Characterization of the binding of ^{125}I -labelled succinylated porcine relaxin to human and mouse fibroblasts

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Summary. A biologically-active succinylated porcine relaxin was iodinated by a modification of the Bolton–Hunter method. Fibroblasts cultured from the mouse pubic symphysis and human skin were used to investigate relaxin binding sites. ^{125}I -labelled relaxin binding to both cell types was time- and temperature-dependent. An accelerated rate of labelled hormone degradation (90%) was observed when both cell types were incubated at 37°C .

Specific relaxin binding sites on the mouse and human cells were observed as other peptides, such as insulin, epidermal growth factor, glucagon, hFSH and human prolactin, failed to inhibit relaxin binding. Further results indicate that porcine relaxin is mitogenic to these specific fibroblasts because increasing concentrations (10^{-9} to 10^{-6} M) of this hormone stimulated cell growth *in vitro*. These data suggest that the effect of relaxin at the target tissue level is mitotic in nature.

Introduction

Porcine relaxin isolated from the pregnant sow ovary is a polypeptide hormone of molecular weight 6300 (Sherwood & O'Byrne, 1974). The complete primary structure is known and strongly resembles insulin and insulin-like peptides (Schwabe, McDonald & Steinetz, 1976; James, Niall, Kwok & Bryant-Greenwood, 1977; Schwabe *et al.*, 1977). Multiple biological actions of this hormone have been described and several have been used as the bases of biological assays (Hall, 1960). The collaborative action of oestrogen and relaxin on the mouse interpubic ligament is the most commonly used bioassay (Steinetz *et al.*, 1960). The exact biochemical-cellular events necessary for pelvic modification induced by relaxin are poorly understood. It has been postulated that relaxin may alter the connective tissue ground substance(s) directly by inducing certain enzyme activities, and/or may stimulate fibroblast growth and peptide-synthesizing activity (Hall, 1960).

McMurtry, Kwok & Bryant-Greenwood (1978) have demonstrated that ^{125}I -labelled succinylated relaxin will bind specifically to tissue homogenates of the mouse and guinea-pig pubic symphyses and with fractions of the pregnant rat mammary gland. Further study was warranted to define more clearly at what level relaxin was exerting its biological action. In the present study we examined the interactions of relaxin with fibroblasts cultured from the mouse pubic symphysis and from normal human skin.

Materials and Methods

Relaxin and other hormones

Relaxin was purified from freshly frozen pregnant sow ovaries according to the method of Sherwood & O'Byrne (1974). Biological activity was assessed by the mouse interpubic ligament

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bioassay (Steinetz *et al.*, 1960). The preparation used for this study (designated CM-a' by Sherwood & O'Byrne, 1974) had a biopotency of 1755 GPU/mg and is subsequently referred to as relaxin. It has been shown that there is no loss of biological activity when relaxin is succinylated (McMurtry *et al.*, 1978).

Porcine insulin, proinsulin and glucagon (No. 258-D30-136-4) were generous gifts from Dr R. Chance, Lilly Pharmaceutical Company, Indiana. Human follicle-stimulating hormone (hFSH), LER-907, was kindly provided by the National Pituitary Agency. Epidermal growth factor was prepared by the method of Savage & Cohen (1972) and was a gift from Dr J. Baker, Department of Biochemistry and Biophysics, University of Hawaii. Human prolactin was generously supplied by the Hormone Distribution Program, NIAMDD.

Radioiodination

Since porcine relaxin contains no tyrosine residue, the iodination method of Bolton & Hunter (1973) was employed using the modification previously described (McMurtry *et al.*, 1978). Separation of ^{125}I -labelled succinylated relaxin from unreacted succinimide ester and free ^{125}I was accomplished by passage through a 2 g Sephadex G-25 column pre-saturated with 0.05% gelatin in 0.05 M-phosphate buffer, pH 7.5. Specific activities of 45–60 $\mu\text{Ci}/\mu\text{g}$ were obtained during a 6-month period.

