Progesterone treatment *in vitro* enhances prostaglandin E and forskolin-promoted cyclic AMP production in human endometrial stromal cells*

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Summary. Confluent human endometrial stromal cell cultures were exposed to steroids for up to 72 h and then stimulated with agonists of adenylate cyclase for 60 min. Neither steroid alone or in combination had a significant effect on cyclic AMP production. However, when stromal cell adenylate cyclase was stimulated with a receptor-dependent agonist (prostaglandin E), or with forskolin (which acts at a post-receptor site), progesterone in oestradiol-primed cells markedly enhanced ($P < 0.05$) the effect of both agonists. The presence of phenol red, a weak oestrogenic compound, in the standard culture medium was sufficient to allow the progesterone effect to be manifest. Moreover, while oestradiol alone had no significant effect on prostaglandin E or forskolin-stimulated cyclic AMP production, the simultaneous exposure of cells to oestradiol and progesterone was the most effective treatment. Short-term incubation (up to 120 min) with progesterone had no effect on agonist-induced cyclic AMP accumulation, indicating that progesterone elicits its effect by the classic nuclear mechanism of action. It is suggested that the potentiation by progesterone of prostaglandin E-promoted production of cyclic AMP represents an important aspect of the functional role progesterone plays in the preparation of the endometrium for implantation.

Keywords: endometrium; stromal cell; prostaglandin E; forskolin; cyclic AMP; man

Introduction

Cyclic AMP has been frequently implicated in various aspects of uterine function. In the myometrium, substances that promote relaxation have been shown to activate the adenylate cyclase–cyclic AMP signal transferring system and a cause and effect relationship between the latter and former phenomena has been suggested (Korenman & Krall, 1977). In the endometrium, cyclic AMP was shown to decrease oestrogen binding (Fleming *et al.*, 1982) and to be involved in the decidual reaction, possibly by mediating the action of prostaglandin (PG) E-2 (Kennedy, 1983). In turn, the uterine adenylate cyclase–cyclic AMP effector system is influenced by the female sex steroids by (i) regulating membrane receptors coupled to the adenylate cyclase–cAMP system (Roberts *et al.*, 1984; Boulet & Fortier, 1988), (ii) affecting one or more of the post-receptor components of this effector system (Tanfin & Harbon, 1987; Riemer *et al.*, 1988) including cyclic AMP-dependent protein kinase (Miyazaki *et al.*, 1980), or (iii) influencing the activity of cyclic AMP phosphodiesterase, the enzyme that inactivates cyclic AMP (Kofinas *et al.*, 1987). Although most of the available evidence indicates that steroid hormones bring about their effects in higher vertebrates via the classical intracellular regulation of protein synthesis, a direct interaction with

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the membrane-bound adenylate cyclase has also been reported (Szego & Davis, 1967; Bergamini *et al.*, 1985). To learn more about the interaction of steroids and cyclic AMP we investigated the influence of oestradiol and progesterone on cyclic AMP production in human endometrial stromal cell cultures under unstimulated conditions and when the cells were exposed to PGE or forskolin, two known activators of adenylate cyclase in a wide variety of cell types.

Materials and Methods

Chemicals. Iodinated cyclic AMP ($[^{125}\text{I}]\text{Sc-cAMP-TME}$, sp. act. 210–270 Ci/mmol) was purchased from Dupont-NEN (Boston, MA, U.S.A.). All other chemicals, media and reagents were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.).

