Vol. 170, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1990 Pages 214-222

CYCLIC AMP RESPONSE TO RECOMBINANT HUMAN RELAXIN BY CULTURED HUMAN ENDOMETRIAL CELLS — A SPECIFIC AND HIGH THROUGHPUT IN VITRO BIOASSAY

David T.W. Fei, Mary C. Gross*, Julie L. Lofgren,
Marina Mora-Worms, and Anthony B. Chen

Department of Medicinal & Analytical Chemistry, and *Assay Services,
Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080

Received May 31, 1990

SUMMARY: A specific and high throughput 96-well format bioassay for recombinant human relaxin (rhRLX) has been developed using human endometrial cells (NHE cells). rhRLX caused a time- and dose-dependent stimulation of cyclic AMP (cAMP) with 1/2 maximal activity of 3.56±0.65 ng/ml (n=30). The range of the standard curve was 0.39 to 25 ng/ml with interplate precision of 17 and 22% CV for high and low controls respectively. The cAMP response requires forskolin and 3-isobutyl-1-methylxanthine, and is enhanced by prostaglandin E_2 and $F_{2\alpha}$. The NHE cells do not respond to A or B chains of rhRLX, or a whole array of hormones. Preincubation of rhRLX with specific monoclonal antibody completely abolished the cAMP response. This bioassay has been used to determine the biological activity of several manufactured lots of recombinant human relaxin.

INTRODUCTION: Relaxin (RLX)) exerts a profound effect on the reproductive tract during pregnancy. Its biological actions include widening of the pelvis (1,2), softening of the pubic symphysis ligament (1,3,4) and inhibiting myometrial contractility (5). Human relaxin (hRLX) has been purified from corpora lutea of patients with ectopic pregnancies (6) and quantitated in serum from non-pregnant and pregnant women (7,8). Relaxin receptors have been detected in the uterus, placenta and seminal fluid (9,10). It has been reported that RLX stimulates the production of cAMP in mouse pubic symphysis (11), rat uterus (12,13), rat uterine tissue slices (14), rat myometrial cells (15), rat anterior pituitary gland (16) and newborn rhesus monkey uterine (NRMU) cells (17). In order to reliably quantitate an in-vitro biological activity for the development of rhRLX as a pharmaceutical, we have made use of the fact that human uterine endometrial cells can also produce cAMP in response to RLX (18) to develop a specific and high throughput bioassay.

MATERIALS & METHODS: **NHE Cell Culture:** Cultures of human endometrial cells (NHE cells) were derived from a collagenase-treated uterus isolated from a premenopausal woman who had a hysterectomy for reasons other than uterine disease. Tissue preparation and cell dispersion were performed according to the procedure of Casey and co-workers. (19) Cells used in this study were taken between passage 13 and passage 22, and were maintained in Falcon T175 flask (3028)

0006-291X/90 $1.50

containing 50 ml culture medium of Ham's F12/Dulbecco's modified Eagles' media (1:1) supplemented with 10% heat-inactivated newborn calf serum, HEPES (24 mM), glutamine (2 mM), Penicillin (100 U/ml) and Streptomycin (100 µg/ml) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. These cells were optimally split every 2 days at a 1:2 ratio when 95-100% confluency was reached. After passage 25, the growth rate of the cells were conspicuously slowed and eventually ceased.

Peptides and Steroids: Recombinant human RLX, relaxin A chain and relaxin B chain were produced and purified by Dr. Ernst Rinderknecht at Genentech, Inc. Protein concentrations were determined by quantitative amino acid analysis. Porcine relaxin was a generous gift from Dr. G. Tregear (Howard Florey Institute of Experimental Physiology and Medicine, University of Melbourne). Protropin-Growth Hormone (rhGH), Activase-tPA, Actimmune-Interferon γ (rHuIFNγ), IGF-I and TGF-β were products of Genentech, Inc. Arginine vasopressin, oxytocin, ACTH, glucagon, angiotensin II, HCG, TRH, EGF, insulin, fibroblast growth factor were purchased from Peninsula Laboratories. β-estradiol, progesterone, prostaglandin E_2, prostaglandin $F_{2\alpha}$, pindolol, isoproterenol, β-mercaptoethanol, forskolin and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Sigma.

