Demonstration of some of the physiological properties of rat relaxin

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Summary. Relaxin was extracted from the ovaries of pregnant rats. Material possessing uterine relaxing activity in vitro was eluted in three peaks from Sephadex G.50 columns. The 'G3 peak' material eluting in a position comparable to that of porcine relaxin inhibited myometrial activity of rats in vivo, improved the rate of rise of pressure of intrauterine pressure cycles in vivo, and, when administered to rats following progesterone and oestrogen priming, increased the distensibility of the cervix in vitro. This material also stimulated inter-puber ligament formation in the mouse. This rat relaxin material therefore exhibits biological actions in the rat similar to those previously assigned to porcine relaxin.

Introduction

Relaxin is a polypeptide hormone which to date has been shown to have three main biological actions, i.e. inhibition of uterine contraction (uterine relaxing factor activity), elongation of the interpubic ligament and softening of the cervix (for review see Porter, 1979a).

Because large amounts of the polypeptide can be extracted from the corpora lutea of the pregnant sow (Hisaw & Zarrow, 1950) most studies have utilized porcine relaxin. However, relaxin exhibits species specificity (Porter, 1979a) and ovarian extracts from pregnant rats show little or no cross-reactivity in a radioimmunoassay (RIA) which we developed for porcine relaxin based on that of Bryant-Greenwood & Greenwood (1979). It may be important therefore to isolate relaxin from each species of experimental interest in order to confirm its physiological actions in those species and to develop specific assays.

During our work of extracting and purifying rat relaxin from the ovaries of pregnant rats, details of the purification of rat relaxin by a different method and of an homologous RIA were published by Sherwood & Crnekovic (1979) and Sherwood (1979). We have therefore extended our findings by examining the physiological actions of rat relaxin.

Materials and Methods

Extraction of rat ovaries

Groups of 20–30 female Wistar rats from a closed colony at the University of Bristol were mated at regular intervals. On Days 18, 19 or 20 of pregnancy the animals were killed by decapitation and trunk blood was collected into ice-cold heparinized tubes. The reproductive tracts were then removed and placed on solid CO₂. When blood was not required the animals were killed by breaking their necks. Plasma obtained from the blood was extracted immediately for relaxin by the method of Albert & Money (1946). Tissue from the reproductive tract was either used immediately or stored at −20°C.
The relaxin extraction method was based largely on that of Sherwood & O’Byrne (1974). All procedures were carried out at 0–4°C unless otherwise stated. Tissue was chopped with scissors and homogenized for 3 × 5 sec at top speed with an ILADO X-10/20 laboratory disperser (Scientific Instrument Centre, London) in a total of 10 volumes ice-cold acid acetone (9 ml conc. HCl: 60 ml distilled water: 140 ml acetone). The homogenate was stirred with a magnetic stirrer for 16–18 h. The homogenate was then centrifuged for 15 min at about 10 000 g. The supernatant was decanted and filtered through glass wool. The filtrate was added to 6 volumes of acetone at −20°C, stirring constantly for 10 min. The precipitate was allowed to settle for about 1 h in the cold when most of the supernatant was poured off. The remaining mixture was filtered on a Büchner flask through a porosity-3 sintered-glass Büchner funnel and the precipitate was washed with copious ice-cold acetone. It was dissolved in distilled water and freeze-dried before storing desiccated at 4°C. Samples of all extracts were taken for in-vitro testing for uterine relaxing activity.

Purification of rat ovarian extract

About 40–60 mg extract were dissolved in 7 ml 0.2 M-ammonium acetate (pH 6.8–6.9) and layered onto a 120 × 2.4 cm (i.d.) column of Sephadex G50 (fine) and equilibrated with the same buffer. The column was eluted with this buffer, the flow of which was controlled by means of a peristaltic mini-pump (Schuco Scientific Ltd, London). The column effluent was monitored for protein concentration by measuring its absorbance at 280 nm (ISCO UA5; MSE, Scientific Instruments, Crawley, Sussex) before collecting fractions every 20 min (6.5 ml). Each fraction was freeze-dried three times to remove all traces of ammonium acetate before taking a sample for activity tests. Active fractions were pooled in peaks, freeze-dried again and stored desiccated at 4°C.

