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HORMONAL SENSITIVITY OF ADENYLATE CYCLASE FROM HUMAN ENDOMETRIUM: MODULATION BY ESTRADIOL

C. M. BERGAMINI[1]*, F. PANSINI†, S. BETTOCCHI JR†, V. SEGALA†, F. DALLOCCHIO*, B. BAGNI‡ and G. MOLLICA†

*Department of Biochemistry and †Obstetrics and Gynecology, ‡Service of Nuclear Medicine, University of Ferrara and Sanitary Local Unit No. 31, 44100 Ferrara, Italy

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Summary—In human endometria, a membrane-bound adenylate cyclase is present, which is recovered in high yield in a low-speed particulate fraction. Neither the specific activity of the enzyme nor the response to modifiers that act through the regulatory subunit of the complex, are modified during the proliferative or secretory phase of the cycle. Surprisingly, we found that *in vitro* treatment of secretory endometrial membranes with 17β-estradiol stimulates 3- to 4-fold the activity of adenylate cyclase. However this response does not occur on proliferative membranes. The activation by estradiol is independent of the presence of guanylylimidodiphosphate and is additive to that of the nucleotide. Possibly, as the consequence of the phenomenon, the concentration of cyclic AMP is significantly higher in curretage samples obtained from patients during the secretory rather than in the proliferative phase of the cycle. To our knowledge this is the first evidence of a target-cell membrane-directed effect of sex steroids in humans.

INTRODUCTION

The action of steroid hormones on target cells depends on the interaction of the hormones with cytoplasmic receptor proteins. The hormone–receptor complex which is formed, is further processed and transferred to the nucleus where it triggers the synthesis of specific proteins [1–3]. This model is based on a large body of evidence and is believed to be valid in all systems studied until now. Exceptions are represented by oocytes of *Xenopus laevis* which are known to undergo the second meiotic division under the influence of progesterone. In this system, the hormone acts directly at the membrane level through an inhibition of adenylate cyclase [4–6]. The resulting decrease of intracellular cAMP is apparently the direct stimulus for the maturation of the oocyte [5]. In addition, reports have appeared that demonstrate the ability of steroid hormones, acting at the plasma membrane level, to influence adenylate cyclase, or to increase the cyclic nucleotide content of target cells [7–10]. This event would be of interest because of the suggested role of cyclic nucleotides as mediators of growth promoting stimula [11].

In the light of these findings we investigated the properties of adenylate cyclase in human endometrium, a typical target tissue of sex steroid hormones. The present report suggests that estrogens directly affect this enzymatic system, selectively, in particulate fractions prepared from secretory endometria.

[1]Correspondence to: Professor C. M. Bergamini, Istituto di Chimica Biologica, Università di Ferrara, Via Borsari 46, 44100 Ferrara, Italy.

EXPERIMENTAL

Materials

Buffers, theophylline, hormones, sodium fluoride, enzyme substrates and creatine phosphokinase were obtained from Sigma (St Louis, MO). Enzyme-grade sucrose was purchased from B.D.H. (Poole, England). Other chemicals were obtained through Merck (Darmstadt, F.R.G). Cyclic AMP was measured via radiommunoassay using an ^{131}I antibody-based kit from New England Nuclear (Boston, MA.).

Tissue collection

Samples of human endometria (100–300 mg each) were obtained by diagnostic curretage and classified into proliferative or secretory. The dating was performed by histological examination [12] and confirmed by determination of the serum concentration of estradiol and progesterone by radioimmunoassay with the use of kits by Radioisotope Service (Würenlingen, Switzerland) and by Diagnostic Products Corporation (Los Angeles, CA), respectively. Immediately upon scraping, tissues were immersed in liquid nitrogen and stored at −50°C until the time of assay, which was performed within 3 weeks.

Homogenization and isolation of membrane enriched fractions

Homogenates of single tissue samples were prepared in 5 vol (v/w) of 0.25 Sucrose, 25 mM KCl, and 5 mM $MgCl_2$ in 50 mM Tris–HCl buffer at pH 7.4.

The particulate fraction was collected by sedimentation at 600 *g* per 10 min and washed again in the

same conditions. The final precipitate was resuspended with homogenization buffer in 10 times the original weight of the tissue to give an approximate protein concentration of 1–2.5 mg/ml.