Relaxin Bioassay: Twelve thousand ±2,000 NHE cells per well in 100 µl of culture medium were seeded into Falcon 96-well tissue-culture plate, and incubated for 20-24 hr at 37°C in a humidified atmosphere of 95% air and 5% CO_2. RLX standards and samples (100 µl) made up in medium containing 2 µM forskolin[1] and 100 µM IBMX[1] were then added to triplicate wells without removing any medium and incubated for 30 min. at 37°C[2]. At the end of the incubation, 100 µl of the culture medium was taken for measurement of extracellular cAMP and the rest discarded. Intracellular cAMP was extracted with 200 µl of 0.1N HCl for 30 min. An aliquot (100 µl) was carefully neutralized with 100 µl 0.1N NaOH, diluted with 300 µl cAMP buffer (50 mM sodium acetate, pH 6.2 with 0.01% thimerosal) and measured by radioimmunoassay. Samples (100 µl) for extracellular cAMP activity were diluted with 400 µl cAMP buffer prior to radioimmunoassay. Sample concentrations (ng/ml) were determined by interpolation on the standard curve where RLX concentration was plotted vs pmoles cAMP per well generated. The data were fitted using a non-linear, least square (4 parameter fit) program.

The radioimmunoassay for measurement of cAMP was based on the competition between unlabeled cAMP (Sigma) either in the standard or sample and a fixed amount of [^{125}I] cAMP Tyrosine Methyl Ester (Dupont), for anti-cAMP antibody (goat anti-cAMP obtained from Cambridge Medical Diagnostics). After overnight incubation of ~18 hours, immune complexes were precipitated with donkey anti-goat IgG (Pel Freez) and PEG 8000 (Sigma, 6% final concentration) and centrifuged. The supernatant was decanted and the pellet was counted in a gamma counter. Sample concentrations were computed from logit/log programs, and expressed in picomoles per milliliter (pmol/ml). The radioimmunoassay has a range of 1.56-50 pmol/ml.

RESULTS: Time Course of Cyclic AMP Generation: Fig. 1 shows a typical time course of cAMP generation in NHE cells at 37°C with 10 ng/ml relaxin. Generation of intracellular cAMP was progressively increased for 15 min, reaching a plateau

---

[1]Final concentration in the well for forskolin and IBMX was 1µM and 50 µM, respectively.

[2]Incubation at room temperature gave higher background and greater variability.

around 30 min and gradually declined to about baseline after 120 min incubation. Concurrent to the increase of intracellular cAMP concentration, there was a gradual increase of cAMP in the extracellular medium. At 30 min incubation, about 15 % of the total cAMP produced (the sum of intracellular and extracellular) was secreted into the medium. At two hours of incubation >90% of the cAMP produced is extracellular while the intracellular cAMP decreased almost to basal level. For measurement of intracellular cAMP, 30 min incubation which gave maximal response was selected for routine assay. Interassay and interplate precision data were obtained under these conditions.

Forskolin and IBMX Requirements: Early studies in NRMU cells (17) have indicated that the response of cAMP to RLX required the presence of 1 μM forskolin and 50 μM IBMX. In the present system using 12,000 cells/well, the presence of both forskolin and IBMX were also essential for the cAMP response (Fig. 2). In the absence of either forskolin or IBMX, cAMP generation was barely detectable. A significant response was observed only when more NHE cells (100,000 cells/well) were used (data not shown). Hence, for all experiments described below, NHE cells were treated with RLX in the presence of 1 μM forskolin and 50 μM IBMX.

