Rapid changes in specific estrogen binding elicited by cGMP or cAMP in cytosol from human endometrial cells

(estradiol binders/molybdate/cyclic nucleotides/endometrial cells/divalent cations)

Honorée Fleming, Rosalyn Blumenthal, and Erlio Gurpide

Departments of Obstetrics, Gynecology and Reproductive Science, of Biochemistry and of Physiology, Mount Sinai School of Medicine, New York, New York 10029

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ABSTRACT

Addition of cGMP to cytosol of human endometrium or to cells of the endometrial cancer line HEC-1 produced severalfold increases in specific estrogen binding (EB) levels. This effect was maximal with 1 μM cGMP in the presence of 0.1 mM isobutylmethylxanthine (a phosphodiesterase inhibitor) during incubations with [3H]estradiol. In contrast, cAMP decreased EB levels under similar conditions. The effects of cyclic nucleotides on EB levels were complete in <15 min in the presence of Mg2+, Mn2+, or Ca2+. The EB sites generated by the addition of cGMP during labeling of cytosol with 10 nM [3H]estradiol were found to sediment in the 8S and 4S regions of low-salt glycerol gradients. No changes in EB levels were observed when cyclic nucleotides were added to cytosol depleted of ATP by preincubation at 4°C for 3 hr, but responsiveness was restored by addition of exogenous ATP. The ATP requirement and the pattern of dependence of cyclic nucleotide actions on divalent cation concentrations suggest that cGMP and cAMP effects may be mediated by kinases and may involve phosphorylations. Another possibility is that the cyclic nucleotides interact allosterically with the binder in the presence of ATP. Addition of sodium molybdate, ATP, and GTP to homogenates of endometrial tissue or HEC-1 cells produces increases in EB levels similar to those obtained by the addition of cGMP. However, these compounds are much less active when added to cytoplasm or cytosol. On the basis of these and other observations, it is hypothesized that molybdate, ATP, and GTP affect EB levels primarily by increasing cGMP concentrations through processes involving a plasma membrane-bound guanylate cyclase.

We previously reported that the addition of cGMP to homogenates of normal human endometrium or endometrial adenocarcinoma cells (HEC-1 line) produced severalfold increases in the levels of specific estradiol (E2) binders sedimenting in the 8S and 4S region of low-salt glycerol gradients. Addition of sodium molybdate to intact or homogenized endometrial cells or endometrial tissue caused a similar elevation in specific estrogen binding (EB) levels which could not be explained by stabilization of the binders after cell disruption, a mechanism usually proposed to account for molybdate effects on steroid binding. Addition of ATP or GTP to the homogenates also increased specific E2 binding levels, whereas cAMP was found to decrease binding and to counteract the effects of cGMP, molybdate, GTP, and ATP (1).

These results, together with earlier findings of rapid fluctuations in the concentration of E2 binders in vivo (2, 3), suggested that binding sites might be subject to reversible modifications. By mechanisms similar to the phosphorylation/dephosphorylation reactions implicated in the regulation of glucocorticoid (4, 5) and progesterone binding (6).

It was initially found that ATP, GTP, cGMP, and MoO₄²⁻ are all equally capable of generating E2 binders when added to cellular homogenates (1). In this report, we demonstrate a fundamental difference between the mode of action of cGMP and the other compounds. Molybdate, ATP, and GTP increase cytoplasmic E2 binding when added to whole-cell homogenates, but not when added to cytosol, in the absence of plasma membranes whereas cGMP is equally effective whether added to whole-cell homogenates or directly to cytosol. cAMP also is effective in the cytosol. In this study, we have attempted to characterize the reactions responsible for cGMP-dependent generation and cAMP-dependent inactivation of cytosolic E2 binding sites.