Organ-bath tests for uterine relaxing activity

Pro-oestrous virgin rats were used. Each animal was killed by a blow on the head and the uterus was removed quickly into ice-cold Krebs–Henseleit buffer (g/l: NaCl 6.9; KCl 0.35; CaCl₂ 0.6 H₂O 0.55; MgSO₄ 7H₂O 0.29; NaHCO₃ 2.1; KH₂PO₄ 0.16; glucose 2.00), pH 7.5. Fat was trimmed off, the cervix and ovaries removed and the uterine horns opened longitudinally. Each horn was cut into 6 strips approximately 3 mm × 6 mm long which were kept in ice-cold buffer until used. Platinum/iridium electrodes were inserted into each end of the strip and the assembly placed in a 10 ml organ-bath containing Krebs–Henseleit buffer warmed to 37°C and oxygenated with 95% O₂/5% CO₂. One electrode was held rigidly and the other attached to an isometric strain gauge (Ormed, Welwyn Garden City, Herts; or Grass, Quincy, Massachusetts, U.S.A.). When all strips were set up a load (~0.5 g) was applied to each and electrical stimulation at 50 Hz was applied for 4 sec every 45 sec at an amplitude of 4 V by means of an AC stimulator. Tension was recorded on a Washington (BioScience, Sheerness, Kent) or Tekman 200/1 (Tekman Electronics Ltd, Bicester, Oxfordshire) pen recorder. The strips were allowed to equilibrate for 15 min. After this time the load was increased stepwise up to 3 g. The tension during this procedure fell and the load was finally reduced to 1 g before washing out. The strips were then ready for use.

After 10 successive contractions of uniform tension had been recorded, a sample of test material dissolved in up to 0.5 ml Krebs–Henseleit buffer (or in 0.1 ml distilled water) was introduced into the bath. After a further 10 contractions had been recorded the bath was washed out 3 times with fresh buffer. If the sample did not affect the activity of the strip another sample was introduced after at least a further 10 control contractions had been recorded. If, however, the sample had evoked an inhibitory response the uterine strip was discarded in order to avoid any problems with tachyphylaxis.
Results were calculated as the mean reduction in the amplitude of the 10 contractions recorded after introduction of the sample divided by the mean amplitude of the preceding 10 control contractions, and expressed as percentage inhibition.

**Mouse pubic ligament test**

Virgin female mice of the MFI strain were primed with 5 µg oestradiol-17β dipropionate (Sigma (London) Chemical Co., Poole, Dorset) administered s.c. in 0.1 ml corn oil on Day 0 and allocated at random into 5 groups. On Day 7 the 37 mice were treated with (i) vehicle, (ii) known doses of porcine relaxin standard (Warner-Lambert W1164—lot 8; 150 GPU/mg) or (iii) the partly-purified extract from pregnant rat ovaries, dissolved in 0.1 ml 1% benzopurpurin (Raymond A. Lamb, London). The mice were killed with ether 24 h later and the pelvic girdles were dissected free of soft tissue. The length of the interpubic ligament was estimated with the aid of transillumination and a dissecting microscope (Steinetz et al., 1960). Uteri exhibiting no macroscopic evidence of oestrogen priming were rejected. There were 6–9 animals in each group and mean differences from the control group values were compared by Student’s *t* test.