Characteristic of the Bioassay: Using the information stated above, a representative dose-response curve for cAMP generated vs rhRLX concentration is shown in Figure 3. The range of the standard curve was 0.39 to 25 ng/ml (65.5 pmol to 4190 pmol). Seeding at cell density in the range of 10,000 to 14,000 per well did not

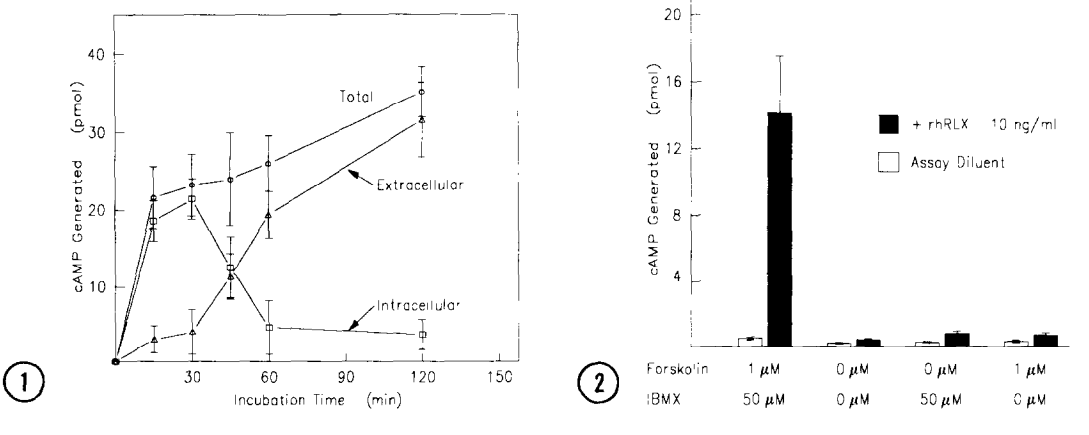

Fig. 1. Time course of cAMP generation by 10 ng/ml of rhRLX in NHE cells seeded at 12,000±2,000 cells/well. At the times indicated 100 μl culture medium were taken and measured by RIA (extracellular cAMP, triangle). Then the rest of culture medium was discarded, intracellular cAMP extracted and measured by RIA (square) as detailed in the Materials and Methods. Total cAMP generated (circle) was the sum of extracellular and intracellular cAMP. Each value is the mean ± S.D. of triplicate determinations.

Fig. 2. Effect of 1 μM forskolin and 50 μM isobutylmethylxanthine on cAMP generation by 10 ng/ml rhRLX in NHE cells.

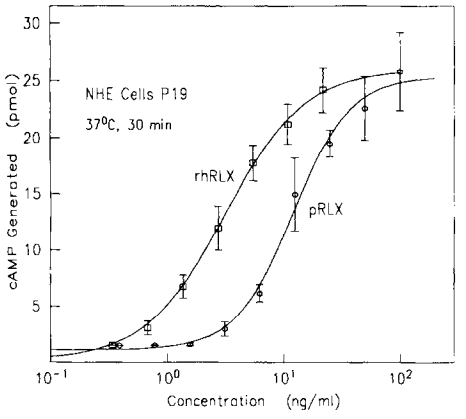

Fig. 3. Intracellular cAMP response in human endometrial cells (NHE). Recombinant human relaxin (rhRLX) was represented by open squares, and porcine relaxin (pRLX) by circles.

significantly alter the slope and 1/2 maximal response of the bioassay (data not shown). The assay was reproducibly sensitive to measure 0.78 ng/ml, two standard deviations above basal level (or 0.19 ng/ml in the well). Routinely, three 96-well plates were performed in one assay. Each plate contained a relaxin standard curve plus two controls (high and low) which were freshly diluted on the day of the assay. From 10 separate assays and a total of 30 plates, the variability of various parameters of the assay were tabulated (Table I). The precision of the assay (coefficient of variance) as calculated from all 30 plates (interplate variability) is 17.3 and 22.3 percent for high and low controls respectively. By comparing the mean of three plates for each assay, the precision is improved (interassay variability) to 8.0 and 14.4 percent for the same controls respectively. The 1/2 maximal response for rhRLX occurred at 3.56±0.65 ng/ml. In all assays, the basal response of NHE cells in the presence of forskolin and IBMX was below the detectability of the cAMP assay (1.56 pmol/ml). Pure native porcine relaxin also stimulated cAMP production in this bioassay with a similar intrinsic activity as human RLX but was less potent (1/2 maximal activity of 11.9±2.1 ng/ml, n=3).