MATERIALS AND METHODS

Cell Culture. HEC-18 cells (1) were grown to confluence in T-125 plastic culture flasks (Corning) in Ham F-10 medium (Flow Laboratories) supplemented with 10% calf serum (Flow Laboratories), glucose at 4.0 mg/mL, insulin (Squibb) at 10 μg/mL, and 1% antibiotic/antimycotic mixture (GIBCO). Cultures were carried out in a humidified atmosphere of 5% CO₂/95% O₂ at 37°C. Cells were harvested by treatment with 1.5 mM EDTA in Hanks balanced salt solution for 15 min at room temperature. The cells were pelleted and resuspended in medium.

Surgical specimens of fresh human endometrial tissue were obtained from patients undergoing vaginal hysterectomy or dilatation and curettage procedures. A sample of fresh tissue, fixed in 10% formalin and stained with hematoxylin and eosin, was dated according to Noyes et al. (7).

E₂ Binding Assay. The cell or tissue sample was disrupted by glass/glass homogenization in 30% (vol/vol) glycerol/1 mM MgCl₂/10 mM Tris, pH 8.0/0.1 mM diithiothreitol (GMTD buffer). The broken cell preparation was centrifuged at 500 × g for 5 min. The supernatant (cytoplasm) was then centrifuged in an SW 65 rotor at 105,000 × g for 60 min to obtain cytosol. Aliquots of homogenate, cytoplasm, and cytosol were incubated at 4°C for 30 min with an equal volume of Hanks balanced salt solution containing [6,7-³H]E₂ (52 Ci/mmol; 1 Ci = 3.7 × 10¹⁰ Bq; New England Nuclear) with or without a 100-fold excess of diethylstilbestrol (DES), with additions of molybdate, GTP, ATP, cGMP, or cAMP as specified for each experiment. The total assay volume was 50 μL. The samples were rapidly frozen. Upon thawing, 350 μL of GMTD buffer was added to each sample to facilitate handling. Each sample was then incubated for 20 min at 4°C with 0.25% dextran-coated charcoal and the suspension was centrifuged at 1,500 × g for 20 min.

Abbreviations: EB, specific estrogen binding; E₂, estradiol; GMTD buffer, 30% (vol/vol) glycerol/1 mM MgCl₂/10 mM Tris, pH 8.0/0.1 mM diithiothreitol; ibuMeNan, isobutylmethylxanthine; DES, diethylstilbestrol.
Radioactivity in 100-µl aliquots of the supernatant was measured by liquid scintillation spectrometry. The counts were normalized to the amount of protein present in each tube, and specific binding was calculated from the difference between counts in tubes with and without DES.

Glycerol Density Gradient Analysis. Continuous 16–41% glycerol gradients (total, 3.4 ml) were prepared in 10 mM Tris, pH 8.0/12 mM thioglycerol/1.5 mM EDTA (TTE buffer) containing 50 mM KCl. Aliquots of cytosol containing labeled E2 binders and diluted with an equal volume of TTE buffer were layered on the top of the gradient and centrifuged at 4°C for 22 hr at 105,000 × g. The bottom of the tube was pierced and 100-µl fractions were collected and assayed for radioactivity. Five marker proteins—alcohol dehydrogenase (7.8 S), bovine serum albumin (4.6 S), ovalbumin (3.5 S), Hb (3.0 S), and horse heart cytochrome (2.2 S)—were layered on a separate gradient and run in parallel. Location of the marker proteins was determined by protein assay. Linear regression analysis of relative mobility provided a sedimentation coefficient of the markers that was performed for each experiment; correlation coefficients were consistently >0.97.

ATP Measurements. Cellular ATP concentrations were determined according to the method of Stanley and Williams (8) as modified by Guffanti et al. (9). ATP was extracted from cell homogenates with 30% perchloric acid and the extract was neutralized with 1 M KOH. Aliquots of the supernatant were assayed by the firefly luciferase method. Initial rates of light flashes were measured in a Beckman LS-230 spectrometer for 6-sec intervals with the coincidence switch off, using a voltage window adequate for 3H measurements. Assays were done in a phosphate/arsenate pH 7.4 buffer containing 0.1 M KH2PO4, 0.1 M arsenate, 40 mM MgSO4, and 40 mM MgCl2. The GMDT buffer used for the steroid binding assay was used as a control in each experiment to ensure that there was no chemical interference by any component in the buffer. Rates corresponding to ATP standard were determined for each experiment with the sodium salt of ATP and a fresh firefly lantern extract (Sigma). The assay was linear over the range 1–100 nM ATP.

