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Early Involvement of Cyclic Nucleotides in the Artificially Stimulated Decidual Cell Reaction in the Mouse Uterus

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ABSTRACT

The role of cyclic nucleotides in triggering the decidual cell reaction (DCR) was investigated. All studies involved ovariectomized mice treated with estradiol and progesterone to prepare the uterus for implantation. The right uterine horn was injected intraluminally with chemicals or was physically traumatized. Cyclic AMP levels in the uterus increased slightly during the hormone priming schedule and markedly after the deciduogenic stimulus. Injection of 10 μl of sesame oil produced a 10-fold increase in cAMP in the right horn within 15 min. There was no change in the cAMP level in the left horn. Cyclic GMP levels increased 6-fold during the progesterone phase of the priming schedule and another 7-fold within 15 min after sesame oil injection. These changes in cGMP levels occurred in both uterine horns. Treatments found to produce a DCR were intraluminal injections of sesame oil, mineral oil, and cholera toxin (5 μg). Glycerol injection, followed by ligation of the uterus, and crushing of the uterine horn also produced a DCR. All of these treatments elevated the levels of cAMP in the right uterine horn. Sesame oil and mineral oil injections were the only deciduogenic treatments which altered cGMP levels in the uterus. These studies suggest that cAMP functions in triggering the uterine DCR and that cGMP does not.

INTRODUCTION

The artficially stimulated decidual cell reaction (DCR) is widely used as a model for the study of changes occurring in the uterus during implantation (Shelesnyak, 1957; DeFeo, 1967; Glasser and Clark, 1975). The transformation of uterine stromal cells to decidual cells can be stimulated in hormone treated ovariectomized mice by either trauma or intraluminal injection of chemical stimuli. Rapid proliferation of decidual cells does not begin until approximately 35 h after stimulation (Ledford et al., 1976). This growth occurs primarily in discrete masses and ultimately results in a 20-fold increase in uterine horn weight. The reaction of the uterus to artificial stimulation closely resembles uterine changes occurring during natural pregnancy. The major advantage of the artificially stimulated DCR is that it permits one to study events occurring within minutes after stimulation.

Cyclic nucleotides (cAMP and cGMP) mediate the action of several hormones and growth factors responsible for controlling cell differentiation and proliferation. Goldberg et al. (1975) have proposed that these two cyclic nucleotides act in opposition to control cell metabolism—the yin-yang hypothesis. A slight increase in cAMP levels has been observed by Leroy et al. (1974) in the traumatized rat uterus. The purpose of this investigation has been to study the involvement of cAMP and cGMP in the triggering of the decidual cell reaction.

MATERIALS AND METHODS

ICR mice (Flow Laboratories) weighing approximately 25 g were ovariectomized. One week after ovariectomy, animals were started on a hormone regimen developed by Miller and Emmens (1969). Three daily subcutaneous injections of 0.1 μg 17β-estradiol in 0.1 ml sesame oil were followed by two days of no treatment. Animals were then given daily subcutaneous injections of 6.7 ng 17β-estradiol and 1 mg progesterone in 0.1 ml sesame oil for the remainder of the experiment. Six hours after the third estradiol-progesterone injection, the right uterine horn was stimulated by trauma or a 10 μl intraluminal injection through the ovarian end of the uterine horn.

Animals were killed by cervical dislocation. Uterine horns were dissected out and immediately frozen on dry ice for cyclic nucleotide determinations. The tissue samples were kept frozen on dry ice until extraction. Individual horns were homogenized in 1 ml of cold 1 percent perchloric acid with a Polytron PCU-2 (Brinkman Instruments) at a setting of 5 for 10 seconds. Tracer amounts of ^{14}C-cAMP and ^{3}H-cGMP were included in the homogenization for the deter-

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mination of cyclic nucleotide recoveries. Homogenates were centrifuged at 12,000 *g* for 15 min at 4°C. The supernatants were neutralized with 6 N KOH and centrifuged at 12,000 *g* for 15 min. The second supernatants were applied to columns (25 × 6 mm) of Dowex AGl-X8 anion exchange resin (formate form) equilibrated with 0.1 N formic acid. The columns were washed with 10 ml of 0.1 N formic acid. The cAMP fraction was then eluted with 10 ml of 2 N formic acid, and the cGMP fraction eluted with 10 ml of 4 N formic acid. The two cyclic nucleotide fractions were collected and lyophilized.