Specificity: The specificity of the cAMP response to RLX was examined by neutralizing antibody studies. Preincubation of RLX (5 ng/ml) with serially diluted

Table I: Variability of the Cyclic AMP Response to Relaxin in NHE Cells

		Controls (ng/mL)		1/2 Max		Maximal response
		Low	High	(ng/mL)	Slope	(pmol)
Interplate Variability (n=30)	mean	1.01	3.23	3.56	1.78	24.1
	SD	0.22	0.56	0.65	0.32	4.7
	%CV	22.3%	17.3%	18.3%	17.9%	19.3%
Interassay Variability (n=10)	mean	1.01	3.22	3.55	1.79	24.1
	SD	0.14	0.26	0.54	0.19	4.2
	%CV	14.4%	8.0%	15.1%	10.5%	17.6%

monoclonal Ab (A8) raised against a RLX analogue (17) leads to complete abolition of biological activity (Fig. 4). Under the same conditions, the non-specific monoclonal Ab 504 raised against gp120 envelope glycoprotein of human immunodeficiency virus has no effect on relaxin's ability to stimulate cAMP production. Neither rhRLX A chain, nor rhRLX B chain nor A chain mixed with B chain (1:1) elicits a cAMP response in this bioassay (Fig. 5a). Reduction of rhRLX into chains by heating at 98°C for 3 min in the presence of 3% β-mercaptoethanol completely abolished the cAMP response, whereas rhRLX treated by the same conditions without reduction retained its biological activity (Fig. 5b). When NHE cells were tested with an array of hormones, growth factors, steroids and drugs (namely, insulin, 58 μg/ml; IGF-I, 50 μg/ml; fibroblast growth factor, 1 μg/ml; TGF-β, 0.1 μg/ml; tPA, 1 μg/ml; rHuIFNγ, 20 μg/ml, HCG, 100 IU/ml; and rhGH, vasopressin oxytocin, ACTH, glucagon, angiotensin II, thyrotropin releasing hormone, EGF, hydrocortisone, β-estradiol, progesterone, all at 10 μg/ml; and pindolol, 25 μg/ml) in no instance was a cAMP response elicited. The NHE cells, however, did respond to more than 1,000 fold above the physiological concentration of prostaglandin E_2 and prostaglandin $F_{2\alpha}$, and minimally to isoproterenol at 25 μg/ml (data not shown).

Potentiation of Cyclic AMP Generation by Protaglandins: When prostaglandin E_2 or $F_{2\alpha}$ were incorporated into the culture medium at a concentration which did not elicit a cAMP response there was an enhanced generation of cAMP by rhRLX in NHE cells. Fig. 6 represents the time course of cAMP generation when either 5 ng/ml prostaglandin E_2 (6A) or 5 μg/ml prostaglandin $F_{2\alpha}$ (6B) were added together with 5 ng/ml rhRLX to the NHE cells. The cAMP generated by the combination of

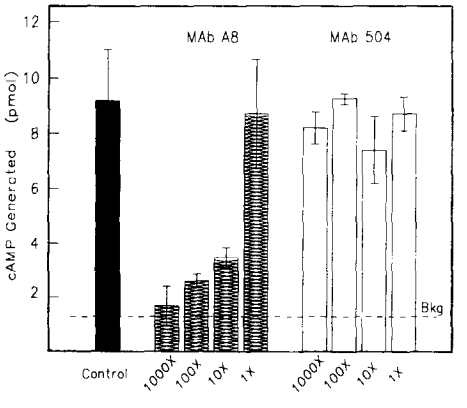

Fig. 4. Neutralization of cAMP response by preincubation of rhRLX with monoclonal antibody to RLX. rhRLX (5 ng/ml) was preincubated for 1 h at 37°C with either assay diluent (filled column), or various dilutions of MAb A8, a monoclonal antibody specific for RLX (hatched column) and a nonspecific monoclonal antibody MAb 504 (open column) prior to the bioassay. Each column represents the mean and S.D. of triplicate determinations. 1X represents an antibody concentration of 25 ng/ml. Bkg represents background cAMP levels (1.56 pmol) of the assay.