Protein Assay. Concentration of protein in cytosol preparations from fresh human endometrium or HEC-1 cells and solutions of internal markers for gradient centrifugations were determined with a Bio-Rad protein assay kit based on the colorimetric method of Bradford (10).

RESULTS
cGMP at 1 mM, but not 20 mM Na2MoO4, 1 mM ATP, or 1 mM GTP, increased EB levels when added to cytoplasm prepared by low-speed centrifugation of secretory tissue homogenates) during labeling with 80 nM [3H]E2 with or without 8 µM DES at 4°C (Fig. 1). Addition of any of these compounds to the corresponding cell homogenate produced 2- to 7-fold increases in specific [3H]E2 binding (1). Similar increments relative to basal EB levels, which vary from culture to culture and specimen to specimen as previously reported (2, 3, 11), were obtained with several cultures of HEC-1 cells and specimens of endometrial adenocarcinoma (four experiments). The slight effect of ATP or GTP added to cytosol or cytoplasm in the experiment shown in Fig. 1 and in some of the other experiments does not appear to be significant and was always less than 10% of the effect observed when these compounds were added to homogenates.

These observations confirm and extend our previous findings (1) which indicated that components of the nucleus/plasma membrane pellet removed during the preparation of cytosol and cytosol are involved in the generation of E2 binding sites by molybdate when whole cells or homogenates are labeled with [3H]E2 at 0–4°C. This effect is different from the known protective or stabilizing action of molybdate added to cytosol under conditions that otherwise result in a loss of E2 receptors—e.g., temperatures >1°C or the long periods (>12 hr in buffer solution) required for gradient centrifugation analysis.

It is shown in Fig. 1 that the generation of binding sites by ATP and GTP also requires factors that sediment during low-speed centrifugation. In contrast, cGMP can exert a stimulatory action on E2 directly when added to cytosol.

cAMP, originally found to lower EB levels in homogenates of endometrial tissue and HEC-1 cells (1), is also capable of affecting EB when added to cytosol of HEC-1 cells (Fig. 2). The effects of cGMP and cAMP on EB levels were concentration-dependent. In the presence of 0.1 mM isobutylmethylxanthine (BuMeXan), a phosphodiesterase inhibitor, effects of cyclic nucleotides on specific estrogen binding were noted at 1 nM and became maximal at about 1 µM.

Both the activation* and inactivation of E2 binding sites required divalent cations but the reactions differed with regard to optimal cation concentration (Fig. 3). The extent of activation in response to 1 µM cGMP continued to increase as the concentration of Mg2+ or Mn2+ was increased up to 100 mM. In contrast, inactivation of estrogen binding in response to 1 µM cAMP was maximal at 2–5 mM Mg2+ or Mn2+ and declined at greater concentrations of these cations. The maximal effects obtained with Ca2+ were smaller than the maximal generation

*The term "activation" is used in this article to indicate an increase in the number of specific E2 binding sites provoked by various agents and it does not imply changes in the DNA binding capability of the estrogen–receptor complex.
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![Graph of Cytosolic EB (pmol/mg protein) vs. [GMP] or [CAMP] (nM)]

**Fig. 2.** Effects of increasing concentrations of GMP and cAMP on E2 binding in HEC-1 cell cytosol. HEC-1 cells were homogenized in 1 ml of GMTD buffer containing 0.1 mM iBuMeXan. Cytosol was prepared and labeled as in Fig. 1; GMP or cAMP was added to obtain the concentrations indicated in the figure. The averages of duplicate assays in a single experiment are shown, and these data are representative of results obtained from two similar experiments with HEC-1 cells and one with normal proliferative endometrium.

or inactivation achieved in the presence of Mg" or Mn". These results demonstrate that the effects of cGMP and cAMP on EB levels are mediated by processes influenced differently by each of these cations.

