In Vitro Cell. Dev. Biol. 26:647-656, June 1990

0883-8364/90 $01.50+0.00

INCREASE IN CYCLIC AMP LEVELS BY RELAXIN IN NEWBORN RHESUS MONKEY UTERUS CELL CULTURE

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(Accepted 26 March 1990; editor David W. Barnes)

SUMMARY

A novel relaxin sensitive cell line of apparent smooth muscle origin has been established from a newborn rhesus monkey uterus (NRMU). NRMU cells respond to relaxin, in the presence of 1 μM forskolin, by producing intracellular adenosine 3′, 5′-cyclic monophosphate (cAMP). The increase in cAMP levels is dose, time and cell density dependent, reaching peak levels at 10 min when cells are seeded at 1×10^5 cells/well. Specificity was demonstrated by neutralization of the relaxin activity with anti-relaxin monoclonal and polyclonal antibodies, degradation of cAMP in the presence of phosphodiesterase, and confirmation of the absence of cGMP. Three synthetic analogs of human relaxin generated a dose-related cAMP response as did synthetic native human relaxin. Natural relaxin purified from human corpora lutea tissue also generated a response similar to synthetic human relaxin. Porcine and rat relaxins also increased levels of cAMP. Insulin, but not IGF I or IGF II, was capable of increasing cAMP levels in NRMU cells, however, 200 ng/mL were required to achieve cAMP levels comparable to 6.25 ng/ml relaxin. Combinations of relaxin with insulin, IGF I or IGF II did not increase cAMP levels above levels obtained with relaxin alone. The effect on NRMU cells of other hormones, growth factors and drugs potentially present in cell culture systems or serum samples was evaluated. In combination with relaxin, oxytocin significantly decreased the cAMP production below the levels induced by relaxin alone, whereas progesterone and prostaglandin E_2 resulted in additive increases in cAMP. These data suggest that the NRMU cell line is an appropriate target tissue for studying relaxin-mediated biological responses *in vitro* as well as functioning as the primary component of a relaxin *in vitro* bioassay.

Key words: relaxin; cyclic AMP; newborn rhesus monkey uterus cells.

INTRODUCTION

The exclusive action of cyclic adenosine 3′,5′-monophosphate (cAMP) is thought to be the activation of cAMP-dependent protein kinases (13,20). cAMP-dependent protein kinases have been described in the uterus and are present in both the myometrium (18) and the endometrium (29,30). The earliest report associating relaxin with increased tissue cAMP levels was that of Braddon in 1978 (1), which described relaxin-dependent changes in murine pubic symphysis cAMP levels. Cheah and Sherwood (6) reported a correlation between biodistribution and cAMP production in ovariectomized rats identifying uterine horns and the cervix as the target tissues of relaxin. Subsequent studies by Judson et al. (15) and Sanborn et al. (31,32) demonstrated elevated uterine cAMP levels in rat uterine strips in the presence of the phosphodiesterase (PDE) inhibitor 3-isobutyl-l-methylxanthine (IBMX). More recently, Hsu et al. (12) described the effect of relaxin treatment on cAMP levels in rat primary myometrial cell cultures in the presence of forskolin, a diterpene activator of adenylate cyclase (33).

Only primary uterine cells or uterine tissue sections have been used in relaxin assays based on cAMP measurements. An established cell line responsive to relaxin would provide a standardized test system for assays free of animal to animal variability and cell type heterogeneity (21). In addition, pure cultures of relaxin responsive tissue grown under controlled conditions could be expected to provide both greater sensitivity to relaxin and better reproducibility. The development of such a cell line has been impeded by the difficulty in establishing smooth muscle cell cultures combined with the tendency of such cultures to dedifferentiate over time (4). A cell line derived from newborn rhesus monkey uterus (NRMU) and a relaxin specific assay based on increased intracellular cAMP levels in these cells are described herein.

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Materials and Methods

Synthetic human relaxin. The native forms of human gene-2 relaxin (hRLX-2) and three analogs were prepared by *in vitro* chain combination of A and B chains produced by solid phase peptide synthesis (14). The hRLX-2 (SMM) analog contains a B chain with a serine (S) as the amino terminal residue deletion of the aspartic acid at position 1. A des- methionine derivative of this analog hRLX-2 (SKA) was made by substitution of the two methionines at positions 4 and 5, in the serine amino terminal B chain, by a lysine (K) and alanine (A), respectively. The hRLX-2 (DKA) analog contains the full length B chain with the same methionine substitutions as described for hRLX-2 (SKA). All *in vitro* chain combined human relaxins have previously been shown to have equivalent biological activity with themselves and porcine relaxin (14) as measured by the murine pubic symphysis (35) and rat uterine contractility bioassays (34).

Protein content was determined by quantitative amino acid analysis and relaxin was stored lyophilized and dessicated at temperatures below −20° C. Standards were prepared fresh daily by diluting a stock solution of 0.1 μg/mL hRLX-2 (SKA) or (DKA) in cAMP assay diluent (F12/DMEM, 0.1% BSA, 0.01% Tween 80 and 24 m*M* HEPES).

Human corpora lutea relaxin. Natural hRLX was extracted from corpora lutea (CL) of patients with ectopic pregnancies at 7 weeks gestation as described in reference (8). Briefly, tissue was extracted with 70% acetone containing 0.15 *M* hydrochloric acid. Immunoreactive hRLX measured by ELISA (24) was precipitated from the extract by the addition of 5 volumes of cold acetone to a final concentration of 95%. Relaxin was further purified by gel filtration on a Biogel P-10 column, followed by affinity chromatography (8) using monoclonal antibody MAb RLX-2. The hRLX thus obtained was estimated to be a 6500 dalton peptide by its elution position on P-10 gel filtration, to have an amino terminal sequence identical to hRLX-2.