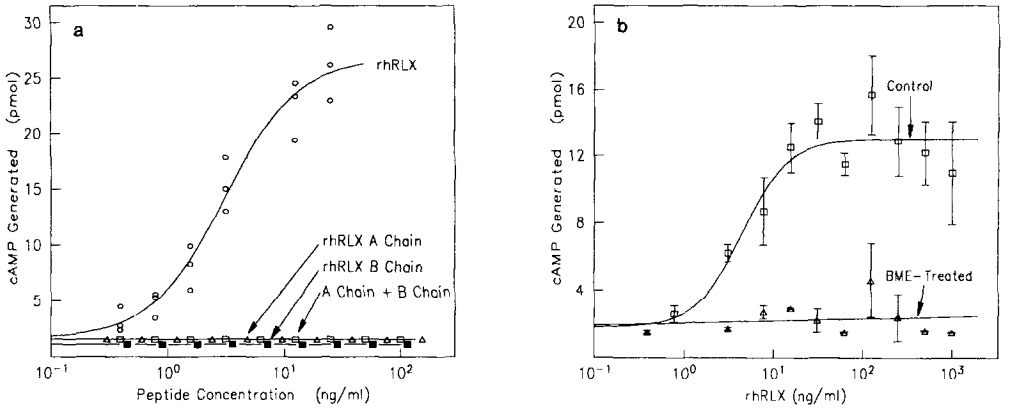

Fig. 5a. Activity of rhRLX A-chain (triangles), rhRLX B-chain (filled squares), or 1:1 rhRLX A-chain mixed with rhRLX B-chain (open squares) in the RLX bioassay. Intact rhRLX is shown by open circles.
Fig. 5b. Abolition of biological activity of rhRLX by reduction into separate chains with β-mercaptoethanol.

prostaglandins and rhRLX was significantly higher ($p<0.001$) than the sum of cAMP generated by individual rhRLX plus prostaglandin.

In vitro Biological Activity of Various Recombinant Human Relaxin Lots: Table II illustrates the 1/2 maximal response of cAMP by five different manufactured lots of recombinant human relaxin produced and purified at Genentech. Preparation 3 was the reference standard. In all instances, the 1/2 maximal response obtained was within two standard deviations of the reference standard.

DISCUSSION: In the present paper, a bioassay specific for hRLX was developed. Based on the early findings that hRLX elicits a cAMP response in NRMU cells (17),

