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Estrogen-Related Increases in Uterine Guanosine 3′:5′-Cyclic Monophosphate Levels

(cyclic AMP/progesterone/estrus cycle)

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ABSTRACT **Endogenous uterine cyclic GMP and cyclic AMP concentrations were monitored at each stage of the estrus cycle; cyclic GMP concentrations were found to be highest and cyclic AMP concentrations lowest during proestrus when plasma estrogen levels have been shown to be maximal. After administration of estradiol-17β or diethylstilbestrol, the concentrations of uterine cyclic GMP in ovariectomized rats underwent a progressive and significant elevation and the levels of cyclic AMP declined significantly below control values when near maximal accumulation of tissue cyclic GMP was achieved. The increase in uterine cyclic GMP concentrations produced by estradiol benzoate administration was prevented when progesterone and the estrogen were co-administered. These results raise the possibility that an enhanced cellular accumulation of cyclic GMP may be involved in the expression of some of the actions of estrogen in uterine tissue.**

It has recently been proposed that guanosine 3′:5′-cyclic monophosphate (cyclic GMP) may serve as a biological regulatory component involved in promoting cellular events antagonistic to those induced by adenosine 3′:5′-cyclic monophosphate (cyclic AMP) (1–4). This concept of the biological importance of cyclic GMP derives primarily from studies in which a relationship has been established between the cellular accumulation of this cyclic nucleotide and the action of polypeptide, neurohormonal, and mitogenic agents whose effects on cell function are believed to result from their interaction at the level of the cell plasma membrane. The possibility that steroid hormone actions might also involve alterations in cyclic GMP metabolism has been raised by the reports that glucocorticoid administration can increase urinary excretion of cyclic GMP (5) and decrease the tissue levels of this cyclic nucleotide in rat lung (1).

Since the findings of Jensen and his associates (6), demonstrating the binding of estradiol to cellular components in estrogen-responsive tissues, the majority of investigations dealing with the mechanism of estrogen and other steroid hormone actions have focused primarily on interactions of this class of agents with intracellular receptor proteins. However, it has also been suggested that the plethora of events associated with estrogen action in the uterus may be mediated in part by an enhanced accumulation of cellular cyclic AMP that has been reported to occur (7) after estrogen treatment. More recent investigations of this phenomenon have failed to confirm the observation (8) or demonstrate that if such increases occur they are probably secondary to the release of catecholamines (9), since they are prevented by administration of β-adrenergic blocking agents. In view of the fact that cell proliferation (10), glycogen deposition (10), and lysosomal enzyme release (11) are characteristic responses of uterine tissue to estrogen, an increase in uterine cyclic AMP concentration would seem to be an unlikely concomitant of such hormone-induced events since cyclic AMP is generally thought to be inhibitory to these cellular processes. On the other hand, cell proliferation has been shown to be associated with enhanced cellular accumulation of cyclic GMP (2) and lysosomal enzyme release to be stimulated upon exposure of cells to cyclic GMP (12, 13). It would seem more reasonable, therefore, to expect that estrogen action in uterine tissue might be associated with an increase in the levels of uterine cyclic GMP rather than those of cyclic AMP.

In the experiments to be reported, this possibility was investigated, and it was found that release and/or administration of estrogen *in vivo* promotes consistent and significant increases in uterine cyclic GMP concentrations while having variable, but usually, opposite effects on the levels of cyclic AMP.

MATERIALS AND METHODS

Female Holtzman rats (150–250 g) were utilized in these studies. Animals used for the *in vivo* effects of estradiol-17β and diethylstilbestrol were ovariectomized 1 week before use and uteri were analyzed by *Method 1*. Those utilized for the *in vivo* effects of estradiol benzoate and progesterone were ovariectomized 3 months before use. The stages of the cycle of the intact rats were determined by vaginal smear examination. Tissues from the latter two studies were analyzed by *Method 2*. Animals were killed by decapitation; the uteri were freed of fat, excised, and prepared for assay by one of the two methods.

Diethylstilbestrol obtained from Eli Lilly was administered subcutaneously (sc) in 0.1 ml of sesame oil; estradiol benzoate from Steraloids and progesterone from Nutritional Biochemicals Co. were administered subcutaneously separately or together in 0.2 ml of sesame oil. Estradiol-17β from Schwartz-Mann, dissolved in ethanol and diluted with polyethylene glycol 400 (Fischer) and water (1:4.5:4.5, on a volume basis) was given intraperitoneally (ip). Radioactive cyclic AMP and cyclic GMP antigens (2′-*O*-succinyl [^{125}I]tyrosine methyl esters of the nucleotides) and corresponding antisera were obtained from Collaborative Research Inc. (Waltham, Mass.).

