.16	0.83 ± 0.05	0.92 ± 0.10
Vagina	2.36 ± 0.31	1.24 ± 0.46	0.74 ± 0.05	4.21 ± 1.19	1.14 ± 0.12	1.08 ± 0.14
Bladder	15.4 ± 7.74	6.92 ± 0.85	13.85 ± 2.13	11.14 ± 2.25	5.08 ± 1.09	5.24 ± 0.96
Jejunum	2.02 ± 0.43	2.49 ± 0.57	1.12 ± 0.14	1.17 ± 0.22	4.96 ± 2.13	3.74 ± 0.42
Pancreas	2.91 ± 1.16	0.79 ± 0.23	0.55 ± 0.02	0.71 ± 0.13	1.12 ± 0.09	1.07 ± 0.12
Liver	2.87 ± 1.18	0.66 ± 0.21	0.48 ± 0.02	0.61 ± 0.11	1.14 ± 0.08	1.03 ± 0.11
Kidney	3.61 ± 0.87	1.95 ± 0.65	1.11 ± 0.09	2.45 ± 0.77	7.20 ± 0.81	8.82 ± 1.02
Lung	1.08 ± 0.29	0.65 ± 0.03	0.68 ± 0.03	1.23 ± 0.42	0.88 ± 0.13	1.02 ± 0.04
Diaphragm	0.78 ± 0.29	0.41 ± 0.05	0.39 ± 0.01	0.41 ± 0.03	0.49 ± 0.05	0.43 ± 0.03
Heart	0.49 ± 0.12	0.34 ± 0.02	0.39 ± 0.03	0.38 ± 0.02	0.50 ± 0.01	0.48 ± 0.04
Ileum	0.87 ± 0.21	1.73 ± 0.68	3.32 ± 0.98	1.08 ± 0.58	1.00 ± 0.18	0.76 ± 0.06
Spleen	1.89 ± 0.71	0.55 ± 0.14	0.50 ± 0.05	0.48 ± 0.07	0.58 ± 0.07	0.55 ± 0.03
<i>n</i>	5	5	5	5	5	5

Values are means $\pm$ SEM.

Fig. 2. Ratios of ^{125}I content of tissue compared with ^{125}I content of blood at 120 min after injection of $5 \mu\text{Ci } ^{125}\text{I}$ -labelled relaxin for (a) uterus and (b) cervix in rats treated daily with corn oil only for 1 (five rats) or 2 days (five rats), $0.4 \mu\text{g}$ oestradiol benzoate kg^{-1} for 1 (five rats) or 2 days (five rats), $0.4 \mu\text{g}$ oestradiol benzoate kg^{-1} plus 4 mg progesterone kg^{-1} for 1 (five rats) or 2 days (five rats) or $40 \mu\text{g}$ oestradiol benzoate kg^{-1} for 2 days (five rats) with administration of $50 \mu\text{g}$ unlabelled relaxin just before injection of ^{125}I -labelled relaxin (four rats). Histograms show means $\pm$ SEM. Significant differences from corn-oil treated control: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, except for oestradiol benzoate-treated rats ($40 \mu\text{g kg}^{-1}$) given unlabelled relaxin which are compared with oestradiol benzoate-treated rats which have not received unlabelled relaxin.

Nonreproductive tissues. Values for ratios of ^{125}I content in tissues compared with ^{125}I content of blood at 120 min were similar in nonreproductive tissues from rats treated with corn oil for 1 or 2 days to those values shown in Table 1. Treatment with oestradiol benzoate (0.4 or $40 \mu\text{g kg}^{-1}$ for 1 or 2 days) or $0.4 \mu\text{g}$ oestradiol benzoate kg^{-1} plus 4 mg progesterone kg^{-1} did not affect ^{125}I -labelled relaxin uptake in nonreproductive tissues, nor were values significantly reduced by administration of $50 \mu\text{g}$ unlabelled relaxin before injections of ^{125}I -labelled relaxin (data not shown).