Fibroblast culture

Mouse fibroblasts were cultured from explants of the pubic symphyses of oestrogen-primed mice (30 g). The pubic symphyses were removed aseptically, minced, and placed in a solution of Hams F.12 medium, pH 7.2–7.4, 20% calf serum containing penicillin (100 $\mu\text{g}/\text{ml}$), streptomycin (100 $\mu\text{g}/\text{ml}$) and amphotericin (Fungizone: 1.25 $\mu\text{g}/\text{ml}$). All materials were obtained from Gibco. After the tissue was explanted in a 75 cm^2 plastic flask (Falcon Plastics) the tissue was allowed to incubate at 37°C in an atmosphere of 95% O_2 , 5% CO_2 . After approximately 2 weeks, there were sufficient cells in the primary culture to transfer to secondary cultures. Transfer was effected by trypsinization for 5 min at 25°C. The hormone-binding studies were conducted on cells obtained from the 2nd to 15th transfer. Cultures were maintained in Hams F.12 medium, supplemented with 10% calf serum.

Cultures of human skin fibroblasts were established from punch biopsies of 3 mm diameter of normal healthy skin from patients attending the dermatology clinic at Tripler Army Hospital. The sites were commonly the forearm, upperarm, scalp and nose. The areas selected were free from disease except the nose specimen which came from a patient with rhinophyma. The biopsies were handled in the same manner as that described for the mouse pubic symphyses explants.

Binding studies

For binding studies, confluent monolayers were washed twice with buffer, Dulbecco's modified Eagle's medium (DMEM) containing 0.1% bovine serum albumin (BSA) pH 7.5, and removed with a rubber policeman. Cells were suspended in buffer solution and diluted to a concentration of $2\text{--}5 \times 10^6$ cells/100 μl buffer. Binding studies were carried out in 12 $\times$ 75 mm glass tubes in the following proportions: 100 μl cells ($2\text{--}5 \times 10^6$ cells), 50 μl of a solution of labelled relaxin at 1 ng/ml, 200 or 250 μl buffer, and 50 or 0 μl unlabelled relaxin at specified concentrations. Binding assays were conducted in duplicate at 4 and 37°C. Simultaneous incubations were run in the presence of excess unlabelled relaxin (5 μg) to determine non-specific binding. Bound and free ^{125}I -labelled relaxin were separated by centrifugation at 2000 g followed by two rinsings with cold buffer. The precipitate was counted in a Packard autogamma counter. The specific binding or net binding of labelled relaxin was obtained by subtracting from total

binding the amount of labelled hormone bound in the presence of high concentration (5 μg) of unlabelled hormone.

Measurement of cell growth

For the growth experiments, cells were plated in 30-mm diameter dishes (Falcon Plastics) at approximately $2.5\text{--}3 \times 10^4$ cells per plate in DMEM containing 2.5% calf serum. The medium was not changed during an 8-day growth period. The peptide hormones relaxin, trypsinized relaxin (obtained by incubating 100 μg relaxin at 37°C in a 1% trypsin solution for 8 h) and human prolactin were dissolved in sterile phosphate-buffered saline (PBS), pH 7.5, and added in 50 μl volumes on Days 1, 3, 5, and 7. On Day 8 the dishes were washed with DMEM trypsinized for 3 min at 25°C , and counted in a haemocytometer.

Results

The net and non-specific binding of labelled relaxin (125 pg/ml) to human fibroblasts derived from the upperarm, as a function of time and temperature, is shown in Text-fig. 1. Maximal binding (9.7%) was obtained at 37°C after a 30-min incubation. The binding was similar regardless of the site of origin of the cells. Very similar binding curves were obtained when mouse pubic symphyseal fibroblasts were used; maximal binding of 12% was reached after 30-min incubation at 37°C which remained constant to the end of the incubation (240 min). At 4°C maximal binding was much less than that at 37°C . In both cell systems, the non-specific binding increased sharply at 37°C , suggesting that the labelled hormone was being degraded at this temperature. To investigate this, an aliquot of the supernatant was removed at the end of 240 min and reincubated with some new cells for 60 min at 37°C . This resulted in less than 3% of the label binding to the cells. A second test was carried out to help ascertain whether degradation of the label had occurred. An aliquot was put through a Sephadex G-25 column, the elution profile showed that over 90% of the radioactivity was free iodide. Hence, degradation of the label at 37°C had occurred in the presence of live cells whilst incubation of medium with label alone showed that there was no degradation.

Text-fig. 1. The amount of ^{125}I -labelled relaxin bound to human skin fibroblasts (upperarm) after incubation at 4°C and 37°C . Net binding (●—●) was obtained by subtracting non-specific binding (○—○) from total binding.

The effect of pH on the binding of labelled relaxin to human and mouse fibroblasts *in vitro* was also investigated. The pH of the buffer (DMEM + 0.1% BSA) was adjusted using 6 N-HCl.