Relaxin specific monoclonal and polyclonal antibody production. MAb RLX-2 (IgG_1,k) was produced by immunizing a BALB/c female mouse as follows: 20 μg of hRLX-2 (SKA) was injected subcutaneously in complete Freund's adjuvant on Day 0; 20 μg was injected subcutaneously and intramuscularly in incomplete Freund's adjuvant on Day 7; 20 μg of hRLX-2 (SKA) in PBS was injected intraperitoneally on Day 14; 10 μg hRLX-2 (SKA) was dried onto a nitrocellulose disc and implanted intraperitoneally on Day 21 followed by a prefusion intraperitoneal implant with 5 μg on a nitrocellulose disc on Day 35. The spleen was removed three days later and fused with the mouse myeloma cell line P3X63-AG8.653 (16) using polyethylene glycol 4000 and a spleen cell to myeloma cell ratio of 4:1 (26). Cells were plated at a density of 4×10^5 cells/well in HAT selection media (22) into 96 well plates. Hybridoma supernatants were screened with hRLX-2 (SKA) using the ELISA format. Briefly, hRLX-2 (SKA) was coated onto 96 well plates overnite at 4° C or 1 h at 37° C at 100 ng/well in PBS. Wells were blocked with PBS + 0.1% gelatin (Biorad, Richmond, CA) for 1 hour at 37° C and then washed with PBS + 0.05% Tween 20 (PBST) and incubated with 50 μL of hybridoma supernatant for 1 h at 22° C. Plates were washed with PBST and incubated with a 1:4000 dilution of goat anti-mouse IgG coupled to horseradish peroxidase (Tago, Burlingame, CA). The plates were again washed with PBST and 100 μL of o-phenylenediamine dihydrochloride (Sigma, St. Louis, MO), 1 mg/mL in 0.05 *M* citrate/phosphate buffer pH 5.0, was added to each well. The reaction was stopped after 30 min by the addition of 100 μL of 2.5 *M* sulfuric acid, and read on a multiscan platereader (Flow, McLean, VA) at 492 nm. Positive fusion wells were cloned twice by the limiting dilution method. Ascites were produced (28) and antibody purified over a protein A (Fermentech, Inc., Edinburgh, Scotland) sepharose column (26) and stored at 4° C in PBS with 0.02% sodium azide. Sodium azide was removed from the purified IgG solutions by dialysis in PBS prior to each *in vitro* assay.

MAb RLX-6 (IgG_1,k) was produced by immunizing a BALB/c female mouse by intraperitoneal implant with 100 μg of hRLX-2 (DKA) dried onto a nitrocellulose disc on Day 0. This was followed by an identical implant on

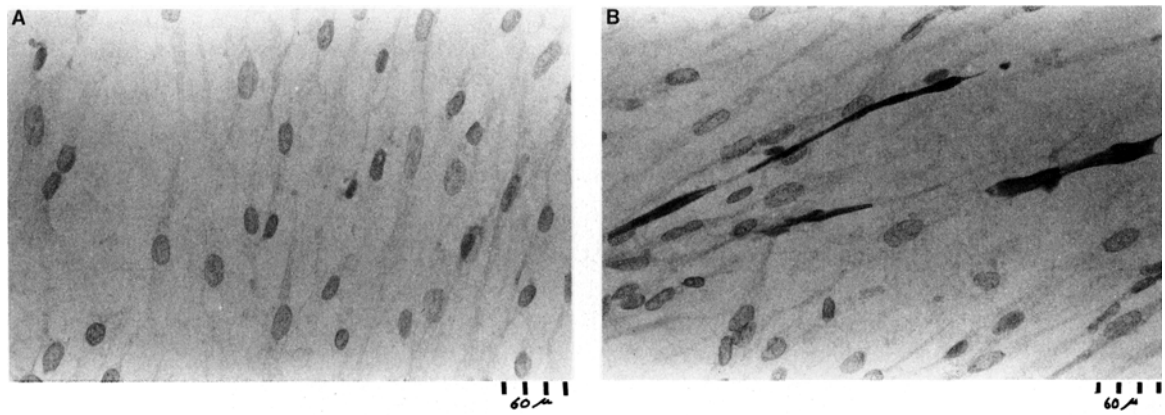

FIG. 1. Indirect immunocytochemical staining (PAP) with monoclonal antibody CGA-7 (anti-smooth muscle actin) of FS-4 cells (*A*) and NRMU cells (*B*).

Day 21 and a prefusion intrasplenic boost of 20 μg on Day 28. Three days later the spleen was removed and fused as described above.

Affinity purified goat anti-relaxin antibody (goat PAb) was produced by immunizing with 250 μg of hRLX-2 (DKA) in complete Freund's adjuvant IM and SQ at multiple sites followed by bimonthly injections of 250 μg of hRLX-2 (DKA) in incomplete Freund's IM. The titer of the antiserum was followed by ELISA as described above. The antiserum was immunoaffinity purified on hRLX-2 (SKA) bound to sepharose and eluted in 10 m*M* glycine HCl with 0.5 *M* NaCl at pH 2.5. The eluate was dialyzed in PBS and stored at −20° C with 0.01% thimerosal. The purified anti-relaxin polyclonal antibody was dialyzed in PBS prior to the in vitro assay.

NRMU cell culture. The uterus of a newborn rhesus monkey delivered by Caesarian section was obtained immediately after delivery. The uterus was stripped of mysentery, slit longitudinally, minced into 1 mm pieces, then washed 3× with cold Hank's balanced salt solution (HBSS). The minced tissue was placed in a dissociating solution of 0.1 percent trypsin in HBSS with a magnetic stirrer for 2 h at room temperature. The suspension was centrifuged at 200 ×*g* for 10 minutes, supernatant removed, and the cells resuspended in Eagles' minimum essential medium (EMEM) containing 10 percent FBS, non-essential amino acids, 10 m*M* HEPES, 10 U/mL penicillin, 10 μg/mL streptomycin, and 20 U/mL nystatin.