Cell culture. Endometrial curettings were obtained from premenopausal women undergoing hysterectomy unrelated to this work. Part of the fresh tissue was fixed for histological examination and the rest was taken to the laboratory on ice in a sterile vial containing Dulbecco's modified essential medium (DMEM) supplemented with D-glucose (4 g/l), Hepes (2.383 g/l), NaHCO_3 (2.7 g/l), pyruvic acid (0.11 g/l), non-essential amino acids (1%, v/v) and antibiotics (penicillin-streptomycin, amphotericin B, 1%, v/v) and insulin-transferrin-sodium selenite (1%, v/v). Endometrial cells were isolated in a manner similar to that described by Satyaswaroop *et al.* (1979). The endometrial tissue was washed several times in DMEM, blood clots and mucus were removed and then the tissue was minced into 1–3 mm³ pieces. The entire procedure was carried out under aseptic conditions in a laminar flow hood. The endometrial pieces were treated with 0.25% (w/v) solution of collagenase (Type 1; 350 U/mg) containing lima bean trypsin inhibitor in DMEM for 1 h in a 5% CO_2 –95% air atmosphere incubator at 37°C. At 15-min intervals the contents were agitated with a sterile pipette. The collagenase-treated endometrium was first strained through a 297- μm mesh to retain any of the undigested tissue and mucus material. Next, the filtrate was passed through a 105- μm sterile mesh. If the endometrium was secretory it was passed through a 70- μm mesh and if it was proliferative, it was passed through an additional 41- μm mesh so that stromal cells could be separated from glands. The filtrate containing stromal cells was centrifuged at 800 g for 15 min. The cells were washed with DMEM and centrifuged twice. A portion of the cell suspension was taken for cell counting. Cell viability using the 0.1% trypan blue exclusion method was consistently greater than 90%.

Cells ($1\text{--}5 \times 10^6$) were plated in 75 cm² sealed plastic flasks in DMEM containing 20% heat-inactivated fetal calf serum (FCS) and incubated in a 37°C 95% air–5% CO_2 environment. Medium was changed 1–2 h after plating when only stromal cells were attached to the plastic, and every 2–3 days thereafter. Confluence was routinely reached within 7–10 days. In all experiments, once confluence was reached, FCS was removed from the medium and the monolayer was washed several times. Harvesting of cells was accomplished by rinsing T-flasks at least twice with Ca–Mg-free buffer and then treating them with a 0.05–0.075% collagenase solution for 10–12 min at 37°C.

In experiments in which the monolayer cultures were treated with steroids for up to 72 h before harvesting, cells were allowed to recover at 37°C for at least 2 h after collagenase treatment, because in preliminary experiments we observed that such a recovery period optimized cAMP responses to agonists. The cells were then pipetted into 10 x 75 mm culture tubes at a concentration of 100 000 viable cells/ml and stimulated with forskolin or PGE in the presence of the cyclic AMP-phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (MIX) at concentrations shown in the tables and figures for 1 h in a shaking water bath at 37°C. The experiments were terminated by placing culture tubes immediately on ice for 5–10 min and then freezing at –20°C.

In other experiments, cells were harvested after confluence was reached and distributed (10^5 /tube unless otherwise stated) into 10 x 75 mm culture tubes containing 1 ml FCS-free DMEM with various steroid concentrations. After a specified interval (30 min–72 h), culture tubes were centrifuged at 800 g, the supernatant was discarded and the cells were resuspended in 0.5–1 ml medium containing the test compounds. Experiments were terminated as described above.

Frozen cells were thawed and refrozen twice. Culture tube contents were vigorously mixed and centrifuged and aliquants of the supernatant were used for cyclic AMP radioimmunoassay.

Assay of cyclic AMP. Cyclic AMP radioimmunoassay was carried out by a modification of the method described by Brooker *et al.* (1979). The assay solution contained 100 μl of sample or standard, 50 μl of $[^{125}\text{I}]\text{Sc-cAMP-TME}$ (10 000 c.p.m./assay tube) as the labelled ligand, and 150 μl antiserum (final dilution 1:250 000). The range of the cyclic AMP standard curve was between 1 and 250 fmol. After an overnight incubation at 4°C, the bound and the free radioactivity was separated by ammonium sulphate precipitation (1.6 ml 60% saturated at 20°C), using gamma-globulin (100 μl of 5 mg/ml in 0.9% (w/v) NaCl) as protein carrier. After centrifugation, the supernatant was discarded, and the radioactivity of the precipitate was measured in a Packard gamma counter. The recovery of added $[^3\text{H}]\text{cyclic AMP}$ was 88–90%. Intra-assay and interassay coefficients of variations were <10%.