Cyclic AMP was assayed by a modification of the competitive protein binding method of Gilman (1970). Binding protein was obtained for Cal Biochem, dissolved in 20 mM phosphate buffer, pH 6.0, and frozen in 2 ml aliquots. The binding reaction was allowed to proceed for at least 120 min at 4°C. Protein-bound cAMP was collected on Millipore filters (HAWP 2500, Millipore Corp.). The filters were then dissolved in 1 ml of methyl cellosolve. Ten milliliters of toluene based scintillation cocktail (5 g PPO/1, 0.4 g POPOP/1): methyl cellosolve (3:1) were added and samples were counted in a Packard liquid scintillation spectrometer.

Cyclic GMP was assayed using a radioimmunoassay kit obtained from Collaborative Research (Waltham, Mass.) according to the instructions provided by the manufacturer.

Decidualization of uterine horns was determined 72 h after stimulation. Horns stimulated with sesame oil increased in weight about five-fold by that time (Ledford et al., 1976). Occasionally, an injected horn showed a single deciduoma at the ovarian end. This was considered a result of trauma from the injection and was scored as negative. On the other hand, horns with several deciduomata along their length were scored as positive.

RESULTS

The specificity of the cyclic nucleotide assays and the efficiency of the extraction procedures were established for uterine tissue. The recoveries of cAMP and cGMP were consistently 65–85 percent and 50–60 percent respectively. Some uterine extracts were, in early studies, treated with phosphodiesterase to test the specificity of the competitive binding assays. Duplicate tissue samples were prepared; one was treated with cyclic nucleotide phosphodiesterase. Samples were then assayed for cAMP using the competitive protein binding assay. The samples that had been treated with phosphodiesterase had no measurable cAMP. An analogous experiment was done to test the specificity of the cGMP assay with similar results. The cross-reactivity of cAMP and cGMP in the opposite binding assays was investigated. Amounts of the opposite nucleotide up to 1000 times that encompassed by the standard curves did not exhibit any binding in the assays. In the cAMP binding assay, the standard curve was linear between 2 and 20 pmoles, and samples were diluted such that only the linear portion of the curve was used in determining the amount of cAMP present in the samples. The cGMP radioimmunoassay curve was linear between 0.5 and 10 pmoles and this was the region used for assays on unknowns. Tissue dilution curves of cAMP and cGMP fractions were run to assure linearity in the region of the normal assay dilution. The mean of replicate assays, agreeing within ± 5 percent, was used to calculate each value.

Cyclic AMP levels in uterine horns were measured daily during the priming regimen. The hormone injections were given at 9 a.m. daily and the animals killed at 3 p.m. for cyclic nucleotide determinations. The left and right uterine horns were assayed independently. Results are shown in Fig. 1. At the beginning of the priming regimen, each uterine horn contained approximately 60 pmoles of cAMP. During the priming with 17β-estradiol, the horn weights doubled and the total cAMP contents of each horn increased slightly. At 3 p.m. on the 8th day of priming, the right uterine horn was stimulated by the intraluminal injection of 10 μl of sesame oil. Fifteen minutes after