The cell preparation was filtered through sterile gauze then seeded at 5×10^5 cells/well in 6 wells of a Costar tissue culture cluster dish. Medium was changed 24 h after plating. The cultures were maintained at 37° C in an atmosphere of air (95 percent) and CO_2 (5 percent). A mixed cell population was observed microscopically with the fusiform/myometrial smooth muscle cell type predominating and a small population of cells with epithelial-like morphology also present. Cultures were confluent after 9 d and were subcultured following trypsinization, using a split ratio of 1:2. With each successive passage, the percent of epithelial-like cells decreased. By passage 6 the cells displayed the myometrial smooth muscle characteristics of fusiform morphology with oval nuclei, growing in parallel arrays (3,4). At this point, the culture medium was changed to F-12/DMEM supplemented with L-glutamine 2 m*M*, 10 percent FBS, 24 m*M* HEPES, 50 U/mL penicillin and 50 μg/mL streptomycin (F-12/DMEM/10 percent FBS) and the split ratio increased to 1:3. A cell bank was prepared at passage 15 and stored in liquid nitrogen.

Comparative immunocytochemical staining. The comparative immunocytochemical staining was performed using CGA-7, an ascites fluid monoclonal antibody specific for smooth muscle actin from Enzo Biochem (New York, NY) (5,11). Cells were grown to confluency on Lab Tech four chamber plastic slides (Nunc, Naperville, IL). Following fixation in methanol at −20° C, cells were stained using a standard peroxidase anti-peroxidase (PAP) procedure (36). The anti-smooth muscle actin was diluted 1:500 for use. The cell passage numbers for both NRMU and FS-4 were approximately 20.

Relaxin treatment and cAMP assay. NRMU cells were suspended in F-12/DMEM/10 percent FBS at 5×10^4 cells/mL and were seeded 2 mL/well into 6-well tissue culture plates. Cells were incubated at 37° C in 5 percent CO_2 for 18–24 h. This resulted in a 50–70 percent confluent monolayer containing 1.0 (± 0.2) × 10^5 cells/well at the time of assay. Spent medium was removed and cells were rapidly washed twice with 2 mL/well F-12/DMEM. F-12/DMEM was removed and 0.5 mL of 0.1 m*M* IBMX was added to each well. Plates were covered and incubated at 37° C, 5 percent CO_2 for 30 min. Following this incubation, 0.25 mL of 4 μ*M* forskolin and 0.25 mL relaxin diluted in F12/DMEM, 0.1% BSA, 0.01% Tween 80 and 24 m*M* HEPES were added. Appropriate cell, forskolin and relaxin controls were included in each assay. The final concentration of forskolin was 1 μ*M*/well. The plates were rotated gently then incubated at 37° C, 5 percent CO_2 for 15 min.

Following incubation, medium was removed and plates were irradiated in a microwave oven at the high setting, 1 minute per 2 plates, to release intracellular cAMP and inactivate phosphodiesterases (23). Plates were placed on ice and 0.5 mL of 0.2 *M* sodium acetate was added to each well with a rotating motion to wet the entire cell surface. After covering, the plates were placed in a Revco freezer at −70° C for a minimum of 30 min. For measurements of extracellular cAMP, medium was removed from plates, added to the sodium acetate buffer and stored at −70° C until time of assay.

TABLE I

EFFECT OF CELL SEEDING DENSITY ON cAMP PRODUCTION OF NRMU CELLS IN RESPONSE TO RELAXIN[a]

Cell Seeding Density (cells/well)	Treatment	pmol cAMP/well	pmol cAMP/mg protein
1×10^4	cell control	63.2 ± 6.2[b]	2590 ± 254
	forskolin control	60.6 ± 1.8	2484 ± 73
	relaxin	89.00 ± 14.0	3648 ± 573
5×10^4	cell control	57.2 ± 4.4	565 ± 43
	forskolin control	55.8 ± 0.8	551 ± 8
	relaxin	202.6 ± 10.8	2000 ± 106
1×10^5	cell control	57.0 ± 9.2	258 ± 41
	forskolin control	85.2 ± 6.4	386 ± 29
	relaxin	301.6 ± 25.8	1365 ± 117
5×10^5	cell control	56.2 ± 4.6	58 ± 5
	forskolin control	84.0 ± 4.2	87 ± 4
	relaxin	576.0 ± 69.0	599 ± 71
1×10^6	cell control	92.4 ± 20.6	62 ± 14
	forskolin control	104.8 ± 14.6	70 ± 10
	relaxin	729.6 ± 115.0	488 ± 77

[a] NRMU cells were seeded at the densities shown and cultured for 18 h. Cell control = serum-free medium; forskolin control = 1 μ*M* forskolin; relaxin = 5 ng/mL relaxin + 1 μ*M* forskolin. cAMP was measured according to the assay described in Materials and Methods.

[b] Mean ± SD ($n = 3$).

To perform the cAMP assay (Amersham Corp., Arlington Heights, IL), plates were brought to room temperature. The assay was performed according to the procedure of the manufacturer. It is based on the competition between unlabeled cAMP and a fixed quantity of tritium labeled cAMP for binding to a bovine muscle binding protein which has a high specificity for cAMP (10). The amount of labeled cAMP-protein complex formed is inversely related to the amount of unlabeled cAMP present in the assay. The concentration of cAMP in unknown samples is determined by comparison with a linearized (transformed) standard curve. A comparable assay for cGMP (Amersham, Corp.) was performed in specificity experiments.

The following reagents were used in the characterization of the NRMU cell cAMP response. Epidermal growth factor (EGF) and insulin-like growth factor II (IGF II) were purchased from Collaborative Research, Inc. (Bedford, MA). Insulin-like growth factor I (IGF I) was purchased from ICN (Lisle, IL). The following reagents were obtained from Sigma Chemical Company (St. Louis, MO): hCG, oxytocin (OT), β-estradiol 17-valerate, progesterone, hydrocortisone, 6 α-methyl-17 α-hydroxy progesterone acetate (MPA), PDE 3′:5′-cyclic nucleotide, prostaglandin E_2 (PGE_2) sodium acetate and IBMX. Forskolin was purchased from Calbiochem (San Diego, CA) and Protropin® (hGH) was obtained from Genentech, Inc. (South San Francisco, CA). Insulin (bovine) was purchased from Canada Packers (Ontario, Canada).