Adenylate cyclase assay

Adenylate cyclase activity was assayed in a final volume of 370 μl. The reaction mixture contained (final concentration) 2 mM ATP, 2 mM creatine phosphate, 6 mM theophilline, 3 mM $MgCl_2$, 10 mM NaCl, 100–200 μg of membrane proteins and 40 μg of creatine kinase in 50 mM Tris–HCl, pH 7.4, along with the additions (Gpp(NH)*p*, NaF, hormones) detailed in the single experiments.

Steroid hormones were added from stock solutions in ethanol, taking care to always keep the final ethanol concentration lower than 0.1% (v/v). The reaction was started by addition of ATP and was run at 30°C for 20 min. The reaction was stopped by boiling and the samples were treated as described in [13]. Briefly the reaction mixture was frozen and thawed after addition of 1.5 ml of distilled water. After centrifugation to remove denaturated proteins, the cyclic AMP produced was measured by RIA on a 100 μl aliquot.

Recovery of cAMP was checked to be greater than 95%. Results are presented as pmoles of cAMP produced/min/mg of membrane protein.

Measurement of tissue content of cAMP

For measurement of the tissular concentration of cAMP proliferative and secretory endometria were homogenized in 2.0 ml of ice-cold 10% (w/v) TCA with a mechanically driven Teflon-glass Potter–Elvehjem apparatus. After removal of denaturated proteins by centrifugations, the supernatant was neutralized with solid $CaCO_3$ [14] and used for the radioimmunological determination of cAMP. Results are referred to the protein content of the sample.

Protein assay

The concentration of protein in the membrane suspensions and the TCA treated pellet of the extracted tissues was measured by the method of Lowry *et al.* [15], with bovine serum albumin as reference protein.

Statistical analysis was performed by Wilcoxon test for unpaired and paired observations.

RESULTS

Identification and subcellular distribution of adenylate cyclase

When incubated with ATP and an ATP-regenerating system (creatine phosphate plus creatine phosphokinase) sample of homogenates from human endometria were found to catalyze the synthesis of cAMP. The reaction is catalyzed by a membrane-bound enzyme as demonstrated by the subcellular distribution of activity in differential sedimentation experiments.

During both phases of endometrial cycle over 95% of activity is recovered in a low-speed microsomal fraction containing 45% of nucleotidase activity of the original extract with a 5-fold increase of specific activity.

Only marginal activity is recovered in a small particulate fraction obtained by centrifugation of the low-speed supernatant at 10,000 ***g*** per 10 min and no activity is detected in the supernatant. The low-speed (600 ***g***) particulate fraction displays a specific activity 6- to 8-fold higher than that of the initial homogenate. Under these assay conditions, the reaction rate is linear with time up to 40 min, even under conditions of maximal stimulation, and is dependent on the quantity of protein added, up to at least 0.5 mg/ml of membrane protein. Although an extensive investigation of the affinity of the enzyme for the substrate, ATP, has not been carried out, it must be observed that the concentration employed is saturating. Indeed, the activity is not modified by raising the concentration of the nucleotide up to 5 mM. The enzyme is dependent on divalent cations for activity. Addition of EDTA to a concentration of 5 mM completely abolishes the activity. In the presence of 5 mM $MgCl_2$ the same activity is obtained as under our standard conditions. $MgCl_2$ can be substituted for by $MnCl_2$, with a 2- to 4-fold increase of activity as compared to the assay performed in the presence of the magnesium salt, but not by the calcium salt, which cannot support activity.

Catalytic properties of adenylate cyclase. Influence of modulators

The catalytic properties of endometrial adenylate cyclase are summarized in Table 1. The basal activity, i.e. that displayed by the enzyme in the absence of effectors in the reported standard assay conditions, is comparable throughout the cycle, at about 4 and 6 pmol/min/mg of membrane protein. The activity is very sensitive to the addition of effectors which are supposed to act via the regulatory G/F subunit [16]: sodium fluoride stimulates the activity to the same extent in both phases of the cycle, to a 8- to 10-fold. The concentration for maximal activity, 5 mM, was the same in both cases and the enzyme was invariably inhibited by higher concentrations of the salt. Also the addition of the non-hydrolizable GTP analog, guanylylimidodiphosphate, Gpp(NH)*p*, modulates