**Experiment 1: the action of pregnant rat ovarian extract on myometrial activity in vivo**

Rats that had littered were ovariectomized and equipped with latex balloons filled with 0-45 ml distilled water (Porter & Downing, 1978). Intrauterine pressure was then recorded for several days. After continuous myometrial activity uninterrupted by periods of quiescence had been recorded for 24 h, each animal was anaesthetized with pentobarbitone sodium (Sagatal: May & Baker Ltd, Dagenham, Essex; 5 mg/100 g body weight i.p.) and the jugular, trachea and the carotid vessels were cannulated. When stable uterine activity had been observed for at least 30 min following the disturbance of anaesthesia, 10 µg partly-purified rat ovarian extract dissolved in 0.05 ml saline (9 g NaCl/l) were administered i.v. At 60 min after recovery from this injection, a second identical dose was given and 10 min after the response began 5 mU oxytocin (Syntocin: Sandoz Products Ltd, Feltham, Middx) i.m. 0.05 ml saline were administered i.v. After recovery from these treatments and after a further 60 min of stable uterine activity had been recorded, rat ovarian extract was again administered, followed 10 min later by 5 µg prostaglandin (PG) F-2α (Lutalyse: Upjohn Ltd, Crawley, Sussex) i.v. in 0.05 ml saline. When the uterine activity had stabilized again the animals were killed without recovery from anaesthesia. The results were analysed by means of Student’s paired *t* test.

**Experiment 2: the effect of pregnant rat ovarian extract on rate of rise of pressure of intrauterine pressure (IUP) cycles**

Rats were prepared as in Exp. 1 and anaesthetized. The rate of rise of pressure of IUP cycles was measured as follows. The chart speed was increased from 3 mm/min to 3 mm/sec for 3 contractions (a) before administration of 10 µg partly-purified ovarian extracts and (b) during the period of recovery from uterine quiescence induced by ovarian extracts. ‘Overall’ rates of rise were estimated from the momet pressure exceeded the resting pressure until peak pressure was reached. ‘Maximum’ rates of rise were estimated by drawing a tangent to the steepest segment of the pressure cycle record. Results were expressed in mmHg sec⁻¹ and were analysed by a paired analysis of variance with replicates (Armitage, 1971).

**Experiment 3: the effect of partly-purified rat ovarian extract on the rat cervix**

Virgin Wistar rats, 180–230 g body weight, were ovariectomized bilaterally under tribromoethanol anaesthesia (2-25%, 1 ml/100 g body weight). All animals were treated for 13
days with oestradiol-17β and progesterone (s.c. in 0.05 ml corn oil) according to the following regimen: Days 1–4 (inclusive) 0.05 µg oestradiol + 1.0 mg progesterone; Days 5–8, 0.1 µg oestradiol + 1.0 mg progesterone; Days 9–11, 0.1 µg oestradiol + 0.5 mg progesterone; Day 12, 0.5 µg oestradiol + 0.25 mg progesterone; Day 13, 1.0 µg oestradiol + 0.1 mg progesterone. Four of the 11 rats also received 5 µg partly-purified rat ovarian extract s.c. in 0.05 ml 1% benzopurpurin daily from Day 7, whereas the remainder (controls) received benzopurpurin only.

The animals were killed by a blow on the head 12 h after the last injection. The cervix was removed and placed in ice-cold saline. Linen threads were passed through each cervical canal and the cervix suspended between two hooks held by an isometric stand (Harvard Instruments, Massachusetts). One hook was fixed and the other attached to a force-displacement transducer (Grass FT 30C). The tissue was immersed in a 10 ml organ bath containing Krebs–Henseleit buffer at 37°C, oxygenated with 95% O₂/5% CO₂. After 15 min equilibration, the resting tension was set by increasing the distance between the two hooks until the tension could be recorded (Tekman TE 200 pen recorder) at 8 cm/g sensitivity. At lower sensitivity the cervix was then extended in 1 mm increments, at 30 min intervals, for a total of 8 mm. The total tension developed at each 1 mm extension and the tension recorded after 30 min adaptation at each new length were compared for the control and experimental animals by Student’s t test.

**Results**

**Extraction**

Ovarian extracts invariably showed high uterine relaxing activity but no extracts of pregnant rat plasma, cervix, maternal placenta or inter-placental uterus possessed such activity when tested in the organ bath either as crude extracts or after partial purification. Both types of extracts of fetal placenta however inconsistently exhibited positive but low uterine relaxing activity.

The ovarian extract was a grey/white powder obtained in yields of about 6–8 mg/g wet weight of ovarian tissue extracted.