Antibody mediated in vitro relaxin neutralization. Purified antibody was diluted to the appropriate concentration in relaxin diluent, and incubated with an equal volume of hRLX-2 at 20 ng/mL for 60 min. at 37° C. A 0.25 mL sample of this mixture was added to the NRMU cells in triplicate wells and the assay performed as described above.

Statistical analysis. Differences in cAMP levels generated in response to relaxin and other agents were compared by one-tailed, unpaired T-test.

Protein assay. Total protein of cultured cells used in cAMP assays was determined by the method of Bradford (2).

Results

NRMU cells. The plating efficiency of the NRMU cells at the passage level routinely used for the cAMP assay is 80%. The population doubling time during log growth is 36 h and the maximum cell density achieved in plateau is 6.5×10^4 cells/cm^2. NRMU cells do not grow in soft agar and do not form foci. These cells have been tested and shown to be free of mycoplasma by both biochemical and culture assay methods.

Immunocytochemical staining of NRMU and FS-4 confluent cell cultures using smooth muscle actin monoclonal antibody CGA-7 is shown in Fig. 1. The spindle shaped NRMU cells demonstrate staining of thin filamentous structures within the cytoplasm. The selective staining of only a portion of the cells is consistent with the observations of Fager et al. (9) who showed that proliferating human arterial smooth muscle cell cultures had a much greater percentage of cells staining with CGA-7 than growth arrested cells, and Charbord et al. (5) who demonstrated staining variability in adherent cells in long term marrow culture based on culture age and degree of confluence. No staining of any FS-4 fibroblasts was observed.

Assay optimization. NRMU cells were seeded at densities from 1×10^4 to 1×10^6 cells/well in 6-well tissue culture plates to determine the optimal culture density for maximum cAMP response to relaxin. Eighteen hours after plating, cells were washed, incubated with relaxin doses in serum-free medium containing 1 μ*M* forskolin for 15 minutes, then assayed for intracellular cAMP. Table 1 shows the correlation of cAMP production with cell density and dose. The pmol cAMP/well increased with increasing cell density, whereas the cAMP/mg protein decreased. Similar results were obtained when cells were preincubated with a PDE inhibitor, 0.1 m*M* IBMX, for 30 minutes prior to the addition of relaxin and forskolin. Based on criteria of interassay and intra-assay reproducibility, as well as compatibility with the sensitivity of the cAMP binding assay, the 1×10^5 cells/well seeding density was selected as optimal.

Since levels of cAMP activity produced by relaxin alone were undetectable in this assay, the effect of forskolin

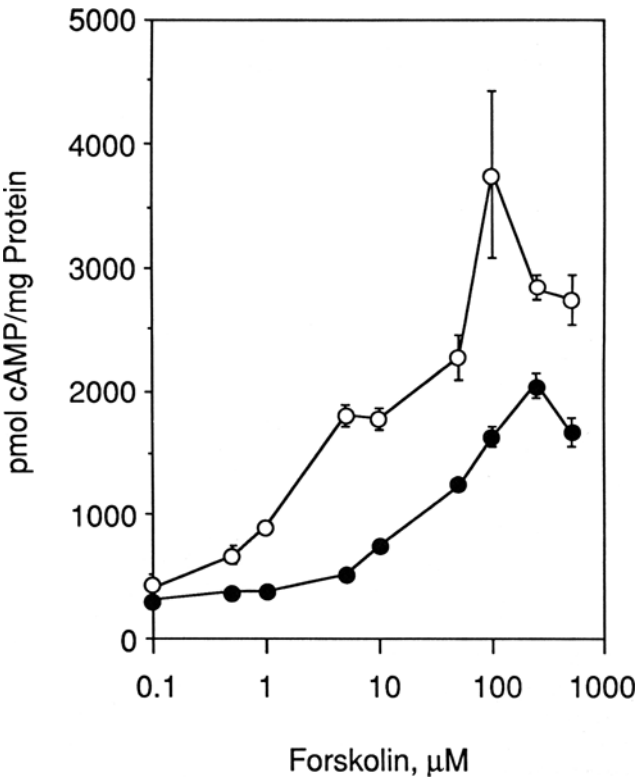

FIG. 2. Effect of forskolin (●) and forskolin + relaxin (○) on cAMP response. A titration of forskolin over a final concentration range from 0.1 to 500 μ*M* was performed in the presence and absence of 5 ng/ml relaxin. The half-maximal dose of forskolin alone was 50 μ*M*, however, the dose giving the optimal forskolin + relaxin:forskolin ratio was 1 μ*M*. This dose of forskolin was used for all subsequent experiments ($n = 3$).

TABLE 2

TIME COURSE OF cAMP ELEVATION IN NRMU CELLS IN RESPONSE TO RELAXIN

Treatment[a] Relaxin (ng/mL)	Time in Minutes[b] 1	5	10	15	30	60
0.00	96 ± 7.8[c]	109 ± 7.4	130 ± 7.8	133 ± 8.4	109 ± 27.2	128 ± 8.5
0.25	118 ± 18.2	144 ± 25	190 ± 62	202 ± 5	214 ± 15.8	153 ± 9.6
5.00	162 ± 30	213 ± 30	553 ± 33	534 ± 166	484 ± 90	206 ± 9
10.00	109 ± 18.8	289 ± 78	564 ± 129	605 ± 19.9	610 ± 107	184 ± 20

[a]Treated in the presenc eof 1 μM forskolin.
[b]Incubation time at 37° C.
[c]Mean pmol cAMP/mg protein ± SD ($n = 3$).

concentration on the production of cAMP in NRMU cells was evaluated both in the presence and absence of relaxin. A titration of forskolin and forskolin + relaxin (5 ng/mL) is shown in Fig. 2. The half maximal dose of forskolin alone was approximately 50 μM, however, the optimal forskolin + relaxin:forskolin ratio, with only minimal stimulation by forskolin alone, occurred at 1 μM forskolin. For subsequent assays, cells were treated with relaxin in the presence of 1 μM forskolin.