Table 1. Basal and stimulated adenylate cyclase activity of proliferative and secretory endometria

Conditions		cAMP (pmol/min/mg) $x \pm$ SE Proliferative	Secretory
Basal		6.1 ± 1.4 (14)	4.4 ± 0.7 (15)
Gpp(NH)*p*	0.1 mM	18.0 ± 3.7 (11)	20.6 ± 5.1 (10)
NaF	5 mM	43.9 ± 7.6 (6)	45.8 ± 9.8 (6)
NaF	20 mM	32.4 ± 5.1 (6)	40.5 ± 2.5 (6)

The number of endometria tested is shown in parentheses.

the activity, leading to a 4- to 5-fold increase in the rate of synthesis of the cyclic nucleotide. Both of these G/F effectors in any case significantly affect the activity to the same degree during the two phases of the cycle.

Hormonal sensitivity of endometrial adenylate cyclase

The next step was to determine the hormonal sensitivity of the enzyme activity. The following hormones were tested through a range of concentrations: prolactin, LH, FSH, adrenalin and glucagon. We were not able to detect any influence by these hormones on the cyclase activity either in the presence or in absence of Gpp(NH)*p*. On the contrary, we observed a significant effect of sex steroids on cyclase activity when estradiol and progesterone were tested *in vitro* on membranes prepared from proliferative and secretory endometria. The data presented in Table 2 demonstrate that high concentrations (10^{-5} M) of progesterone in the absence of Gpp(NH)*p* do not change the activity of adenylate cyclase. In the concomitant presence of progesterone and of nucleotide, the enzyme was stimulated in both phases to roughly the same extent, as when it was stimulated by Gpp(NH)*p* alone. A quite different action is shown by estradiol, which selectively stimulates 3-fold the activity of the membranes prepared from secretory endometrium, even when tested in the absence of Gpp(NH)*p*. In the presence of the nucleotide (10^{-4} M), additive effects of Gpp(NH)*p* and estradiol are evident.

In control experiments, it was verified that the action of estradiol is independent of the solvent concentration. In fact, addition of ethanol to achieve a final concentration of 0.1% does not significantly affect the basal and the Gpp(NH)*p* stimulated activities. Furthermore, the effect of estradiol appears to be quite selective since neither progesterone nor a semisynthetic estradiol analog, ethynylestradiol (10^{-5} M), modify the cyclase activity in membranes prepared from secretory endometria.

Since the reported effects were obtained using only very high concentrations of estradiol (10^{-5} M) we tried to determine the saturation plot for the stimulation of the cyclase activity by estradiol and therefore the concentration for half-maximal stimulation. The results of a representative experiment, run in absence of Gpp(NH)*p*, are presented in Fig. 1. Here, as well as in additional experiments, we observed a concentration-dependent, estradiol-mediated stimulation of cyclase activity in the secretory membranes. The half-maximal effect is observed in the presence of an estradiol concentration of approx. 10^{-8} M.

Table 2. Effect of estradiol (E_2) and progesterone (P) on adenylate cyclase activity of proliferative and secretory endometria in the presence and in absence of Gpp(NH)*p*

	cAMP (pmol/min/mg) $x \pm$ SE	
Conditions	Proliferative	Secretory
Basal	2.6 ± 0.2	2.9 ± 0.2
Gpp(NH)*p*	12.9 ± 1.8*	10.4 ± 1.3*
E_2	1.5 ± 0.2	8.3 ± 1.1*
E_2 + Gpp(NH)*p*	10.5 ± 1.8*	19.0 ± 2.3*
P	2.9 ± 0.3	4.0 ± 1.1
P + Gpp(NH)*p*	13.5 ± 2.0*	11.6 ± 0.2*

Statistical significance (stimulated vs basal): * < 0.05. Experiments were performed as paired observations on 6 endometria for each phase, steroids and Gpp(NH)*p* were added at concentration of 0.01 and 0.1 mM, respectively.