The cells were incubated for 60 min at 37°C, centrifuged and washed as described. Maximal binding was obtained at pH 7.5 with both cell types.

Competition experiments were carried out with labelled relaxin (125 pg/ml) using mouse or human fibroblasts under optimal binding conditions. The displacement of labelled relaxin by unlabelled relaxin was examined over the concentration range 1–10 000 ng/ml. The displacement caused by relaxin was almost the same for human fibroblasts (Text-fig. 2) and mouse fibroblasts (data not shown). A number of other peptide hormones were tested in each system;

Text-fig. 2. Competition for labelled relaxin to human skin fibroblasts by unlabelled relaxin (●) and unrelated peptide hormones; porcine insulin (○), porcine proinsulin (△), and porcine glucagon (⊗), epidermal growth factor (□) and hFSH (×). Cells were incubated in the presence of labelled relaxin and unlabelled hormones in the concentration range 1–10 000 ng/ml for 60 min at 37°C and centrifuged. (Reproduced by permission from *Recent Prog. Horm. Res.* (1979) **34**, p. 205.)

Table 1. Effect of porcine relaxin on mouse fibroblast cell growth

Relaxin conc. ($\mu\text{g/ml}$ medium)	No. of cells $\times 10^{-4}$ *
0	2.61 ± 0.02
0.001	3.98 ± 0.56
0.01	4.31 ± 0.61
0.1	9.82 ± 1.03
1.0	11.94 ± 1.95

Values are mean $\pm$ s.e.m. of triplicate determinations.

* Cells were plated at $2.5\text{--}3 \times 10^4$ cells/plate in DMEM containing 2.5% calf serum. Relaxin was added on Days 1, 3, 5, and 7 at the final concentrations indicated. There was no change of medium during the experiment. On Day 8, cells were removed and counted as described in 'Materials and Methods'.

Table 2. Effect of relaxin on human fibroblast growth, as no. of cells $\times 10^4$ /plate*

Source of fibroblasts	Hormone treatment	Relaxin conc. (M)			
		10^{-9}	10^{-8}	10^{-7}	10^{-6}
Forearm†	CM-a' relaxin	3.1	3.6	4.7	11.3
Upper arm	CM-a' relaxin	1.9	5.3	7.4	13.2
Scalp	CM-a' relaxin	2.3	2.4	7.8	9.2
Nose	CM-a' relaxin	2.5	2.2	3.0	5.2
Forearm†	Trypsinized CM-a' relaxin	3.5	3.7	3.3	4.2
Forearm†	Human prolactin	2.9	3.0	2.6	3.1
Forearm†	None‡	2.6	2.7	2.5	3.0

* Each value is the mean for 3 plates at each hormone concentration.

† Same cell line.

‡ Cells maintained in the presence of 2.5% calf serum plus 50 μ l vehicle (PBS).

there was no inhibition greater than 7% observed with any of these hormones except porcine proinsulin (Text-fig. 2). Competition by proinsulin was less in the mouse fibroblast system than with the human cells.

Previous studies have shown that with elongation of the pubic symphysis, the number of fibroblasts in the tissue increases in relation to other cell types present (Link, Oudet & Petrovic, 1975). The effect of relaxin concentration on mouse fibroblast growth was therefore investigated. At the end of an 8-day growth period, there were three times more cells per plate at high relaxin concentration than were present at the lowest concentration (Table 1). In control plates (medium supplemented with 2.5% calf serum only) no growth occurred over the same period.

Table 2 shows the growth pattern of human skin fibroblasts cultured with increasing concentrations (10^{-9} to 10^{-6} M) of relaxin. Relaxin had a mitogenic effect on cells from the forearm and upperarm and, to a lesser extent, on cells from the scalp and nose. Cells incubated with the same concentration of relaxin which had been previously trypsinized did not increase in numbers. Likewise, human prolactin had no mitogenic effect. No increase in cell numbers was observed at the end of 8 days when the medium contained only 2.5% calf serum.

Discussion

Culture of different cell types has proved to be an invaluable tool for investigating polypeptide hormone actions and hormone receptor activity at the cellular level. Recent reports have demonstrated specific cellular binding of such hormones as insulin (Hollenberg & Cuatrecasas, 1975), epidermal growth factor (Carpenter, Lembach, Morrison & Cohen, 1975) and prolactin (Frantz, Payne, Dombroske & Sonnenschein, 1975). In this study, we report the presence of specific relaxin binding sites on mouse and human fibroblasts. The fibroblasts used in these studies were derived from a known target tissue for relaxin, the mouse pubic symphysis, and from human skin. The latter is a poorly understood target tissue for relaxin.