The time course of cAMP production in NRMU cells in response to relaxin is given in Table 2. The rate of cAMP synthesis was rapid and linear for 10 minutes, reaching a plateau at 10 and continuing to 30 minutes. Fifteen minutes was selected for the routine assay incubation time because the most reproducible inter- and intra-assay values were obtained with this condition.

Table 3 shows a comparison of passage 17 and passage 32 NRMU cAMP measurements from a titration of relaxin. Although the cells are still responsive to relaxin at passage 30, the optimal response for the assay conditions described is obtained with cells between passages 17 and 24 (approximately 45 to 67 population doublings).

Assay sensitivity and reproducibility. The assay is sensitive to .025 ng/mL (final concentration) relaxin. The reproducibility of the assay is shown in Table 4, with the coefficient of variation (CV) calculated for five dose levels from five separate assays. The assay varies by 20% or less at concentrations ≥.25 ng/mL relaxin. Intra-assay variability ranged from CV values of 3% to 30% over a dose range of 0.0 to 10.0 ng/mL relaxin. The variability did not appear to be dose dependent as can be observed by the standard deviations shown in Table 2 for the 15 minute treatment time.

Intracellular and extracellular cAMP determination. The quantitation of intracellular and extracellular cAMP produced by NRMU cells in response to relaxin as a function of time was examined at two dose levels, in the presence of IBMX, a PDE inhibitor. The intracellular cAMP levels rose rapidly in response to relaxin, reaching a plateau at approximately 10 min., and gradually declined after 30 min. (Table 2). In contrast, the concentration of cAMP in the medium was not reproducibly detectable until the 30 minute time point and then rose slowly and began to plateau after 120 min period (Table 5). The results show that not only do NRMU cells respond to the presence of relaxin by the production of cAMP, but also, the cAMP is secreted from the cells in a time dependent fashion.

Specificity: antibody neutralization, PDE treatment and effect of non-relaxin related hormones. The specificity of the cAMP response to relaxin was first examined by antibody neutralization. A 20 ng/mL hRLX-2 preparation was preincubated for 60 min. at 37° C with the appropriate antibody preparations ranging from 0.4 fold to 200 fold molar excesses above the relaxin concentration. A class specific (IgG 1, k) antigenically unrelated MAb and a non-specific, purified goat IgG preparation were run as controls at the appropriate molar ratios. The results are shown in Table 6. Both MAbs RLX-2 and RLX-6 neutralized the relaxin induced cAMP production in NRMU cells. RLX-6 was the more efficient MAb by neutralizing 88% of the cAMP at a 20 fold molar excess over relaxin. The affinity purified goat PAb anti-relaxin neutralized 100% of the cAMP activity at a 10 fold molar excess over relaxin. Both the MAb control and the goat IgG control had no effect on cAMP levels.

To further demonstrate the specificity of the cAMP assay, NRMU cells were treated with 50 ng/mL relaxin in the presence of PDE, the only enzyme known to degrade cAMP or cGMP. Table 7 shows that PDE degrades extracellular cAMP more readily than intracellular cAMP, most likely because of accessibility, and the degree of degradation increases with incubation time.

TABLE 3

RELATIONSHIP OF NRMU CELL PASSAGE NUMBER TO RELAXIN TREATMENT cAMP RESPONSE

Treatment[a] Relaxin Concentration (ng/mL)	NRMU Passage 17	NRMU Passage 32
0	37[b]	19[b]
0.025	114	20
0.5	416	36
5.0	683	55
8.0	800	36
15.0	1075	34

[a]Treated in the presence of 1 μM forskolin.
[b]Mean pmol cAMP/mg protein from duplicate samples of two representative assays.

TABLE 4

INTERASSAY REPRODUCIBILITY OF NRMU cAMP RESPONSE TO RELAXIN[a]

Relaxin Concentration (ng/mL)	pmol cAMP/mg[b]	% CV[c]
0.000	317 ± 100	32
0.025	363 ± 114	32
0.250	573 ± 112	20
2.500	981 ± 154	16
5.000	1080 ± 61	6
10.000	1213 ± 103	9

[a]The pmol cAMP/mg values represent mean values from 5 assays performed over a 3 week period.
[b]Mean ± SD ($n = 5$).
[c]%CV = Coefficient of Variation.

The addition of PDE to relaxin/forskolin-stimulated NRMU cells reduced the extracellular levels of cAMP by 62% after 60 min. and 73% after 90 min. at 37° C.

To ensure cyclic nucleotide specificity, triplicate samples of the relaxin standard curve were tested for cAMP and cGMP production. The cAMP assay demonstrated a dose response over the 0.025 to 10 ng/mL range of relaxin, whereas no changes in cGMP levels were detected over the same range.

The effect of hormones, growth factors and drugs which are potential components of culture systems or serum samples to be assayed for relaxin-induced cAMP activity is shown in Fig. 3. Each preparation was diluted to a dose level likely to be physiological. The cAMP response to hCG (212 IU/mL), MPA (1 μM), and progesterone (130 ng/mL) did not differ significantly from relaxin (6.25 ng/mL) alone ($P > .05$). EGF (10 ng/mL), OT (125 μU/mL), 17 β-estradiol (0.3 mg/mL), hydrocortisone (12.5 μg/mL) and hGH (100 ng/mL) all produced significantly lower ($P < .05$) cAMP than relaxin (6.25 ng/mL). In combination, progesterone + relaxin (Fig. 3) and PGE_2 + relaxin (Table 8) resulted in a significant increase in cAMP response over relaxin alone ($P < .05$), while OT + relaxin significantly decreased the response ($P < .05$). These effects appear to be additive rather than synergistic.