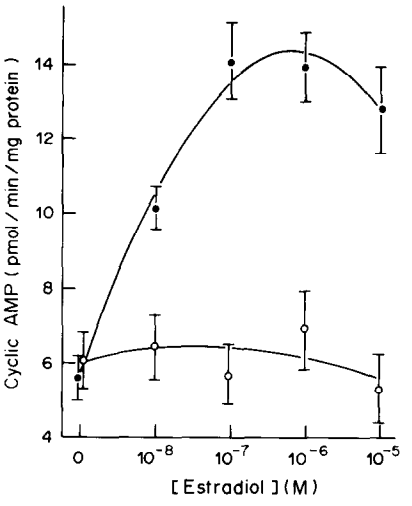

Fig. 1. *In vitro* stimulation of adenylate cyclase activity in human endometrium (○——○, proliferative; ●——●, secretory) by increasing concentrations of estradiol. The error bars are SEM.

Tissular content of cyclic AMP along the endometrial cycle

To elucidate the relevance of our *in vitro* findings, to the *in vivo* conditions, we have decided to measure the tissue content of cyclic AMP during both phases of the cycle, i.e. under conditions where the cells are exposed to relatively high concentrations of estradiol with or without priming by progesterone. The results, presented in Fig. 2, document an impressive increase

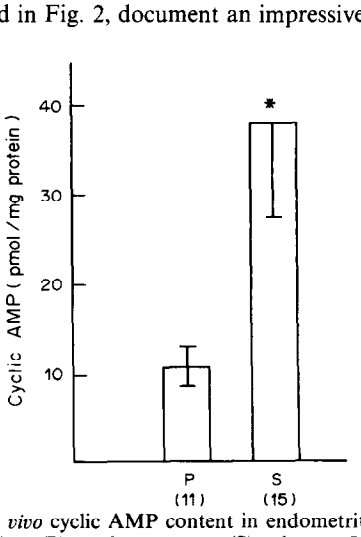

Fig. 2. *In vivo* cyclic AMP content in endometrium during proliferative (P) and secretory (S) phases. Values are mean ± SEM. In the brackets are reported the number of cases. *, $P < 0.01$.

of cAMP in the endometria prepared from the secretory phase in comparison to those obtained from proliferative tissues.

In the secretory phase we obtained values of 37.7 ± 10.0 pmol/mg of protein, while the correspondent value for proliferative endometrium is only 11.4 ± 2.0 pmol/mg of protein (mean $\pm$ SEM).

DISCUSSION

The results in the present report give clear evidence for the existence of a membrane-bound form of adenylate cyclase in human endometria. The activity of the enzyme is influenced to the same extent during the two phases of the cycle, by the usual stimulators, NaF and Gpp(NH)*p*, known to act through an interaction with the regulatory G/F subunit [16]. However, as the data presented in Table 2 suggest, steroid hormones seem to modulate the enzyme activity through a direct action on the membrane.

The activation of adenylate cyclase by estradiol is selective for membranes prepared from endometria in the secretory phase, although the enzyme is present at a constant concentration throughout the cycle. This specific activation is probably the consequence of structural or functional changes of the membrane during the luteal phase of the cycle and could be the reason for the difference in the *in vivo* levels of cAMP in endometrium observed along the cycle, in agreement with the results obtained by Munemura on endosalpinx [17].

If this interpretation is correct, this is the first description of a direct action of 17β estradiol in mammals modifying a membrane-bound enzymatic activity, by a mechanism independent of the classic intracellular receptor-gene activation pathway. However, the present data do not allow us to distinguish between a direct effect of the hormone on the enzyme as opposed to a receptor-mediated process. In favour of the first hypothesis is the observation that the stimulation of adenylate cyclase is independent of the regulatory G/F subunit, which is the usual link for coupling a hormonal receptor to the hormone-dependent adenylate cyclase [16]: in fact, the activation takes place in the absence of Gpp(NH)*p* and is additive to that brought about by the nucleotide, suggesting a direct (allosteric?) action of the hormone on the catalytic components of the cyclase.

In any case, our data point to a possible involvement of cyclic AMP in the physiological changes taking place in the endometrium during the luteal phase. In this perspective, we must remember that cyclic nucleotides have been implicated in short term regulation of hormone binding in target tissues [18, 19], probably by controlling the phosphorylation state of the receptor [20]. Besides this, it is conceivable that cyclic nucleotides participate in the regulation of the activity of protein kinases, which are known to vary during the cycle [21]. They also might play a role in the induction of the decidual reaction, which is easily induced in experimental animals by a single intraperitoneal injection of cholera toxin [22], which in fact, is the most effective stimulator of adenylate cyclase yet identified.

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