Activation or inactivation of estrogen binders occurred rapidly when cytosol of proliferative endometrium was incubated with 80 nM [3H]E2 in the presence of 0.1 mM iBuMeXan and either 1 μM cGMP or 1 μM cAMP (Fig. 4). Levels of specifically bound [3H]E2 were determined at various times of incubation, from 5 to 90 min. cGMP-dependent activation of estrogen binders, as well as binding of [3H]E2 to the newly generated sites, was practically complete at 5 min; cAMP-dependent inactivation was almost complete at 15 min. Binding observed at 90 min in control tubes containing no cyclic nucleotides is also shown.

Fig. 5 presents data on binding of [3H]E2 at 4°C, 30°C, and 37°C, obtained after a 30-min incubation of HEC-1 cell cytosol with the labeled estrogen in the presence of 0.1 mM iBuMeXan and 1 μM cGMP or 1 μM cAMP. No differences were noted in control binding levels or in changes elicited by the cyclic nucleotides at 4°C and 30°C but there was an indication of loss of binding sites at 37°C.

Endogenous ATP was necessary to elicit activation of estrogen binding sites by cGMP or their inactivation by cAMP (Fig. 6). The levels of ATP in cytosol of HEC-1 cells immediately after preparation was 3 nmol/mg of protein, and addition of cGMP or cAMP produced changes in EB levels similar to those shown in Fig. 2, with cyclic nucleotides at 1 μM. The concentrations of ATP in cytosol kept at 4°C for 3 hr declined to undetectable levels, and the responses to cyclic nucleotides were greatly diminished. When ATP was added to the ATP-depleted...
cytosol to obtain 25 nmol/mg of protein, the responsiveness to cGMP and cAMP was restored and the EB levels obtained were approximately the same as those observed when the cyclic nucleotides were added to the original cytosol. Estrogen binding sites sedimenting in the 4S and 8S regions of a low-salt glycerol gradient could be increased by cGMP (Fig. 7). These results were obtained by incubating cytosol from HEC-1 cells with 10 nM \(^{3}H\)E\(_2\) in the presence or absence of 1 \(\mu\)M cGMP and 0.1 mM iBuMeXan. The amount of specifically bound radioactivity in each of these two samples was approximately doubled in cGMP-treated cytosol compared to untreated controls.

**DISCUSSION**

The results reported here demonstrate that cGMP and cAMP can directly affect cytosolic E\(_2\) binding, whereas GTP, ATP, and MoO\(_4^{2-}\) increase EB levels indirectly, perhaps by increasing cGMP concentrations through processes involving a membrane-bound guanylate cyclase (1).

Characterization of the cGMP-dependent generation or activation and the cAMP-dependent inactivation of estrogen binders indicates that the mechanisms involved in these two processes may be similar. Both activation and inactivation require ATP and either Mg\(^{2+}\) or Mn\(^{2+}\) for maximal effects. Both reactions show similar dependence or concentrations of the respective cyclic nucleotides and are rapidly completed, even at 4°C.

The observation that ATP and divalent cations are required for optimal cGMP-related increases in E\(_2\) binders suggests that activation might involve phosphorylations, as has been suggested for the glucocorticoid receptors (4, 5). Auricchio et al. (12) presented evidence for the existence of a cytosolic kinase that can reactivate estrogen binders in the presence of ATP. In fact, progesterone receptor has recently been reported to be phosphorylated in vivo (1) and in vitro (2).