Specificity: human and animal relaxins and structurally related hormones. All of the assay characterization data described herein were generated with hRLX-2 (SKA) or (DKA) analogs. Johnston et al. (14) have reported comparison of the human relaxins to porcine and rat relaxins, as well as to human insulin. Equivalence of bioactivity between the hRLX-2 (SKA) synthetic standard and natural hRLX-2 purified from human CL tissue (8) is demonstrated in Fig. 4. The slopes of the dose response curves, the maximal stimulation, and the concentration at half maximal stimulation [1.9 ng/mL for CL and 2.0 ng/mL for hRLX-2 (SKA)] are comparable for both preparations. In addition to hRLX-2 (SKA), the three other synthetic preparations of hRLX-2 (native, DKA, and SMM) are all capable of generating a dose related cAMP response from NRMU cells (Table 9).

Specificity testing also included the assay of porcine and rat relaxin on NRMU cells. Final concentrations of .25, 2.5 and 25 ng/mL of human, porcine and rat relaxin yielded dose-related cAMP production (Fig. 5). The differences between the activities of human and porcine relaxins over the dose range evaluated were not statistically significant ($P > .05$), whereas the activity of rat relaxin at 2.5 and .25 ng/mL was significantly less than human or porcine ($P < .05$).

Since relaxin is structurally related to insulin (14,17), comparisons of cAMP production in response to insulin, IGF I and IGF II with and without relaxin were evaluated (Fig. 6) for potential inhibition or synergism. At a dose of 200 ng/mL, insulin produced a cAMP response comparable to that observed with 6.25 ng/mL relaxin. No combination of insulin, IGF I or IGF II with relaxin resulted in a cAMP response significantly different than the relaxin response alone.

DISCUSSION

NRMU cells have been cultured and characterized as apparent smooth muscle. This has been shown by specific staining of NRMU cells with a smooth muscle specific anti-actin antibody. In addition, the cells have demonstrated the presence of bundles of myofilaments, dense bodies and microtubules consistent with smooth muscle cell morphology by transmission electron micros-

TABLE 5

TIME COURSE OF INTRACELLULAR AND EXTRACELLULAR DETECTION OF cAMP[a]

Relaxin Dose ng/mL (sample)	Time in Minutes			
	30	60	120	180
0 (intracellular)	150 ± 15[b]	190 ± 7	160 ± 2	101 ± 8
0 (extracellular)	0	0	0	0
0.25 (intracellular)	701 ± 12	356 ± 15	169 ± 24	133 ± 8
0.25 (extracellular)	21 ± 5	179 ± 30	283 ± 30	321 ± 66
2.50 (intracellular)	1196 ± 149	614 ± 18	212 ± 10	177 ± 6
2.50 (extracellular)	119 ± 14	512 ± 57	641 ± 72	544 ± 58

[a]NRMU cells were treated with a final concentration of 0.0, 0.25 or 2.5 ng/mL relaxin. Culture supernatants were removed and simultaneous intracellular and extracellular measurements of cAMP were made at 30, 60, 120 and 180 minutes.
[b]Mean ± SD ($n = 3$).

TABLE 6

NEUTRALIZATION OF cAMP RESPONSE BY RELAXIN-SPECIFIC ANTIBODIES[a]

Antibody	Molar Excess	Percent Neutralization[b]
Goat PAb	10.0X	100
Goat PAb	2.0X	86
Goat PAb	0.4X	0
Goat IgG Control	10.0X	0
MAb RLX-2	200.0X	66
MAb RLX-2	20.0X	0
MAb RLX-6	200.0X	87
MAb RLX-6	20.0X	88
MAb Control	200.0X	0
None (Diluent only)	—	0

[a]Equal volumes of hRLX-2 (20 ng/mL) and antibody at the designated molar excess of hRLX-2 were incubated for 60 min at 37° C. Triplicate samples of each preparation were added to NRMU cells to test for cAMP activity as described above.

[b]Percent neutralization was calculated using the hRLX-2 preparation which received no antibody as 0 percent neutralization and the forskolin/cell control baseline value for 100% neutralization.

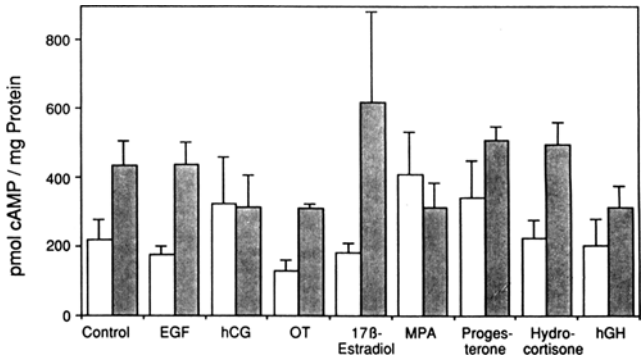

FIG. 3. The effect of related hormones, growth factors and drugs on the production of cAMP by NRMU cells in the presence and absence of relaxin is shown above. All samples were assayed in the presence of forskolin 1 μM. The sample concentrations assayed without (*white bar*) and with (*shaded bar*) 6.25 ng/ml relaxin are as follows: EGF 10 ng/ml, hCG 212 IU/mL, oxytocin (OT) 125 μU/ml, 17 B-estradiol 0.3 mg/mL, MPA 1 μM, progesterone 130 ng/mL, hydrocortisone 12.5 μg/mL and hGH 100 ng/mL. Some of the preparations were reconstituted in EtOH, however, the concentration of EtOH in diluted samples had no effect on relaxin activity.

copy. The cells respond to relaxin in both a dose and time-dependent manner by production of cAMP. This unique cell line provides the opportunity to eliminate the inevitable interassay variability when cAMP is measured in rat uterine strips (15) or rat primary myometrial cell cultures (12). Subsequent to the preparation of the NRMU cell line an NRMU-2 cell line was established from the uterus of another newborn Rhesus monkey. Increased cAMP levels were also seen in this cell line after exposure to relaxin.