Data analysis. Data are presented as the mean $\pm$ standard error. Comparison of data was by Student's *t* test. Statistical significance was accepted at the $P < 0.05$ level in a two-tailed test.

Results

Microscopic examination (phase microscopy and transmission EM) revealed homogenous cultures of stromal cells, identical in appearance to those characterized by other investigators (Liu & Tseng, 1979; Gal *et al.*, 1982) who used isolation and culture conditions similar to those in the present study. Because we observed no differences between cyclic AMP responses of stromal cells obtained from proliferative and secretory endometrium after 7–10 days in culture all data have been combined. In the first series of experiments (Table 1) the influence of oestradiol-17 β and progesterone alone and in combination was evaluated in stromal cells grown in a medium containing phenol red, which has been reported to possess weak oestrogenic activity (Berthois *et al.*, 1986). Exposure to steroids for 48 h had no effect on basal production of cyclic AMP, and oestradiol (10^{-8} M) alone did not potentiate significantly PGE-2- or forskolin-stimulated cyclic nucleotide output by stromal cells. Progesterone (10^{-7} M) on the other hand significantly potentiated cyclic AMP responses to forskolin (1.66-fold) as well as to PGE-2 (1.68-fold), agonists which themselves increased basal cyclic AMP levels by 24-fold and 15-fold respectively. In all subsequent experiments we substituted PGE-1 for PGE-2, for it appeared to provoke a greater cAMP response. The effects of forskolin and PGE-1 were both dose-related and the potentiating effect of progesterone alone or in combination with oestrogen was once again clearly evident (Figs 1 & 2). When the progesterone concentration of the culture medium was kept constant (10^{-7} M) and the concentration of oestrogen was varied (10^{-9} – 10^{-6} M), 10^{-8} M-oestradiol was most effective in allowing the progesterone to enhance cyclic AMP responses to both PGE-1 and forskolin (Fig. 3). Similarly, when oestrogen content of the medium was standardized (10^{-8} M), progesterone was maximally effective between concentrations of 10^{-8} and 10^{-6} M (Fig. 4).

Table 1. Influence of oestradiol-17 β and progesterone on forskolin- and PGE-2-promoted cyclic AMP production by human endometrial stroma cells in culture

Treatment	Cyclic AMP (pmol/ 10^6 cells/60 min)		
	Control	Forskolin (25 μ M)	PGE-2 (28 μ M)
None	3.9 $\pm$ 0.3	92.7 $\pm$ 5.2	57.2 $\pm$ 5.3
Oestradiol (10^{-8} M)	4.4 $\pm$ 0.4	99.1 $\pm$ 6.7	68.2 $\pm$ 9.3
Progesterone (10^{-7} M)	3.6 $\pm$ 0.1	153.7 $\pm$ 12.9*	96.3 $\pm$ 8.5*
Oestradiol + progesterone	4.6 $\pm$ 0.6	184.8 $\pm$ 12.3*	138.0 $\pm$ 9.7**

Stromal cells were grown to confluence in DMEM containing phenol red and 20% FCS. Cells were then washed and cultured for 48 h in DMEM without serum and with or without steroids. After washing, cells were stimulated for 1 h with agonists in the presence of MIX (0.1 mM) or MIX alone (control). Values are mean $\pm$ s.e.m. ($n = 4$).

* $P < 0.05$ vs none or oestradiol alone; ** $P < 0.05$ vs all in the same column.