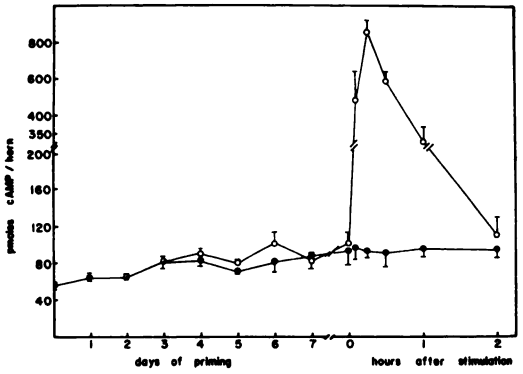

FIG. 1. Cyclic AMP levels in uterine horns during the hormone priming regimen and following sesame oil stimulation of the right uterine horn. On Days 1, 2 and 3 of priming, the animals received 0.1 μg of estradiol (s. c. in 0.1 ml sesame oil). On Days 6, 7 and 8 of priming, animals received 1 mg of progesterone and 6.7 ng of estradiol (s. c. in 0.1 ml sesame oil). On Day 8, an intraluminal injection of 10 μl of sesame oil was given into the right uterine horn. The left horn served as the untreated control. Each point represents the mean of at least 6 animals and bars represent the standard errors of the means. Right horn (○), left horn (●).

stimulation, the cAMP level in the stimulated horn had increased about ten-fold. The cAMP level in the unstimulated horn did not change. Two hours after stimulation, the cAMP level in the stimulated horn had returned to the unstimulated level.

Cyclic GMP levels were measured in the same uterine horns. The results are shown in Fig. 2. Prior to hormone treatment, cGMP levels fell within a range of 0.5 to 0.75 pmoles/horn. The estrogen phase of the priming schedule had no effect upon uterine cGMP levels. However, progesterone treatment resulted in a six-fold increase in uterine cGMP content. Following sesame oil stimulation of the right uterine horn, cGMP levels in both the stimulated and unstimulated horns increased to approximately 40 pmoles/horn within five minutes. The level of cGMP in both horns remained elevated for at least 2 h following stimulation.

The nature of the stimulus producing an alteration in uterine cyclic nucleotide levels and the DCR was investigated. Several possibilities existed including: 1) the hydrophobic interaction of the oil with the uterine membrane; 2) potential mitogenic or estrogenic activity of seed oils; 3) physical effects of the stimulus on the uterus. To test these possibilities, various putative stimuli were studied: 1) mineral oil, a purer, more chemically defined oil; 2) glycerol, a non-hydrophobic liquid with a viscosity similar to sesame oil; 3) dibutyryl cAMP; 4) cholera toxin, an activator of adenylate cyclases, 5) saline and 6) crushing the uterine horn with a hemostat, a physical trauma. Cyclic nucleotide levels were determined on uterine tissue collected 15 min after treatment. The absence or presence of a decidual cell reaction was noted 72 h after treatment.

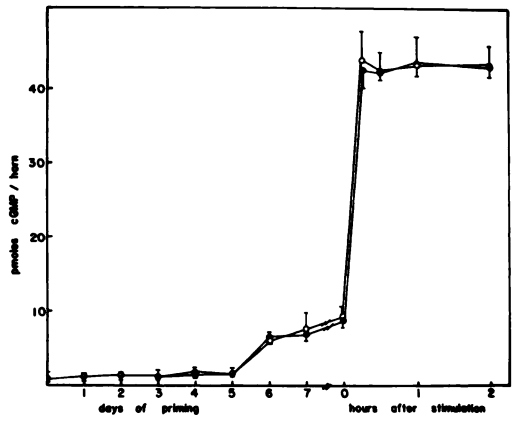

FIG. 2. Cyclic GMP levels in the uterine horns during the hormone priming regimen and following sesame oil stimulation of the right uterine horn. On Days 1, 2 and 3 of priming, the animals received 0.1 μg of estradiol (s. c. in 0.1 ml sesame oil). On Days 6, 7 and 8 of priming, animals received 1 mg of progesterone and 6.7 ng of estradiol (s. c. in 0.1 ml sesame oil). On Day 8, an intraluminal injection of 10 μl of sesame oil was given into the right uterine horn. The left horn served as the untreated control. Each point represents the mean of at least 6 animals and bars represent standard errors of the means. Right horn (○), left horn (●).

Two control experiments were performed to test the effects of anesthesia and surgical procedures on uterine cyclic nucleotide levels and decidualization of the uterus. One group of animals was only anesthetized. The other group was anesthetized and sham-operated. Neither group exhibited an increase in cyclic nucleotide levels or a DCR as shown in Table 1.