The binding of iodinated relaxin by both cell types was time-, temperature- and pH-dependent. Binding was both rapid and reversible. Physiological amounts of unlabelled hormone (10^{-9} M) significantly displaced labelled relaxin. Functionally unrelated hormones such as insulin, glucagon, and hFSH did not compete for relaxin binding to human or mouse fibroblasts. Correspondingly, relaxin (5 μ g/ml) did not compete with the insulin-like peptide, multiplication-stimulating activity, for binding to chick embryo fibroblasts (M. Rechler, personal communi-

cation). The competition for relaxin-binding sites on human fibroblasts by porcine proinsulin, albeit at high concentrations, is difficult to explain since there was complete lack of such competition with porcine insulin. The existence of major structural homologies between insulin and relaxin has been well documented (James *et al.*, 1977; Schwabe & McDonald, 1977; Isaacs *et al.*, 1978). The results suggest that there may be greater structural homologies between relaxin and proinsulin, as shown also in radioimmunoassays for porcine relaxin (Bryant-Greenwood & Greenwood, 1979).

The precise effect of relaxin at the cellular level has been neglected and is controversial. Theories of enzyme activation, specifically of collagenase and alkaline phosphatase, have been postulated. An increase in the peptide-synthesizing capacity of specific cells under the influence of relaxin has also been suggested (Hall, 1960). The study of Wahl, Blandau & Page (1977) would indicate that relaxin *per se* does not alter any enzyme activities necessary for the modifications of the pubic symphysis. Relaxin had minimal effect on collagenase levels and activity in the guinea-pig pubic symphysis. Conversely, the observation that relaxin increases ornithine decarboxylase activity in the mouse pubic symphysis (Braddon, 1978) suggests that certain enzyme(s) may act as an intermediary during symphyseal reconstruction. Another hypothesis for the events of pelvic modification during pregnancy and after relaxin treatment can be suggested from our data. Previous histological studies have shown an increase in the number of fibroblasts present in the mouse pubic symphysis following treatment with relaxin (Talmage, 1946; Hall, 1956) and at parturition (Linck *et al.*, 1975). Our results, in which relaxin stimulated the growth of mouse and human fibroblasts *in vitro*, would indicate that relaxin is mitogenic to these cells. A degree of specificity appears to be present in the stimulatory effect of relaxin on different cell types as relaxin failed to stimulate the growth of rabbit articular chondrocytes (Corvol, Malemud & Sokoloff, 1972). Based on these results and from related experiments, it can be postulated that a dissolution of the symphyseal fibrocartilage occurs first, under the influence of oestrogen (Hall, 1960). With the mitotic effect of relaxin, there is an increasing number of fibroblasts present in the reorganized tissue. Concomitantly, there is an elevation in synthesis of new collagen, also stimulated by relaxin (Frieden & Martin, 1954), resulting in a more pliable, ligamentous cartilage, capable of withstanding and conforming to the stresses of parturition.

We have demonstrated that labelled relaxin is capable of binding specifically to human skin fibroblasts and mouse symphyseal fibroblasts and that it is capable of stimulating the growth *in vitro* of these cells. However, we have yet to demonstrate that this binding has a biological consequence and an alteration in the synthesis of collagen as the end biological response must be shown.

The present results are encouraging and form a further link in the little understood action of relaxin on connective tissue. Others have demonstrated the reduction of collagen and hexosamine content of rat skin (Sobel, 1953) and the increase of elasticity caused by exogenous relaxin in patients with scleroderma (Casten & Boucek, 1958). Hence a specific role for relaxin in non-reproductive tissues and in the non-pregnant state awaits further study with possible significance in connective tissue disorders of unknown aetiology.

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References

- Bolton, A.E. & Hunter, W.M. (1973) The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I containing acylating agent. *Biochem. J.* **133**, 529–539.