**Purification**

Ovarian extract from pregnant rats was purified routinely by gel filtration. A typical result from the Sephadex G50 gel filtration is shown in Text-fig. 1. Three peaks of activity were normally found and these were designated ‘G1’, ‘G2’ and ‘G3’ in order of descending molecular size. The ‘G3 peak’ material was eluted in a position similar to that of porcine relaxin suggesting that the two substances are of similar molecular size (Text-fig. 1). Material from peak ‘G3’ was used in all subsequent experiments.

**Uterine strip tests for uterine relaxing activity**

The electrically driven uterine strip provides a valuable semi-quantitative test system for the presence of uterine relaxing activity in extracts and chromatography fractions. When tension was recorded isometrically it was possible to obtain reproducible recordings of uterine contractions of uniform amplitude. This provided a stable baseline of myometrial activity against which to test for inhibitory properties (Text-fig. 2). That the inhibitory effect observed in the fractions was attributable to relaxin was supported by (1) the similarity of effect with that of porcine relaxin; (2) the virtual exclusion of other known myometrial inhibitors such as steroids, prostaglandins, catecholamines and histamine by the extraction and fractionation procedures; (3) the failure of extracts from the ovaries of non-pregnant rats to inhibit the myometrium; and
Actions of rat relaxin

**Text-fig. 1.** The elution on a Sephadex G50 (fine) column of 40 mg crude extract from the ovaries of pregnant rats. The eluant was 0·2 M-ammonium acetate. The trace shows the protein concentration in terms of absorbance at 280 nm. Uterine relaxing activity is shown by the histograms. Fractions which were tested but had no activity are indicated by the bars below the x-axis. The elution position of porcine relaxin from the same column and the approximate activity (-----) of 3 μg porcine relaxin standard (W1164, lot 8) are shown.

**Text-fig. 2.** Tension development in a uterine strip from a pro-oestrous rat in vitro. The strip was immersed in Krebs–Henseleit buffer at 37°C and stimulated electrically every 45 sec at 4 V amplitude and 50 Hz. The effect of introducing 5 μg ‘G3 peak’ material and the recovery after washing out are shown.

(4) the failure of fractions (in amounts adequate to inhibit the myometrium in the test system by at least 75%) to affect the activity of the rat ileum. The material was also able to induce cervical softening and separate the pubic symphysis, the latter being specific for relaxin.

**Mouse pubic ligament test**

Table 1 shows the increases in the length of the interpubic ligament induced by ‘G3 peak’ material. The ‘G3 peak’ material exhibited a similar activity (w/w) to the standard in this test.
Table 1. Effect of porcine relaxin and the ‘G3 peak’ material extracted from ovaries of pregnant rats on the mouse pubic ligament length

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of mice</th>
<th>Mean ± s.e.m. length of ligament (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (vehicle only)</td>
<td>9</td>
<td>0.21 ± 0.04</td>
</tr>
<tr>
<td>Porcine relaxin standard 2 µg</td>
<td>7</td>
<td>0.31 ± 0.10</td>
</tr>
<tr>
<td>8 µg</td>
<td>7</td>
<td>1.04 ± 0.16**</td>
</tr>
<tr>
<td>‘G3 peak’ material 2 µg</td>
<td>7</td>
<td>0.35 ± 0.07</td>
</tr>
<tr>
<td>8 µg</td>
<td>6</td>
<td>0.90 ± 0.28*</td>
</tr>
</tbody>
</table>

Values significantly different from the control value: *P < 0.02, **P < 0.001.

Experiments 1 and 2: action of ovarian extract on myometrial activity and IUP cycles in vivo

Complete cessation of myometrial activity occurred for about 20 min in each of 7 non-pregnant anaesthetized rats given an i.v. injection of ‘G3 peak’ material (P < 0.001) (Text-fig. 3). Activity recovered thereafter at a higher frequency in individual rats than had been recorded before treatment but, because of variations in the period of total quiescence, average frequency did not exceed control levels until 60–70 min after treatment. During the period of myometrial quiescence the uterus remained responsive to 5 mU oxytocin i.v. (mean frequency of IUP cycles 8.0/10 min ± 1.8; mean maximum amplitude 42.8 ± 7.8 mmHg; n = 4) and to 5 µg PGF-2α i.v. (mean frequency IUP cycles 11.0 ± 3.6; mean maximum amplitude 39.0 ± 9.5 mmHg; n = 4).