Human (G292) and rat (UMR-106) osteosarcoma cell lines also respond to relaxin with an increase in cAMP levels, however, the amount of relaxin necessary to generate this response is one hundred to one thousand fold greater than with NRMU cells. In addition to the increase in cAMP levels, NRMU cells also release plasminogen activator into the culture medium in response to relaxin (data not shown). This is consistent with the physiological events which accompany the remodeling of the uterus and cervix throughout the course of pregnancy, and particularly at term.

The relaxin cAMP assay using NRMU cells described herein is reproducibly sensitive to 0.25 ng/mL with an ultimate sensitivity of 0.025 ng/mL. Specificity has been documented by neutralizing the relaxin activity completely with polyclonal antibodies and substantially with monoclonal antibodies, eliminating activity with PDE, differentiating cAMP from cGMP, demonstrating that other related hormones, growth factors and drugs have minimal, or rarely additive effects at physiologic doses in the presence of relaxin. Although levels of cAMP produced by relaxin alone, without the forskolin treatment, were undetectable in this cAMP binding assay system, measurements with ^{125}I-cAMP in a more sensitive RIA (Amersham Corp.) detected a dose response curve of relaxin alone over a range of 0.1 to 12.5 ng/mL (C. Lai, personal communication). Therefore, it is apparent that the role of forskolin is independent from relaxin in our assay system and serves only to stimulate cells to a constant baseline level to allow the relaxin cAMP effect to be measured by a competitive binding assay.

In the experiments examining the intracellular and extracellular cAMP levels in the presence of IBMX, it is interesting to note that total cAMP activity declined over time, and this decline in total cAMP levels was due

TABLE 7

EFFECT OF PDE ON INTRACELLULAR AND EXTRACELLULAR cAMP LEVELS[a]

Treatment	Intracellular pmol cAMP/mg 60 min.	Extracellular pmol cAMP/mg 60 min.	Extracellular pmol cAMP/mg 90 min.
Cell Control[b]	0	36	8
0 ng/mL Relaxin	256 ± 33	0[b]	0[b]
50 ng/mL Relaxin	479 ± 26	821 ± 105	687 ± 36
50 ng/mL Relaxin + 10 mU PDE	468 ± 38	316 ± 33	187 ± 40

[a]NRMU cells were treated with relaxin or relaxin + PDE with all wells except cell controls containing 1 μM forskolin. Plates were incubated either 60 min. or 90 min. at 37° C ($n = 4$).

[b]Replicate measurements were below the detection limits of the assay.

TABLE 8

ADDITIVE EFFECT OF PGE_2 AND RELAXIN ON THE PRODUCTION OF cAMP BY NRMU CELLS

PGE_2	Relaxin ng/mL	pmol cAMP/mg Protein[a]
0	0	223 ± 15
0	2.5	930 ± 70
2×10^{-10} M	0	639 ± 77
2×10^{-10} M	2.5	1421 ± 95

[a]Mean ± SD ($n = 4$).

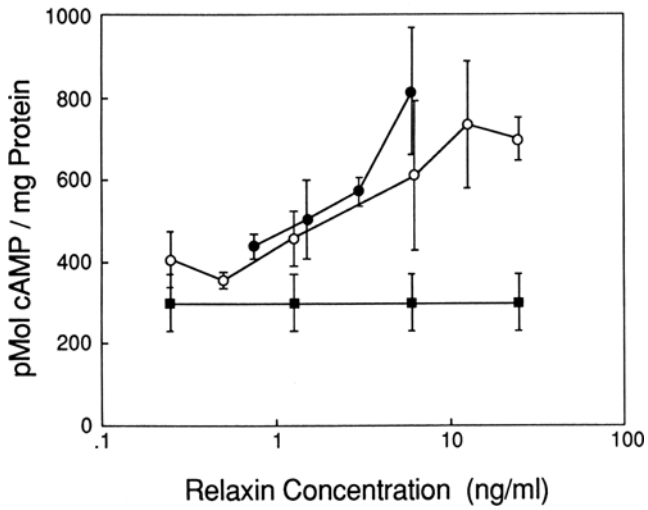

FIG. 4. Comparison of synthetic hRLX-2 (SKA) (○) with natural CL derived hRLX-2 (●). Forskolin control is shown as (■). Cells were incubated 30 min with IBMX, followed by the addition of relaxin and forskolin (1 μm) and a 15 min incubation at 37° C ($n = 3$).

entirely to the disappearance of intracellular cAMP. Extracellular levels of cAMP for the .25 ng/mL relaxin dose increased from 30 to 180 minutes, and for the 2.5 ng/mL relaxin dose from 30 to 120 minutes, after which time they also began to decrease. This decrease in intracellular cAMP appears to be due to two factors: 1. leakage of cAMP from the cell into the medium, and 2. intracellular activity of PDE. The data further show, that the escape of cAMP from the cells is both dose and time dependent. The addition of .25 ng/mL relaxin not only produced considerably lower levels of cAMP than the addition of 2.5 ng/mL relaxin, but also, after 30 minutes of incubation, resulted in the release of only 3% of cAMP into the medium, versus 9% for the 2.5 ng/mL relaxin dose. With increasing time, the permeability of the cell membranes to cAMP increased for cells treated with both low and high doses of relaxin so that at the end of 180 minutes over 70% of the total cAMP was found extracellularly.