- Braddon, S.A. (1978) Stimulation of ornithine decarboxylase by relaxin. *Biochem. Biophys. Res. Commun.* **80**, 75–80.
- Bryant-Greenwood, G.D. & Greenwood, F.C. (1979) The specificity of radioimmunoassays for relaxin. *J. Endocr.* **81**, 239–247.
- Carpenter, G., Lembach, K.J., Morrison, M.M. & Cohen, S. (1975) Characterization of the binding of ^{125}I labelled epidermal growth factor to human fibroblasts. *J. biol. Chem.* **250**, 4297–4304.
- Casten, C.G. & Boucek, R.J. (1958) Use of relaxin in the treatment of scleroderma. *J. Am. med. Ass.* **166**, 319–324.
- Corvol, M.T., Malemud, C.J. & Sokoloff, L. (1972) A pituitary growth-promoting factor for articular chondrocytes in monolayer culture. *Endocrinology* **90**, 262–271.
- Frantz, W.L., Payne, P., Dombroske, O. & Sonnenschein, C. (1975) Binding of ovine ^{125}I prolactin to cultured anterior pituitary tumor cells and normal cells. *Nature, Lond.* **255**, 636–638.
- Frieden, E.H. & Martin, A.S. (1954) Uptake of glycine- $^1\text{C}^{14}$ by connective tissue. I. Effects of estrogen and relaxin. *J. biol. Chem.* **207**, 133–142.
- Hall, K. (1956) An evaluation of the roles of estrogen, progesterone and relaxin in producing relaxation of the symphysis pubis of the ovariectomized mouse using the technique of metachrome staining with toluidine blue. *J. Endocr.* **13**, 384–392.
- Hall, K. (1960) Relaxin. *J. Reprod. Fert.* **1**, 368–384.
- Hollenberg, M.D. & Cuatrecasas, P. (1975) Insulin and epidermal growth factor, human fibroblast receptors related to deoxyribonucleic acid synthesis and amino acid uptake. *J. biol. Chem.* **250**, 3845–3853.
- Isaacs, N., James, R., Niall, H., Bryant-Greenwood, G., Dodson, G., Evans, A. & North, A. (1978) Relaxin and its structural relationship to insulin. *Nature, Lond.* **271**, 278–281.
- James, R., Niall, H., Kwok, S. & Bryant-Greenwood, G. (1977) Primary structure of porcine relaxin: homology with insulin and related growth factors. *Nature, Lond.* **267**, 544–546.
- Linck, G., Oudet, C. & Petrovic, A. (1975) Multiplication des différentes variétés cellulaires de la symphyse pubienne de souris au cours de la croissance et de la première gestation: étude radioautographique à l'aide de la thymidine tritiée. *Bull. Assoc. Anat.* **59**, 467–472.
- McMurtry, J.P., Kwok, S.C.M. & Bryant-Greenwood, G.D. (1978) Target tissues for relaxin identified *in vitro* with ^{125}I -labelled porcine relaxin. *J. Reprod. Fert.* **53**, 209–216.
- Savage, C.R. & Cohen, S. (1972) Epidermal growth factor and a new derivative: Rapid isolation procedures and biological and chemical characterization. *J. biol. Chem.* **247**, 7609–7611.
- Schwabe, C. & McDonald, J.K. (1977) Relaxin: a disulfide homolog of insulin. *Science, N.Y.* **197**, 814–915.
- Schwabe, C., McDonald, J.K. & Steinetz, B.G. (1976) Primary structure of the A chain of porcine relaxin. *Biochem. Biophys. Res. Commun.* **70**, 397–405.
- Schwabe, C., McDonald, J.K. & Steinetz, B.G. (1977) Primary structure of the B chain of porcine relaxin. *Biochem. Biophys. Res. Commun.* **75**, 503–510.
- Sherwood, C.D. & O'Byrne, E.M. (1974) Purification and characterization of porcine relaxin. *Archs rats. Archs Biochem. Biophys.* **46**, 221–231.
- Sobel, A. (1953) The collagen and hexosamine content of the skin of normal and experimentally treated rats. *Archs Biochem. Biophys.* **46**, 221–231.
- Steinetz, B.G., Beach, V.L., Kroc, R.L., Stasilli, N.R., Nussbaum, R.E., Nemith, R.J. & Dun, R.K. (1960) Bioassay of relaxin using a reference standard. A simple and reliable method utilizing direct measurement of interpubic ligament formation in mice. *Endocrinology* **67**, 102–115.
- Talmage, R.V. (1964) Histological effects of estradiol, progesterone and relaxin on the symphysis pubis of the guinea pig. *Anat. Rec.* **96**, 528–529.
- Wahl, L.M., Blandau, R.J. & Page, R.C. (1977) Effect of hormones on collagen metabolism and collagenase activity in the pubic symphysis ligament of the guinea pig. *Endocrinology* **100**, 571–579.

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