Text-fig. 3. Intrauterine (IUP) and carotid arterial (BP) pressure records from an anaesthetized rat treated with three doses of 10 µg ‘G3 peak’ material: (a) shows spontaneous recovery from the treatment, (b) responses to ‘G3 peak’ material followed by 5 mU oxytocin (i.v.) and (c) responses to ‘G3 peak’ material followed by 5 µg PGF-2α intra-arterially.
The rate of rise of intrauterine pressure during IUP cycles was significantly increased during recovery from relaxin (Text-fig. 4). The overall rates of pressure rise were $2.63 \pm 0.54$ mmHg/sec before treatment with the 'G3 peak' material and $6.55 \pm 1.39$ mmHg/sec during the recovery period. The corresponding figures for the maximum rates of rise were $4.74 \pm 0.84$, and $14.26 \pm 2.75$ mmHg/sec. In both cases the differences were statistically significant ($P < 0.005$). Although the mean and maximum amplitudes of IUP cycles fell slightly during the first 10 min, i.e. before complete quiescence occurred after treatment with 'G3 peak' material, during recovery these measures of amplitude were not significantly different from the pretreatment levels.

![Text-fig. 4](Image)

Text-fig. 4. Intrauterine pressure, recorded at a chart speed of 3 mm/sec, from a conscious unrestrained rat. The rate at which pressure rises in the pressure cycles before treatment with 'G3 peak' relaxin is less than that observed in pressure cycles recorded during recovery from relaxin inhibition.

**Experiment 3: effect of 'G3 peak' material on tension development during stretch of the cervix**

As shown in Table 2, the tensions developed initially and after adaptation for 30 min were significantly less ($P < 0.05$, d.f. = 9) in the cervices from animals treated with 'G3 peak' material than those from control animals at all extensions > 1 mm above 'resting length'.

| Table 2. Development of tension (g) in the rat cervix during extension |
|--------------------------|--------------------------|--------------------------|
| Initial                  | After 30 min             |                           |
| Extension (mm)           | Control                  | 'G peak' material         | Control                  | 'G peak' material         |
| 0                        | $0.9 \pm 0.1$            | $0.5 \pm 0.1$            | ---                      | ---                      |
| 1                        | $4.5 \pm 0.5$            | $3.1 \pm 0.8$            | $2.3 \pm 0.5$            | $1.1 \pm 0.2^*$          |
| 2                        | $9.8 \pm 1.4$            | $4.7 \pm 1.0^*$          | $4.9 \pm 0.9$            | $2.2 \pm 0.4^*$          |
| 3                        | $16.2 \pm 2.1$           | $8.1 \pm 2.0^*$          | $8.3 \pm 1.3$            | $3.6 \pm 0.7^*$          |
| 4                        | $24.7 \pm 3.1$           | $13.2 \pm 3.1^*$         | $13.1 \pm 2.0$           | $5.8 \pm 1.4^*$          |
| 5                        | $34.2 \pm 3.5$           | $19.0 \pm 4.8^*$         | $20.0 \pm 3.0$           | $9.4 \pm 2.3^*$          |
| 6                        | $46.8 \pm 4.0$           | $27.9 \pm 6.8^*$         | $30.2 \pm 3.9$           | $15.6 \pm 4.2^*$         |
| 7                        | $57.5 \pm 2.7$           | $38.5 \pm 8.6^*$         | $44.0 \pm 4.0$           | $25.0 \pm 6.9^*$         |
| 8                        | $72.7 \pm 6.1$           | $53.0 \pm 10.0$          | $62.2 \pm 4.6$           | $39.4 \pm 10.3^*$        |

Values are mean $\pm$ s.e.m. for 7 control samples and 4 treated with 'G3 peak' material. *Significantly different from control value, $P < 0.05$.