Method 1. Uteri from animals treated with estradiol-17β or diethylstilbestrol were either frozen immediately upon re-

Abbreviation: sc, subcutaneously; ip, intraperitoneally

moval from the animal or incubated for 30 min at 37° in Munsick's medium (14) maintained at pH 7.5 by a constant flow of 95% O_2 and 5% CO_2 before they were frozen in Freon 12, cooled to −150° in liquid nitrogen, and stored at −90°. Frozen uteri were powdered in mortars cooled in liquid nitrogen (in a cold room at −20°) and extracted by a method described (15) with perchloric acid containing a trace amount (2000 cpm) of purified tritiated cyclic GMP (ICN) to monitor recovery. Formic acid was added to the $KHCO_3$-neutralized supernatant to achieve a final concentration of 0.05 M, and the solution was passed over a column (0.5 × 2.5 cm) of Bio-Rad AG1-X8 (100–200 mesh) in the formate form. The cyclic AMP was removed with 10 ml of 1 N formic acid (16), and the cyclic GMP with an equal volume of 4 N formic acid. The eluates were evaporated to dryness on a Buchler Evapomix. The cyclic GMP fraction was then dissolved in 2 ml of distilled water and further purified on a column (1 × 4 cm) of QAE-Sephadex A25 (Pharmacia) by the procedure of Schultz *et al.* (17). The pH 6.0 ammonium formate eluate containing the cyclic GMP was passed through a column (1 × 4.5 cm) of Bio-Rad AG50W-X8 (100–200 mesh) in the hydrogen form to remove ammonium ions, and the eluate plus a 2-ml water wash of the column were evaporated to dryness. The cyclic AMP and cyclic GMP fractions were dissolved in 0.5 ml of pH 7.5 buffer containing 25 mM Tris · HCl, 2 mM $MgCl_2$, and 0.5 mM EDTA. Two aliquots (40 μl for cyclic AMP and 100 μl for cyclic GMP) of each sample were incubated at room temperature for thirty minutes with phosphodiesterase (Boehringer-Mannheim Corp.) diluted 1:100 in the reaction mixture. All tubes (i.e., with or without added phosphodiesterase) were then heated at 90° for 3 min and placed in an ice bath to cool before the addition of reagents for cyclic nucleotide analysis. Cyclic AMP was analyzed by the method of Gilman (18), but the binding reaction was conducted at pH 7.5 instead of pH 4. Cyclic GMP was analyzed by a modification (described below) of the procedure of Steiner *et al.* (19).

Method 2. Excised uteri from the intact rats or uterine horns from estrogen- and/or progesterone-treated ovariectomized rats were immediately frozen in Freon 12 at dry ice temperature and weighed. A trace amount (3000 cpm) of either purified tritiated cyclic AMP or purified tritiated cyclic GMP was added to 3-ml aliquots of cold 10% trichloroacetic acid for recovery purposes. The frozen tissue was transferred to the trichloroacetic acid solution (20) and sonicated with a Polytron (PT 10ST) homogenizer at a pulse frequency of 7300 Hz for 30–60 sec. The insoluble material was removed by centrifugation and analyzed for protein content. The supernatant fraction was extracted with three 5-ml portions of diethyl ether to remove the trichloroacetic acid and then chromatographed on a column (0.5 × 2.5 cm) of AG1-X8 in the formate form. The cyclic AMP was removed with 8 ml of 1 N formic acid, the cyclic GMP with 10 ml of 4 N formic acid, and the eluates were evaporated to dryness on a Buchler Evapomix. Both cyclic AMP and cyclic GMP were determined by radioimmune assay.

Radioimmune Assays. The radioimmune assays were conducted in the following manner: the samples containing cyclic GMP or cyclic AMP, ^{125}I-labeled antigen, antiserum, and 50 mM sodium acetate buffer, pH 6.2 (final volume 500 μl) were equilibrated overnight (15–20 hr) at 0°. The incubated samples were diluted with 3 ml of cold 50 mM sodium acetate buffer and immediately poured over an HAWP 02500 (0.45 μm) Millipore filter. The sample tube was washed twice with 3 ml of the same buffer and the washings were passed through the filter. The filter was finally washed with an additional 3 ml of buffer. The filters were dried and the ^{125}I-labeled antigen–antibody complexes retained were determined directly in a Packard gamma counter or, after addition of a scintillant (Beckman TLA), in a Beckman scintillation spectrometer.