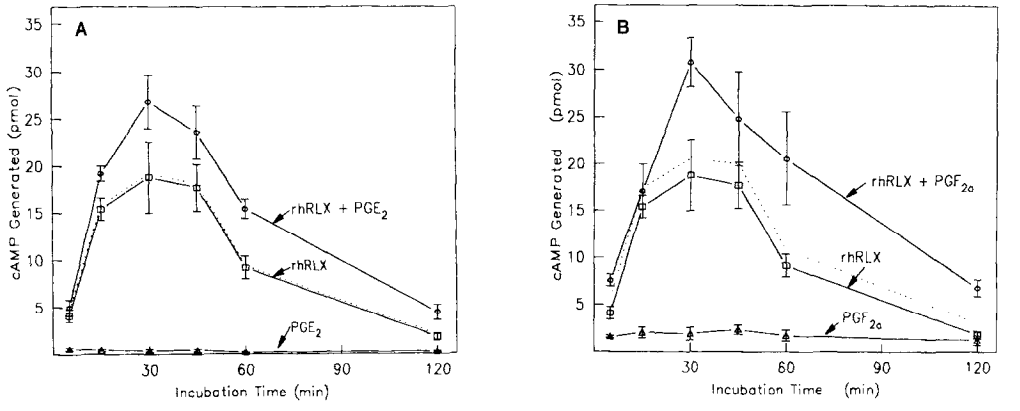

Fig. 6. Potentiation of cAMP response to 5 ng/ml recombinant human relaxin (square) by prostaglandin E_2, 5 ng/ml (A) and prostaglandin $F_{2\alpha}$, 5 μg/ml (B). Dotted lines represent the additive values of cAMP generated by rhRLX plus PGE_2 or $PGF_{2\alpha}$, while the circle represents the actual values of cAMP measured.

Table II: 1/2 Maximal Response (ng/ml) for Various Preparations of Recombinant Human Relaxin in the Generation of cAMP in NHE Cells

	1/2 Max (ng/ml)				
Assay Number	Preparation 1	2	3	4	5
1	2.90	3.50	4.00	4.70	4.84
2	3.65	5.30	2.74	5.70	2.80
3	5.10	5.00	3.87	3.40	2.79
mean	**3.90**	**4.60**	**3.54**	**4.60**	**3.48**
S.D.	1.10	1.00	0.69	1.20	1.20
n	3	3	3	3	3

the current bioassay utilized homologous human endometrial cells to measure the biological activity of rhRLX. The assay was improved from a 6-well format to a high throughput 96-well format with good precision throughout the range of 0.78 to 25 ng/ml. The response was both dose and time-dependent. Preliminary data indicate that two other cell lines derived from endometrium and myometrium of a different patient were also responsive to rhRLX in the generation of cAMP. Of the cell lines tested positive, the NHE cell line is the most responsive one requiring only 12,000 cells/well at the start of seeding. Chen and co-workers (18) have reported that porcine RLX elicits cAMP in human endometrial epithelial glandular cells when 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone, a phosphodioesterase inhibitor was included in the incubation medium. These investigators also indicated that there was great variability of response to porcine RLX by various cell line preparations with the proliferative phase of human endometrium being most responsive. In the present system, purified porcine RLX was less potent than hRLX with 1/2 maximal activity of 11.9 ng/ml. Both forskolin, an activator of adenyl cyclase, and IBMX, an inhibitor of phosphodiesterase, were essential to this cAMP response by relaxin (Fig. 3). In the absence of forskolin and IBMX, more NHE cells (100,000/well) were required to observe the cAMP response to RLX (data not shown). It is tempting to postulate that other agents that activate adenyl cyclase may potentiate the effect of relaxin.

One interesting finding is the cAMP accumulation in the extracellular media. Similar findings in NRMU cells have been previously reported (17). By prolonging the incubation time, measurement of extracellular cAMP concentration may offer a convenient way of screening cells that respond to RLX. The data shows that by 120 minutes of incubation, over 90% of generated cAMP was secreted into the extracellular medium. It is possible that RLX may act as a paracrine hormone which exerts its action on other target tissues via the secreted cAMP.

It is intriguing to find that the addition of either prostaglandin E_2 and $F_{2\alpha}$ in the culture medium enhanced the production of cAMP by relaxin (Fig. 6). Both prostaglandin E_2 and $F_{2\alpha}$ were present in high concentrations during labour and spontaneous abortion (20). It has been proposed that prostaglandin play a central role in initiating myometrial contractions and the onset of labor in women (21,22).

These effects were in direct antagonism to RLX which inhibits the contraction of myometrial tissue (5). The biological significance of this enhanced cAMP reponse to relaxin by prostaglandins is currently being investigated.