**Discussion**

These experiments demonstrate for the first time that rat relaxin exerts physiological effects in the rat similar to those that have previously been described only for porcine relaxin. The rat preparation also stimulated pubic ligament formation in mice, a property specific for relaxin. The
ability of rat relaxin to inhibit myometrial activity in vivo and in vitro, and to soften the uterine cervix is consistent with the reports of McMurtry, Kwok & Bryant-Greenwood (1978), Mercado-Simmen, Bryant-Greenwood & Greenwood (1980) and Cheah & Sherwood (1980) that these tissues possess receptors for relaxin as evidenced by their binding of $^{125}$I-labelled relaxin. These findings support the suggestion (Porter, 1979b) that relaxin may play an important role in the preparation of the uterus and the cervix for parturition in the rat. However, the report of Mercado-Simmen et al. (1980) that the number of high-affinity binding sites per mg protein in the uterus declines sharply from about the 17th day of gestation raises doubt about this hypothesis. The problem must await further study for it is uncertain whether or not this finding was attributable to receptor occupancy. The numbers of animals in the late gestation groups were also very small, and no relaxin binding was detected in the uterus of ovariectomized rats, although we have demonstrated apparently high sensitivity of the uterus of such animals in vivo to relaxin inhibition (Porter, Downing & Bradshaw, 1979).

The finding that the rate of rise of intrauterine pressure was significantly elevated after treatment suggests that relaxin improves myometrial co-ordination. This conclusion is based on theoretical (Coren & Csapo, 1963) and experimental (Csapo & Takeda, 1965) evidence that the rate of development of pressure within a uterus is determined by the degree of electrical synchronization within the myometrium. The finding that rat relaxin has this property is consistent with similar findings reported for porcine relaxin (Downing, Bradshaw & Porter, 1980), and lends additional support to the contention that relaxin may mediate some of the actions of oestrogens on the uterus (Steinetz, Beach, Byle & Kroc, 1957) because oestradiol is also known to enhance the rate of rise of intrauterine pressure (Downing, Lye, Bradshaw & Porter, 1978), amongst other actions resembling those of relaxin (Steinetz et al., 1957).

The ability of rat relaxin to soften the cervix contributes to the growing evidence (Porter, 1981) that relaxin is implicated in cervical softening pre partum. However, the degree of softening achieved, even in conjunction with a steroid treatment regimen designed to simulate the endocrine environment of pregnancy, was much less than occurs naturally at term. This suggests that other factors or mechanisms may also be involved. The ‘G3 peak’ material evoked an increase in the length of the interpubic ligament of the oestrogen-primed mouse which appeared to be dose-dependent. The increases were of a similar magnitude to those caused by comparable doses of the Warner-Lambert porcine relaxin standard so that the ‘G3 peak’ material also appeared to contain about 150 GPU relaxin activity/mg. Larkin (1974) has suggested that rat and porcine relaxins may produce dose-response curves which have different slopes, but our data suggest that highly purified rat relaxin is no more active on the mouse pubic symphysis than is relatively impure porcine relaxin. This is in agreement with the findings of Larkin (1974) and of Sherwood (1979), although the explanation is unclear.

In conclusion this report is the first demonstration that relaxin extracted from a particular species exerts the major biological actions ascribed to the hormone in the same species, and therefore justifies further investigation of the significance of this hormone in the normal reproductive functions of the animal.

We are grateful to the Agricultural Research Council for financial support (Grant AG7/77) and to the Lalor Foundation for a fellowship to S.J.D. Warner-Lambert Research Laboratories, Morris Plains, New Jersey, kindly provided the sample of porcine relaxin.

References


Received 10 November 1980

Note added in proof

Further purification of rat relaxin ‘G3 peak’ material by ion exchange chromatography on a carboxymethyl cellulose (Whatman CM52) column during the course of this work yielded a single sharp peak possessing uterine relaxing activity. From an identical column porcine relaxin eluted in 2 or 3 broad peaks at a higher salt concentration.

This finding is in contrast to the report of Sherwood (1979) who obtained 2 peaks of rat relaxin but is supported by the report of Walsh & Niall (1980) who, using a different extraction method, have obtained rat and porcine relaxins as single molecular forms. Present evidence therefore indicates that there is only a single rat relaxin.

References