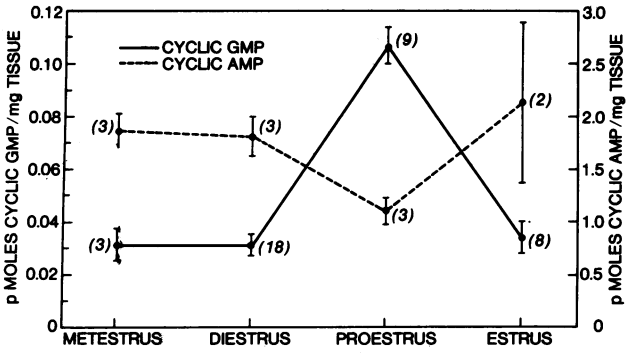

FIG. 1. Cyclic nucleotide levels ($\bar{x} \pm$ SEM) in the uterus of the intact rat during the estrus cycle. Nucleotide levels were determined by *Method 2*. Significance of cyclic GMP differences: proestrus compared to diestrus, $P < 0.001$; proestrus compared to estrus, $P < 0.001$; proestrus compared to metestrus, $P < 0.001$. Significance of cyclic AMP differences: proestrus compared to diestrus, $P < 0.05$; proestrus compared to estrus, not significant; proestrus compared to metestrus, $P < 0.05$. All differences among diestrus, estrus, and metestrus are not significant for either cyclic GMP or cyclic AMP (two-tailed *t*-test).

RESULTS

Changes in Uterine Cyclic Nucleotide Levels During the Estrus Cycle. Endogenous levels of uterine cyclic nucleotides were monitored in rats during various stages of the estrus cycle (Fig. 1). Cyclic GMP levels were essentially the same at metestrus, diestrus, and estrus but increased over three-fold ($P < 0.001$) during proestrus. In contrast, cyclic AMP levels at proestrus were significantly ($P < 0.05$) lower than at metestrus and diestrus. One hormonal characteristic of proestrus that distinguishes it from other stages of the cycle is that the circulating plasma levels of estrogen rise to their maximum during this period (21).

Effects of Estradiol-17β and Diethylstilbestrol Administration In Vivo. Uterine cyclic nucleotide levels were determined after a single injection of estradiol-17β (1 μg, ip) to 250-g ovariectomized rats (Fig. 2). Significant increases in cyclic GMP levels were seen 90 min (2-fold) and 120 min (3-fold) after estrogen treatment; at the same times, cyclic AMP concentrations decreased significantly (30 and 40%, respectively) below control values. At earlier time intervals (15–60 min), no significant alteration occurred in the level of either cyclic nucleotide.

In another series of experiments (Fig. 3), uteri, removed at different intervals after the injection of diethylstilbestrol (100 μg in oil, sc) to 200-g ovariectomized rats, were stabilized for a 30-min period in Munsick's medium before being frozen and prepared for cyclic nucleotide analysis. Under these conditions the levels of cyclic GMP were somewhat elevated (about 56%) at the earliest time periods tested (i.e., 30 and 60 min) after diethylstilbestrol injection, but statistically

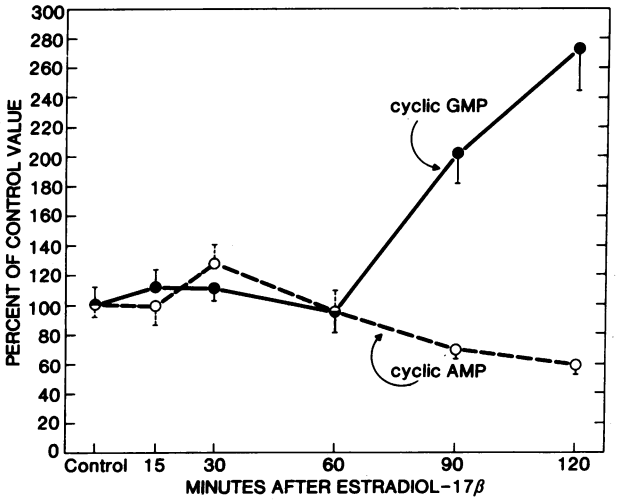

FIG. 2. Time course of changes in uterine cyclic GMP and cyclic AMP concentrations after estradiol-17β administration (1 μg, ip) to 250-g ovariectomized rats. The cyclic nucleotide levels are expressed as percentage of control values ($\bar{x} \pm$ SEM). Animals were killed at the times indicated after estradiol-17β administration, and cyclic nucleotide levels were determined by *Method 1*. Cyclic GMP levels increased significantly at 90 ($P < 0.01$) and 120 ($P < 0.001$) min; cyclic AMP levels decreased significantly at 90 ($P < 0.01$) and 120 ($P < 0.01$) min (two-tailed *t*-test).