The advantage offered by this assay becomes apparent by a comparison with the alternative assays, rat uterine strip contractility and the mouse interpubic ligament

TABLE 9

COMPARISON OF SYNTHETIC HUMAN RELAXINS: ANALOGS TO NATIVE[a]

Relaxin Treatment (ng/mL)	hRLX-2 pmol cAMP/mg Protein			
	Native	DKA	SKA	SMM
0.000	570	570	570	570
0.025	967	605	650	580
0.250	856	1004	920	911
2.500	1061	1443	1434	1799

[a]NRMU cells were treated with native human or relaxin analogs in the presence of forskolin (1 μM) and assayed for intracellular cAMP levels as described in Materials and Methods. Mean values are shown ($n = 3$).

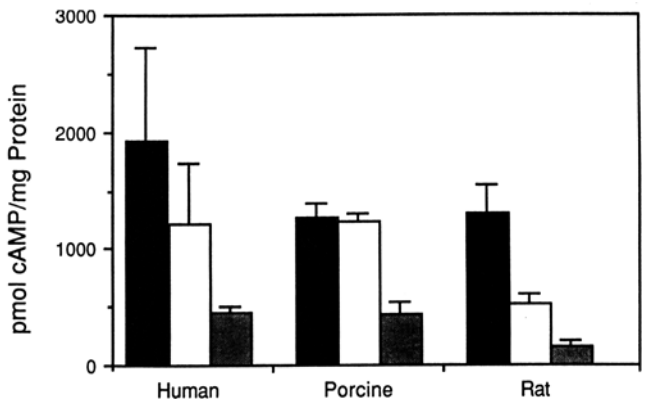

FIG. 5. Comparison of three species of relaxin. Human, porcine and rat relaxin were assayed at 25 (*black bar*), 2.5 (*white bar*) and .25 (*shaded bar*) ng/mL doses. Cells were preincubated for 30 min with IBMX, then relaxin and forskolin (1 μM) were added and incubated 15 min at 37° C. ($n = 4$).

dilation. Although data generated with synthetic hRLX-2, human analogs and porcine relaxin on all three assays correlate well, the NRMU cell cAMP assay requires far less material (on the order of 10^3 fold for a complete dilution curve with acceptable error), is more rapid, economical and reproducible. This was demonstrated when showing the biological equivalence of synthetic hRLX-2 and natural CL hRLX-2. Given the limited amounts of CL for purification, such a result was impossible to generate with the previous bioassays. The availability of synthetic relaxin has permitted the standardization of this assay allowing interassay as well as intra-assay comparisons to be made with some degree of confidence.

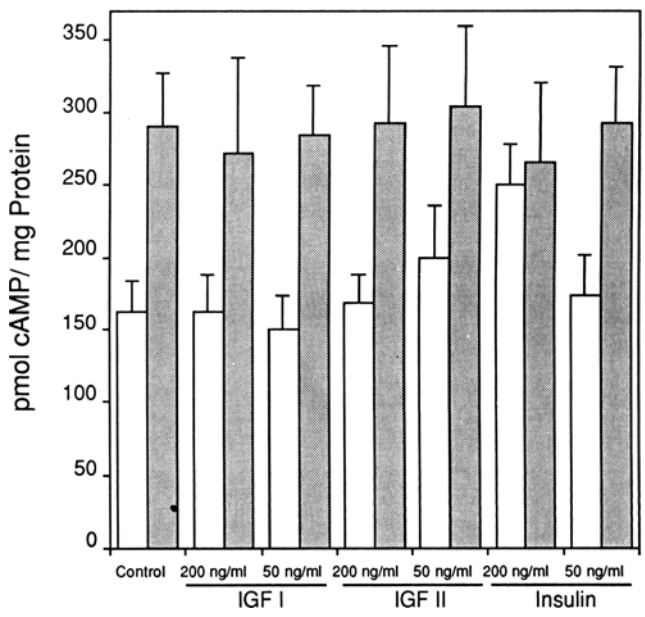

FIG. 6. Determination of insulin, IGF I and IGF II activity. Insulin, IGF I and IGF II were assayed at 50 and 200 ng/mL concentrations in the absence (*white bars*) and presence (*shaded bars*) of 6.25 ng/mL relaxin. All samples were assayed in the presence of 1 μM forskolin ($n = 4$).

The role of cAMP in uterine relaxation continues to be controversial (17,19) due to the low cAMP levels detected, relative to other cell culture cAMP-steroidogenesis systems described in the literature. However, Partridge et al. (27) and Moyle et al. (25) have shown in other systems that only part of the generated cAMP may be required for full activation of protein kinase or steroidogenesis. Cole and Garfield (7) have shown that the permeability of gap junctions in uterine smooth muscle may be regulated by cAMP and physiologically relevant agonists such as relaxin. They hypothesize that control of this permeability may be important for the regulation of intercellular communication and uterine wall contractility during pregnancy and parturition. Whether cAMP production is an actual mediator of relaxin activity or a secondary component, sensitive and reproducible quantitation of biologically active relaxin can be achieved with the NRMU smooth muscle cells.

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The authors wish to thank Dr. Geoffrey Tregear of the Howard Florey Institute, University of Melbourne, Melbourne, Australia for purified porcine and rat relaxin, Genentech, Inc. colleagues Toni Klassen for MAb RLX-2 and RLX-6, Abbie Celnicker for affinity purified goat anti-relaxin, John Burnier for synthetic peptides, Monique Carver for cAMP and cGMP comparisons, Sharon Apperson for assistance in assay development, Stan Hansen, Robin Taylor and Dr. Denny Liggitt for immunocytochemical staining procedures, and Dr. David Phillips of the Population Council, New York for electron micrography. The authors wish to acknowledge Dr. Mary Martin and Dr. Robert Jaffe, Department of Obstetrics, Gynecology and Reproductive Sciences, University of California, San Francisco, CA, for rhesus monkey fetal tissue.

EDITOR'S STATEMENT

This paper details a smooth muscle cell line that is responsive to relaxin and provides a useful assay system for the hormone, as well as providing a model system for the study of the mechanisms of relaxin action.