Experiment D: influence of tolerance on uptake of relaxin

Reproductive tissues. At 3 h after termination of infusion relative uptake of ^{125}I -labelled relaxin was similar in rats given an i.v. infusion of saline or relaxin at $20 \mu\text{g kg}^{-1} \text{h}^{-1}$ for 40 h

Fig. 3. Ratios of ^{125}I content of tissue compared with ^{125}I content of blood at 120 min after injection of $5 \mu\text{Ci } ^{125}\text{I}$ -labelled relaxin for (a) uterus and (b) cervix at 3 h or 12 h after termination of infusion in rats given an i.v. infusion of saline at 0.15 ml h^{-1} (3 h, five rats; 12 h, six rats; 3 h with administration of $50 \mu\text{g}$ unlabelled relaxin just before injection of ^{125}I -labelled relaxin (four rats), or relaxin at $20 \mu\text{g kg}^{-1} \text{h}^{-1}$ (3 h, five rats; 12 h, six rats) or $50 \mu\text{g kg}^{-1} \text{h}^{-1}$ (3 h, five rats; 12 h, six rats) or 3 h with administration of $50 \mu\text{g}$ (five rats) or $100 \mu\text{g}$ (four rats) unlabelled relaxin just before injection of ^{125}I -labelled relaxin). Histograms show means $\pm$ SEM. Significant difference from saline-infused rats at appropriate time after cessation of infusion: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

for both uterus and cervix. At 3 h after termination of infusion of relaxin at $50 \mu\text{g kg}^{-1} \text{h}^{-1}$, uterine and cervical uptake of ^{125}I -labelled relaxin was significantly reduced compared with uteri and cervixes from saline infused rats ($P < 0.02$, $P < 0.01$, respectively) and rats which received relaxin infusion at $20 \mu\text{g kg}^{-1} \text{h}^{-1}$ ($P < 0.02$, $P < 0.05$, respectively, Fig. 3a, b). By 12 h after termination of infusion, uterine and cervical uptake of ^{125}I -labelled relaxin was similar in rats given an infusion of saline and in rats given an infusion of relaxin at $20 \mu\text{g kg}^{-1} \text{h}^{-1}$ or $50 \mu\text{g kg}^{-1} \text{h}^{-1}$. Administration of $50 \mu\text{g}$ or $100 \mu\text{g}$ unlabelled relaxin just before injection of ^{125}I -labelled relaxin significantly reduced uterine and cervical ^{125}I -labelled relaxin uptake in both saline-infused rats ($P < 0.001$) and in rats given an infusion of relaxin at $50 \mu\text{g kg}^{-1} \text{h}^{-1}$ ($P < 0.01$).

Table 3. Effect of glibenclamide (20 mg kg⁻¹), phentolamine (10 mg kg⁻¹) or vehicle (0.2 mol NaOH l⁻¹ in 4% glucose or saline, respectively) on ratio of ¹²⁵I-labelled relaxin content of tissue compared with ¹²⁵I-labelled relaxin content of blood in reproductive and nonreproductive tissues from ovariectomized rats

Treatment	Vehicle for glibenclamide	Glibenclamide	Vehicle for phentolamine	Phentolamine
Tissue				
Uterus	1.78 ± 0.10	1.67 ± 0.09	1.62 ± 0.14 ^a	1.13 ± 0.13 ^a
Cervix	1.88 ± 0.17	1.71 ± 0.21	1.72 ± 0.23	1.25 ± 0.13
Bladder	2.98 ± 0.18	2.55 ± 0.32	3.12 ± 0.70 ^b	1.03 ± 0.24 ^b
Jejunum	4.69 ± 0.53	2.45 ± 0.75	4.52 ± 1.47 ^c	0.90 ± 0.07 ^c
Ileum	2.24 ± 0.85	1.19 ± 0.53	0.65 ± 0.03	0.57 ± 0.05
Diaphragm	0.63 ± 0.07	0.65 ± 0.04	0.57 ± 0.06	0.42 ± 0.03
Heart	0.46 ± 0.03	0.46 ± 0.03	0.45 ± 0.03	0.41 ± 0.01
Lung	1.58 ± 0.09	1.78 ± 0.15	1.48 ± 0.23	1.16 ± 0.13
n	5	5	5	5

Values are means ± SEM. Values with the same superscripts are significantly different: a, b, c, *P* < 0.05, (Student's *t* test).