significant increases were not recorded until 3 hr, when the concentration of cyclic GMP had more than doubled. At 12 and 24 hr after the single, relatively large dose of diethylstilbestrol, increases in cyclic GMP concentration of 4- and 8-fold, respectively, were found.

Small increases (15–30%) were also noted in uterine cyclic AMP levels after diethylstilbestrol treatment, which may

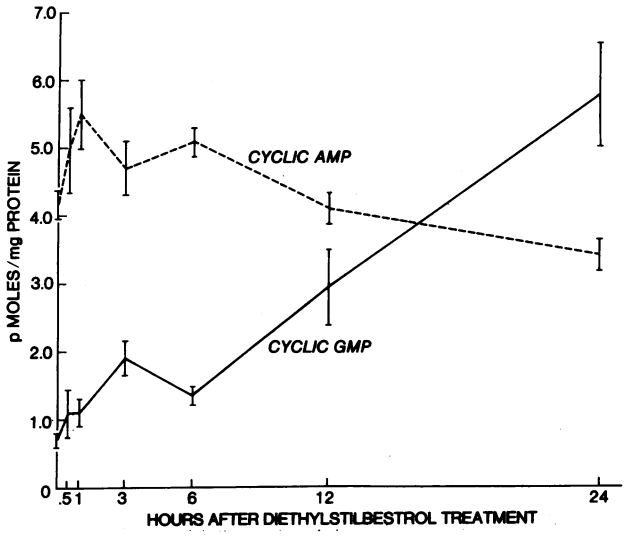

FIG. 3. Time course of changes in uterine cyclic GMP and cyclic AMP concentrations ($\bar{x} \pm$ SEM) after administration of diethylstilbestrol (100 μg in sesame oil, sc) to 200-g ovariectomized rats. Animals were killed at the times indicated, and cyclic nucleotide levels were determined by *Method 1*. Cyclic GMP levels increased significantly above controls at 3 ($P < 0.01$), 6 ($P < 0.01$), 12 ($P < 0.001$), and 24 ($P < 0.001$) hr; cyclic AMP concentrations were significantly higher at 1 hr ($P < 0.05$) and lower at 24 hr ($P < 0.05$) than control levels (two-tailed *t*-test).

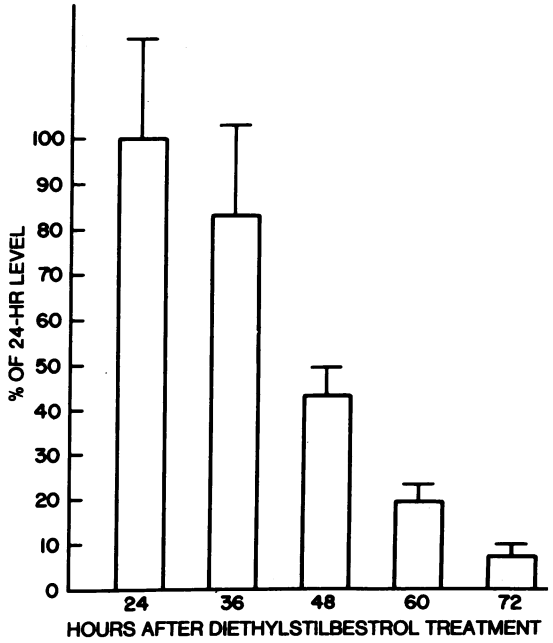

FIG. 4. The changes in uterine cyclic GMP levels after a single injection of diethylstilbestrol (100 μg, in oil, sc) to 200-g ovariectomized rats. The cyclic nucleotide levels are expressed as the percentage ($\bar{x} \pm$ SEM) of the maximum levels existing 24 hr after estrogen treatment. Cyclic nucleotide levels were determined by *Method 1*. The cyclic GMP levels at 60 and 72 hr are significantly decreased from the 24-hr value ($P < 0.02$; two-tailed *t*-test).

correspond to the rise in uterine cyclic AMP reported by Szego and Davis (7). The increase in uterine cyclic AMP was relatively transient compared to the changes observed in cyclic GMP concentration, and by 24 hr, when the levels of cyclic GMP were maximal, there was a significant decline (18%) in the uterine cyclic AMP.