In conclusion, a specific and high throughput bioassay for RLX has been developed. Hormones such as insulin and IGF-I which have structures similar to relaxin have no effect on the cAMP production. The assay is reproducibly reliable in measuring the in vitro biological activity of rhRLX. The general assay format may be adopted in other cell lines where RLX receptors were demonstrated to be present.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Ernst Rinderknecht for the production and purification of recombinant human relaxin, relaxin A chain and relaxin B chain; Dr. Brian Fendly and Ms. Toni Klassen for raising specific monoclonal antibody to relaxin; Dr. Susan Kramer and Ms. Ursula Gibson for isolating and initiating the NHE cell lines; Ms. Monique Carver and Cora Lai for performing the early 6-well format bioassay; Assay Services for performing cAMP assay; and Dr. Richard Vandlen for his interest and support in making this study possible.

REFERENCES

1. Kroc, R.L., Steinetz, B.G., and Beach, V.L. (1959) Ann.N.Y. Acad.Sci. 75, 942-980.
2. O'Byrne, E.M., Sawyer, W.K., Butler, M.C., and Steinetz, B.G. (1976) Endocrinology 102, 1641-1644.
3. Steinetz, B.G., Beach, V.L., and Kroc, R.L. (1957) Endocrinology 61, 271-280.
4. Ferraiolo, B.L., Cronin, M., Bakhit, C., Roth, M., Chestnut, M., and Lyon, R. (1989) Endocrinology 125, 2922-2926.
5. Schwabe, C., Steinetz, B.G., Weiss, G., Segaloff, A., McDonald, J.K., O'Byrne, E.M., Hochman, J., Carriene, B., and Goldsmith, L. (1978) Recent Prog.Horm.Res. 34, 123-199.
6. Shih, A., Goldsmith, L.T., Weiss, G.,, Bourell, J., and Winslow, J. (1989) 71st Annual Meeting of the Endocrinology Society (Abstract No. 1543), Seattle, Washington.
7. Lucas, C., Bald, L.N., Martin, M.C., Jaffe, R.B., Drolet, D.w., Mora-Worms, M., Bennett, G., Chen, A.B., and Johnston, P.D. (1989) J.Endocrinol. 120, 449-457.
8. Stewart, D.R., Celniker, A., Taylor, C., Cragun, J.R., Overstreet, J.W., and Lasley, B.L. (1990) J.Clin.Endocrinol.Metab. (in press)
9. Mercado-Simmer, R.C., Bryant-Greenwood, G.D., and Greenwood, F.C. (1982) Endocrinology 110, 220-226.
10. Sherwood, O.D. (1988) Relaxin (E. Knobil and J. Neill, eds.), pp.585-673. Raven Press, Ltd., New York, NY.
11. Braddon, S.A. (1978) Endocrinology 102, 1292-1299.
12. Sanborn, B.M., and Sherwood, O.D. (1981) Endocrine Research Comm. 8(3), 179-192.
13. Sanborn, B.M., Kuo, H.S., Weisbrodt, N.W., and Sherwood, O.D. (1980) Endocrinology 106, 1210-1215.
14. Judson, D.G., Pay, S., and Bhoola, K.D. (1980) J.Endocrinol. 87, 153-159.
15. Hsu, C.J., McCormack, S.M., and Sanborn, B.M. (1985) Endocrinology 116, 2029-2035.

16. Cronin, M.J., Malaska, T., and Bakhit, C. (1987) Biochem.Biophys.Res. Commun. 148, 1246-1251.
17. Kramer, S.M., Gibson, U.E.M., Fendly, B.M., Mohler, M.A., Drolet, D.W., and Johnson, P.D. (1990) In Vitro Cell.Devel.Biol. (in press)
18. Chen, G., Huang, J.R., and Tseng, L. (1988) Biol.of Reproduction 39, 519-525.
19. Casey, M.L., MacDonald, P.C., Mitchell, M.D., and Synder, J.M. In Vitro Cell. Devel.Biol. (1984) 5, 396-403.
20. Karin, S. (1971) Ann.N.Y. Acad.Sci. 483-497.
21. Thorburn, G.D., Challis, J.R.G., and Currie, W.B. (1977) Biol.of Reproduction 16, 18-27.
22. Thorburn, G.D. (1985) Prostaglandins and the regulation of myometrial activity: a working model (C.T. Jones, ed.), pp.381-402. Academic Press, New York, NY.