Nonreproductive tissues. ¹²⁵I-labelled relaxin uptake in non-reproductive tissues was similar at 3 h and at 12 h after termination of infusion in both saline-infused and relaxin-infused rats and was unaffected by administration of 50 µg or 100 µg unlabelled relaxin just before injection of ¹²⁵I-labelled relaxin (data not shown).

Experiment E: influence of glibenclamide and phentolamine on uptake of relaxin

Uptake of ¹²⁵I-labelled relaxin was similar in both glibenclamide-infused and vehicle-infused rats for reproductive and non-reproductive tissues (Table 3). Animals that received 10 mg phentolamine kg⁻¹ i.v. showed significantly reduced uterine uptake of ¹²⁵I-labelled relaxin (*P* < 0.05) compared with saline-treated rats (Table 3). Cervical uptake of ¹²⁵I-labelled relaxin was not significantly reduced by phentolamine treatment. ¹²⁵I-labelled relaxin uptake was also significantly reduced by phentolamine treatment in bladder (*P* < 0.05) and jejunum (*P* < 0.05).

Discussion

In the study reported here specific uptake of ¹²⁵I-labelled porcine relaxin over 60–120 min in anaesthetized rats was used as an indicator of both relaxin binding to specific receptors and subsequent internalization of the relaxin–receptor complex. Specific binding sites for relaxin have been demonstrated in reproductive tissues *in vitro* (Osheroff *et al.*, 1990; Yang *et al.*, 1992). It appears, however, that receptor density is naturally low in relaxin target tissues, rendering isolation and characterization of receptors for relaxin difficult (Yang *et al.*, 1992). In the current experiments, highly purified porcine relaxin was

radioiodinated and separated from unreacted and hydrolysed reagents by gel filtration. Labelled relaxin retained full biological activity: this was demonstrated in a separate experiment in which porcine relaxin was labelled with 'cold' Bolton–Hunter reagent and its ability to inhibit uterine contractions *in vivo* in conscious rats was confirmed. The study showed specific localization of ¹²⁵I-labelled relaxin, as it could be displaced by unlabelled relaxin, in those tissues considered to be target tissues (uterus, cervix) but not in non-target tissues. It is, therefore, considered that sites of relaxin uptake and internalization were measured.

Treatment of rats with 0.4 µg oestradiol benzoate kg⁻¹ for 2 days resulted in a twofold increase in uterine sensitivity to relaxin as an inhibitor of spontaneous contractions (Downing and Hollingsworth, 1992a, b). In the study reported here, treatment with the same dose of oestradiol benzoate resulted in a significant increase (2.5-fold) in uterine uptake of ¹²⁵I-labelled relaxin, although ¹²⁵I-labelled relaxin uptake in cervix did not increase. Treatment with a high dose of 40 µg oestradiol benzoate kg⁻¹ further increased uterine uptake of ¹²⁵I-labelled relaxin (3.5-fold over controls) and increased cervical uptake of ¹²⁵I-labelled relaxin (fivefold over controls). In contrast, however, to the eightfold increase in uterine sensitivity to relaxin as an inhibitor of contractions produced by the lower dose of oestradiol benzoate plus progesterone treatment (Downing and Hollingsworth, 1992a), this steroid treatment did not increase uterine ¹²⁵I-labelled relaxin uptake above that produced by 0.4 µg oestradiol benzoate kg⁻¹ treatment alone. Similarly, progesterone treatment did not increase uptake of ¹²⁵I-labelled relaxin in the cervix. Progesterone may increase uterine sensitivity to relaxin by enhancement of coupling of relaxin receptors to second messenger systems within the myometrial cell, rather than by increasing relaxin receptor concentration. It is also possible that uptake of ¹²⁵I-labelled relaxin by endometrium, which may not be influenced by oestrogen and