In another experiment conducted under similar conditions (i.e., *in vivo* treatment with diethylstilbestrol followed by a 30-min equilibration incubation of the excised organ), the levels of uterine cyclic GMP were monitored from 24 to 72 hr after a single injection of diethylstilbestrol (100 μg, in oil, sc) (Fig. 4). The concentration of uterine cyclic GMP declined progressively to a level at 72 hr that was only 7% of the peak value at 24 hr.

In subsequent work, both the time course and the magnitude of the cyclic GMP response were found to be similar to those shown in Fig. 3 when the uteri were frozen immediately upon removal from the animal (without incubation) after diethylstilbestrol treatment (22). Calculation of the cyclic nucleotide levels on a wet weight rather than protein basis does not significantly modify the pattern of the cyclic GMP response in any of the experiments with diethylstilbestrol or estradiol-17β.

Effects of Estradiol Benzoate and Progesterone Administration In Vivo. Treatment of ovariectomized rats with estradiol benzoate (1 μg, in oil, sc) for 4 successive days before removal of the uteri for analysis (3–4 hr after the last injection) resulted in a greater than 2-fold increase ($P < 0.01$) in uterine cyclic GMP concentration compared to uteri from control animals (Table 1). The administration of progesterone alone (4 mg) for 4 successive days caused an apparent (but not

TABLE 1. *Effects of estradiol benzoate and/or progesterone on cyclic GMP levels in uteri of ovariectomized rats*

Substance administered	Weight of uterine horn (mg)	Cyclic GMP	
		pmoles/uterine horn	pmoles/mg of tissue
Control (solvent) (sc, 4 days)	83 ± 4 (8)	3.72 ± 0.56 (8)	0.044 ± 0.005 (8)
1 μg of Estradiol benzoate (sc, 4 days)	193 ± 6 (4)	20.6 ± 3.1 (4)	0.108 ± 0.018 (4)
4 mg of Progesterone (sc, 4 days)	111 ± 13 (4)	8.14 ± 2.45 (4)	0.072 ± 0.018 (4)
1 μg of Estradiol benzoate + 4 mg of progesterone (sc, 4 days)	128 ± 11 (4)	4.67 ± 0.22 (4)	0.037 ± 0.004 (4)

Significance of differences among values in column at extreme right: estradiol compared to control, $P < 0.01$; estradiol compared to estradiol + progesterone, $P < 0.01$. All other differences are not significant. Mean values ± SEM with number of observations in *parentheses*.

significant) increase (61%) in the levels of cyclic GMP. However, when progesterone was administered in combination with estradiol benzoate on the 4-day schedule, there was no measurable increase in uterine cyclic GMP levels as there had been when estrogen was administered alone ($P < 0.01$).

DISCUSSION

The observation that uterine cyclic GMP levels are elevated by the administration of estradiol or diethylstilbestrol to ovariectomized rats is consistent with the fluctuations of this cyclic nucleotide found to occur at different stages of the estrus cycle in the rat. Maximum concentrations of cyclic GMP were found during proestrus when estrogen levels reach their peak value (21). There also appears to be good agreement between the absolute (minimal and maximal) levels of cyclic GMP in the uterus of the cycling rat with those found in the ovariectomized rat and its estradiol benzoate-primed counterpart. It is of interest that the amounts of estrogen-induced protein and DNA, and the number of estrogen-receptor sites are also maximal at proestrus (23). It also appears that an inverse relation is demonstrable with regard to fluctuations in cyclic GMP and cyclic AMP levels in this organ; cyclic AMP levels become depressed when the levels of cyclic GMP increase toward maximum after diethylstilbestrol or estradiol administration or when the stage of proestrus is attained. These observations are consistent with the finding that uterine adenylate cyclase activity in the estrus-cycling rat is at its lowest value during proestrus (24).