Implicit in this study was the assumption that progesterone initiates its potentiating effect by classical interaction of intracellular progesterone receptors, which in turn are induced by oestradiol (or the oestrogenic action of phenol red). To obtain additional evidence that the action of progesterone is not a rapid membrane-mediated response, endometrial cells were cultured in medium without phenol red or oestrogen and then exposed for 30 or 120 min to progesterone before stimulation with PGE-1 or forskolin for another 60 min. Short-term exposure to progesterone failed to influence cyclic AMP responses of stromal cells to PGE-1 or forskolin (Table 2), providing supportive evidence for the delayed, nuclear action of progesterone.

Fig. 1. Dose-response study of PGE-1-stimulated cAMP production in endometrial stromal cells without steroid treatment (control) and after exposure to oestradiol or progesterone or the combination of both for 72 h. Values are mean $\pm$ s.e.m. ($n = 4$ experiments). When error bars are not shown these were smaller than the size of the symbols. Values of progesterone and oestradiol + range of treatments at 0.28–28 μ M-PGE-1 concentrations were significantly different from corresponding control or oestradiol treatments ($P < 0.05$ – 0.01). Other experimental conditions as in Table 1.

Fig. 2. Dose-response curve of forskolin-stimulated cAMP production in endometrial stromal cells cultured in DMEM containing phenol red for 72 h without or in the presence of oestradiol or oestradiol + progesterone. Values are mean $\pm$ s.e.m. ($n = 5$). All values of oestradiol + progesterone treatment are significantly ($P < 0.05$) different from corresponding controls or oestradiol treatment.

Fig. 3. Influence of increasing concentrations of oestradiol in combination with a fixed dose (10^{-7} M) of progesterone on forskolin- and PGE-1-promoted cAMP production in stromal cells. Cells were grown in DMEM + 20% FCS + phenol red until confluent (7–8 days). Oestradiol (10^{-9} – 10^{-6} M) was then added to the media for 24 h, followed by progesterone for an additional 48 h. Values are mean $\pm$ s.e.m. ($n = 4$ cultures) of a typical experiment. When error bars are not shown these were smaller than the size of the symbols.

Fig. 4. Influence of increasing concentrations of progesterone (10^{-9} – 10^{-6} M) and a fixed dose of oestradiol (10^{-8} M) on PGE-1- and forskolin-stimulated cAMP production in cultured stromal cells. Experimental protocol as in Fig. 3.

Discussion

The results of this study have provided evidence that progesterone in oestrogenized human endometrial stromal cells significantly potentiated the cyclic AMP-generating effect of PGE and forskolin. The possibility that this effect was due to decreased breakdown of cyclic AMP seems unlikely on the grounds that the incubation mixture contained the cyclic AMP phosphodiesterase inhibitor MIX at concentrations which have been shown to inhibit the enzyme effectively in various

Table 2. Lack of effect of progesterone on cyclic AMP production (pmol/10⁶ cells/60 min) during short-term incubation of human endometrial stromal cells

Duration of treatment	+ Progesterone (10 ⁻⁷ M)			- Progesterone		
	Control	PGE-1 (14 µM)	Forskolin (25 µM)	Control	PGE-1 (14 µM)	Forskolin (25 µM)
30 min	19.2 ± 0.63	38.2 ± 4.76	73.5 ± 15.2	16.3 ± 0.59	35.4 ± 3.64	79.0 ± 3.83
120 min	16.8 ± 0.53	38.8 ± 1.54	77.2 ± 7.83	17.5 ± 0.41	33.8 ± 1.34	76.2 ± 6.71

Stromal cells were grown in T-flasks until confluent in DMEM + 20% FCS, but without phenol red. After harvesting with collagenase cells were washed 3 times. Aliquants of cell suspension (5×10^4) were incubated with or without progesterone in 10×75 mm culture tubes (final volume 0.5 ml). Agonists were added after 30 or 120 min and the incubation was allowed to proceed for 60 min. All tubes contained 0.5 mM-MIX.