progesterone treatment, could be obscuring changes in uptake of ^{125}I -labelled relaxin occurring in the myometrium. Autoradiographic localization of uterine relaxin binding sites, however, indicates that these are predominantly in the myometrium, with little specific binding to endometrium in rats (Osheroff *et al.*, 1990) and mice (Yang *et al.*, 1992). Thus, changes in uterine uptake of ^{125}I -labelled relaxin may accurately reflect changes in myometrial relaxin binding. It has been reported that oestrogen pretreatment is necessary for the action of relaxin in promoting cervical softening and changes in connective tissue (Kroc *et al.*, 1959; Cullen and Harkness, 1960; Downing and Sherwood, 1985c, 1986). Oestrogen may again increase coupling of relaxin receptors with second messenger systems in addition to raising concentrations of relaxin receptors in the cervix.

Prolonged i.v. infusion (40 h) of relaxin ($20\text{ }\mu\text{g kg}^{-1}\text{ h}^{-1}$) results in a significant (eightfold) reduction in uterine sensitivity to relaxin as an inhibitor of uterine contractions which is maintained for 24 h (Downing and Hollingsworth, 1992a). In contrast, no significant change in uptake of ^{125}I -labelled relaxin was observed at either 3 h or 12 h after cessation of infusion in rats given a similar relaxin infusion compared with animals given a saline infusion. Prolonged infusion of relaxin at $50\text{ }\mu\text{g kg}^{-1}\text{ h}^{-1}$ for 40 h, which produces a 130-fold reduction in uterine sensitivity to relaxin as an inhibitor of spontaneous contractions (Downing and Hollingsworth, 1992a), produced only a small reduction (1.5-fold) in uterine uptake of ^{125}I -labelled relaxin at 3 h after termination of infusion. By 12 h after cessation of infusion, uterine uptake of ^{125}I -labelled relaxin was similar to that observed in saline-infused rats. This finding suggests that development of tolerance may not be due primarily to internalization and loss of specific binding sites for relaxin, but may be due to uncoupling of receptors from second messenger systems in the myometrial cell. It is also possible that, in relaxin-tolerant tissues, relaxin is metabolized, and therefore lost, more rapidly.

Glibenclamide, a blocker of ATP-sensitive potassium channels, did not reduce ^{125}I -labelled relaxin uptake in either uterus or cervix, suggesting an indirect mechanism for antagonism by glibenclamide of the inhibitory effect of relaxin on spontaneous uterine contractions (Downing and Hollingsworth, 1991) rather than glibenclamide acting as an antagonist at the relaxin receptor. Phentolamine, however, produced a significant reduction in ^{125}I -labelled relaxin uptake in uterus, although cervical ^{125}I -labelled relaxin uptake was unaffected by phentolamine treatment. A significant reduction in ^{125}I -labelled relaxin uptake in bladder and jejunum suggests that phentolamine nonspecifically inhibits relaxin uptake. Phentolamine, by blockade of α -adrenoceptors in blood vessels, may delay uptake of relaxin by the uterus owing to redistribution of blood flow.

This study demonstrates specific uptake of relaxin in the uterus and cervix of the rat *in vivo* which is increased by oestrogen pretreatment and is decreased in tissues tolerant to relaxin. Comparison of changes of relaxin uptake induced by oestrogen and tolerance with changes in sensitivity to the inhibitory action of relaxin on uterine contractions suggest that not only relaxin binding sites, but also signal-transduction mechanisms are modulated by oestrogen and development of tolerance.

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