The relationship between estradiol and progesterone action in the uterus is a complex one (25). While progesterone action is favored by prior administration of estrogen (an effect believed to stem, in part, from an estrogen-induced increase in the number of progesterone receptors) (26), certain actions of the two steroids are antagonistic (i.e., progesterone can inhibit the effect of estrogen to promote uterine growth and contractility) (27). The fact that the increases in uterine cyclic GMP concentration seen after estradiol benzoate administration were blocked when progesterone was administered with the estrogen, is consistent with the antagonistic roles of the two steroid hormones on growth and contractile function of the uterus. It is generally agreed that selectivity of steroid action is imparted by an initial interaction with specific cytosol receptors (28). It is not possible to conclude from the data presented whether the antagonism between estradiol and progesterone reflected by changes in cyclic GMP levels occurs as a result of an action at the cytosol receptor(s) or stems from a subsequent event related to estrogen and/or progesterone action. The delay of over an hour to achieve significant and maximal increases in uterine cyclic GMP after either estradiol-17β or diethylstilbestrol administration would appear to favor a response deriving from such a subsequent, estrogen-related event (i.e., protein or RNA synthesis). However, our more recent findings that estradiol (10 nM) is capable of raising cyclic GMP levels within 15 min *in vitro* in the rat uterus (22) and estrogen-responsive rat pituitary tumor cells (34) do not favor this interpretation. In addition, we have been able to show (22) that the estrogen-induced increases in uterine cyclic GMP levels occur primarily in the endometrial portion of the organ, which contains less than one-third of the total uterine cyclic GMP (in ovariectomized, nonestrogen-treated animals). Therefore, earlier increases that might occur primarily in the endometrial layer could be partially obscured by the larger pool of myometrial cyclic GMP that appears much less responsive (or unresponsive) to these effects of the hormone at the particular times tested.

By analogy with the action of agents that raise tissue cyclic AMP levels, it would seem reasonable to predict that estrogen may elicit its effect to elevate tissue cyclic GMP concentration by activating guanylate cyclase. Specific activities of guanylate cyclase in whole homogenates and soluble cellular fractions measured with optimal levels of GTP and Mn^{2+} were no different in uteri from estrogen-treated and untreated ovariectomized rats (29). The latter does not eliminate the possibility of an increase induced by estrogen in the rate of cyclic GMP biosynthesis in the intact cell, but indicates that there is probably no induction of guanylate cyclase specific activity. One change that was uncovered, which may account in part for the increase in uterine cyclic GMP levels, was an apparent decrease (40–50%) in the rate of phosphodiesterase-promoted hydrolysis of cyclic GMP. The latter was observed with low-speed supernatant fractions of uterine homogenates prepared from estrogenized animals when micromolar substrate levels of the cyclic nucleotide were used (30). The decrease in the rate of cyclic GMP hydrolysis by phosphodiesterase paralleled the time course of the increases found to occur in the tissue cyclic GMP concentration after diethylstilbestrol treatment.

Considering the numerous alterations induced by estrogen in uterine metabolism and the concepts now emerging with regard to the biological importance of cyclic GMP, what case can be made for an involvement of cyclic GMP in the expression of estrogen action? Perhaps most intriguing is the fact that a number of effects induced by estrogen in uterine tissue are analogous to alterations in cellular function that have recently been shown to be induced by other biologically active agents in association with an elevation of cellular cyclic

GMP concentration and/or by the addition of cyclic GMP (or derivatives) in several other biological systems. Examples of such cellular processes are the stimulation of cellular proliferation (2), lysosomal enzyme release (12, 13), transport of deoxyglucose and amino acids (31), and nuclear RNA synthesis (3). It has recently been shown that a number of agents that stimulate uterine contraction (acetylcholine, prostaglandin $F_{2\alpha}$, and serotonin) elevate levels of cyclic GMP in uterine (1, 3) and other smooth-muscle tissues (17). Again this would not be inconsistent with the known effect of estrogen to increase the spontaneous rhythmic contractile activity of the uterus and enhance the responsiveness of this organ to oxytocic agents. There is, however, no way of determining from the experiments reported here whether the increases in cyclic GMP levels actually result from a direct action of estrogen or an indirect one deriving from the formation and/or release of a neurohormonal substance. As pointed out above, several agents including prostaglandin $F_{2\alpha}$ have been shown to stimulate cyclic GMP accumulation in the uterus, and estrogens have been observed to promote the synthesis of F-prostaglandins in this tissue (32, 33).

It is evident from the present investigation that the action of estrogens on the rat uterus is associated with an increase in cyclic GMP levels. Recent studies by many investigators demonstrating that cyclic GMP may be an important biological regulatory agent make it attractive, therefore, to suggest that this nucleotide may play an important role in the expression of estrogen action. Further exploration in this area will be required before a definitive assessment can be made of the significance of the estrogen–cyclic GMP interrelationship.

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