Values are mean ± s.e. of quadruplicate cultures from 1 of 2 similar experiments.

systems. It seems more likely therefore that progesterone, in combination with an agonist, enhances the synthesis of this second messenger. Because the steroid was about equally effective in potentiating the action of PGE, which is believed to activate the adenylate cyclase-cyclic AMP generating system via a specific receptor-mediated mechanism as well as the action of forskolin which acts at a post-receptor site (Seamon *et al.*, 1981), it is reasonable to suggest that progesterone increased the amount of the adenylate cyclase enzyme or one or more of its regulatory components. This interpretation is consistent with the finding that under unstimulated conditions progesterone did not raise cyclic AMP production, but when the cells were challenged by an activator of the enzyme the generation of the cyclic nucleotide was significantly potentiated. Moreover, the ED₅₀ for the two agonists did not decrease in steroid-treated cells, indicating that the enzyme system has undergone quantitative rather than qualitative changes. It should also be noted that in the present *in vitro* system the concentrations of PGE required to cause significant increments in cyclic AMP output may have been considerably higher than those expected to be found in endometrial cells *in situ*. Riemer *et al.* (1988) found a reduced production of cyclic AMP in myometrial minces of ovariectomized rabbits pretreated with oestrogen that was reversible by progesterone administration. This phenomenon was attributed in part to oestrogen-induced decrease in the stimulatory nucleotide-binding protein (Gs). On the other hand, a decreased cyclic AMP production and an increase in the amount of Gi and a decrease in Gs were observed in the progesterone-dominated rat uterus at Day 12 of gestation (Tanfin & Harbon, 1987).

The physiological significance of the described effect of progesterone is uncertain. As pointed out earlier, cyclic AMP may be involved in a variety of cellular processes that are under the regulatory influence of progesterone. It has been demonstrated in several species of animals that eicosanoids, particularly certain prostaglandins, are involved in increased vascular permeability and the differentiation of stromal into decidual cells in preparation of blastocyst implantation (Evans & Kennedy, 1978; Holmes & Gordashko, 1980; Phillips & Poyser, 1981). In women, presumably under the influence of luteal progesterone, stromal differentiation into predecidual cells occurs during each menstrual cycle in the absence of implantation. Prostaglandins therefore appear to be intimately involved in endometrial physiology. Although the mode of action of prostaglandins in endometrial cells is still largely unknown, there is suggestive evidence, derived from work on other cell types, that the E-series of prostaglandins utilize cyclic AMP as an intracellular messenger. Artificial induction of the decidual reaction is accompanied by a significant increase in uterine levels of cyclic AMP (Rankin *et al.*, 1977; Vilar-Rojas *et al.*, 1982). Because indomethacin, a potent inhibitor of prostaglandin synthesis, can also prevent the rise in cyclic AMP concentrations (Kennedy, 1983), supporting evidence is provided for the involvement of cyclic AMP in prostaglandin-mediated decidual reaction. Moreover, high affinity binding sites for PGE have been detected in endometrial stroma but not in epithelial cells (Kennedy *et al.*, 1983). It seems reasonable

to assume therefore that stromal cells are target sites for PGE and that interaction with specific receptors activates the adenylate cyclase/cyclic AMP generating system. Our observation that progesterone significantly potentiates that important biochemical step is consistent with its proposed role in regulating cellular events in the endometrium.

Alternatively, or in addition to the above, the uterine relaxant effect of progesterone may be mediated partly by increased endometrial production of cyclic AMP in response to PGE or other putative agonists. How the released endometrial cyclic AMP is taken up by myometrial cells is uncertain, but cell-to-cell communication via gap junctions could be one such avenue. Finally, under the synergistic effect of progesterone, PGE may regulate more efficiently via cyclic AMP-dependent steps the activity of enzymes in stromal cells. Alkaline phosphatase activity in rat endometrium has been reported to be elevated by PGE-2 infusion and inhibited by indomethacin, which could be overridden by the administration of PGE-2 or PGF-2 α (Yee & Kennedy, 1988). The results of the present study therefore indicate that one of the actions of progesterone in the human endometrium may be that of enhancing the activation of adenylate cyclase by PGE and possibly other agonists.

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