If a cGMP-dependent phosphokinase is responsible for generation of E\(_2\) binding sites, then binders would be expected to be inactivated by dephosphorylation. Hydrolysis of phosphate esters has actually been related to decreased E\(_2\) binding in two separate studies: it has been shown that endogenous phosphatase activity, associated with endometrial cell nuclei, can inactivate cytosolic E\(_2\) binding (13), and that alkaline phosphatase added to corpus luteum cytosol can inactivate E\(_2\) receptors (9).

Our results indicate that other mechanisms, in addition to dephosphorylation, may cause E\(_2\) binder inactivation. The observation that cAMP in the presence of ATP and Mg\(^{2+}\) causes cytosolic binding levels to decrease suggests that a cAMP-dependent phosphorylation may also reduce E\(_2\) binding levels. It is possible that cGMP and cAMP bind to and differentially affect the activity of a single protein phosphokinase or that each cyclic nucleotide binds to a different kinase. It is particularly interesting that activation of E\(_2\) binding sites in the presence of cGMP increases as the concentration of Mg\(^{2+}\) or Mn\(^{2+}\) is increased to as high as 100 mM, a cationic sensitivity similar to that observed for cGMP-dependent protein kinase (14). In contrast, cAMP-dependent protein kinases, both type I and type II, show peak activities at Mg\(^{2+}\) or Mn\(^{2+}\) concentrations lower

than 5 mM (14), as does the cAMP-dependent reaction that causes E₂ binder inactivation. These observations suggest that the cyclic nucleotides may be activating two different phosphokinases. Opposite effects on binding could be the result of phosphorylation of different sites in the binder or of different proteins associated with the binder.

The evidence that phosphokinases are involved in activation (generation) and inactivation of E₂ binders is still circumstantial and alternative mechanisms could explain the observed effects of cGMP and cAMP. One possibility is that, in the presence of ATP and divalent cations, the cyclic nucleotides interact allosterically with the binder or associated proteins, thereby altering affinity for E₂ or overall stability.

As shown by the data in Fig. 6, intracellular concentrations of ATP appear to be sufficient to support a full effect of cGMP. It therefore is likely that much of the increase in EB levels induced by the addition of ATP to homogenates is due to generation of cGMP through an activation of membrane-bound guanyl cyclase (15). It is possible, however, that some specimens depleted of ATP during prolonged periods of hypoxia before analysis may show an enhancing effect of ATP even when it is added directly to cytosol.

The low levels at which cGMP and cAMP exert significant effects on EB levels [e.g., 1 nM in the presence of iBuMeXan (Fig. 2)] point to a possible physiological role for the cyclic nucleotides in the regulation of levels of estrogen binders in vivo. Variations in cGMP and cAMP levels may be responsible for the reported rapid fluctuations in E₂ binding levels in cultures of endometrial cells (2).

The finding that cAMP and cGMP have opposite effects on EB levels is characteristic of the action of these compounds on various other physiological events (16). It is of particular interest that the concentrations of cGMP and cAMP have been reported to vary in opposite directions during the estrus cycle in mature intact rats; cGMP levels have been shown to be at their highest and cAMP levels at their lowest in proestrus (17), when the concentration of total uterine estrogen receptors is reported to be maximal (18, 19).

It has also been reported that dibutyryl cAMP lowers cytoplasmic and nuclear E₂ binding in induced rat mammary carcinomas (20), a result in intact animals that agrees with our observation in cultured cells and endometrial tissues. Finally, melatonin has been shown to increase cytoplasmic E₂ receptors in uterine tissue of prepubertal hamsters in vivo and in vitro (8). Our results indicate that this effect may be due to the ability of melatonin to increase cGMP levels (21).

The cGMP-induced increase in the concentration of the specific SS binder labeled with 10 nM [3H]E₂ (Fig. 7) suggests that the cyclic nucleotide activates the type of binding usually considered to correspond to the intracellular estrogen receptor as well as a smaller (4S) binder that has been also frequently observed in specimens of endometrial tissue (22).

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