Intraluminal injection of saline produces a DCR in the rat uterus (DeFeo, 1967). Finn has reported, however, that saline is ineffective as a stimulus of the DCR in the mouse (1966). The effects of the saline on cyclic nucleotide levels and uterine decidualization in hormone primed mice are shown in Table 1. The cAMP level in the saline treated horn increased by approximately two-fold. There was no change in the cGMP level and no extensive DCR was observed.

A physical method of stimulating the DCR involves crushing the uterine horn in several places with a small hemostat. When the uterus was stimulated in this manner, the amount of cAMP tripled in the stimulated horn, and there was no change in cGMP (Table 1). A DCR was evident at each site of crushing but not throughout the horn as occurred with the oil stimulus.

The rapid changes in cAMP and cGMP levels following sesame oil stimulation of the DCR suggested an involvement of cyclic nucleotides in the triggering mechanism. This possibility was tested by intraluminal injection of dibutyryl cAMP (5mM) or cholera toxin (5μg) in saline. Dibutyryl cAMP was ineffective as a stimulus of the DCR (Table 1). Cholera toxin injected in the right horn, however, produced a massive DCR in both the right and left uterine horns. It also stimulated an increase in cAMP in the right horn comparable to that observed following sesame oil stimulation. Cholera toxin had no effect on uterine cGMP levels.

The possibility exists that substances in-

TABLE 1. The effect of different treatment on uterine decidualization and cyclic nucleotide levels.

Treatment	cAMP (pmoles/horn)		cGMP (pmoles/horn)		Decidualization[a]	
	Right	Left	Right	Left	Right	Left
Anesthesia	86 ± 11	83 ± 8	8.4 ± .4	8.1 ± .2	−	−
Sham operation	90 ± 12	92 ± 9	7.8 ± .4	8.0 ± .2	−	−
Sesame oil	882 ± 47	91 ± 12	45 ± 8	42 ± 5	+	−
Trauma (crushing)	284 ± 51	88 ± 5	8.7 ± .4	8.3 ± .2	+	−
Saline	198 ± 18	91 ± 10	9.2 ± .7	9.0 ± .4	−	−
Dibutyryl cAMP (5mM)	ND	ND	ND	ND	−	−
Cholera toxin (5 μg)	690 ± 82	79 ± 1	8.2 ± .6	8.3 ± .5	+	+
Sesame oil ligated	792 ± 51	87 ± 10	41 ± 7	40 ± 8	+	−
Glycerol	78 ± 9	83 ± 6	9.8 ± .8	9.4 ± .2	−	−
Glycerol ligated	188 ± 17	198 ± 22	8.5 ± .8	8.7 ± .8	+	+
Mineral oil	820 ± 71	92 ± 11	35 ± 14	39 ± 8	+	−

[a] + = positive DCR; − = negative DCR.

ND = values not determined.

Ovariectomized mice were hormone primed as outlined in Materials and Methods. Cyclic nucleotide levels were measured 15 min after treatment of the right uterine horn. Decidualization was scored 72 h after treatment. Cyclic nucleotide levels represent the mean from at least 6 animals per group ± SEM. Cholera toxin and dibutyryl cAMP (sodium salt) were made up in saline.

jected into the stimulated uterine horn may leak out into the peritoneal cavity and make contact with the unstimulated horn. This could account for the observed increase in cGMP levels in the left uterine horn following sesame oil stimulation of the right uterine horn. To eliminate this type of effect, the ovarian end of the stimulated horn was ligated after injection. As shown in Table 1, the cGMP levels were increased in both uterine horns indicating that leakage was not responsible for cGMP increases in the unstimulated horn.

Glycerol was tested as a potential stimulator of the DCR since it is a liquid with a viscosity similar to that of sesame oil, but is not hydrophobic. Table 1 shows that glycerol is ineffective in triggering a DCR or an increase in cyclic nucleotide levels when injected in the usual manner. However, when the uterine horn was ligated after glycerol injection, a DCR was observed which involved both uterine horns. This DCR was accompanied by increased cAMP levels in both horns. No change in cGMP levels was observed.

In attempting to understand the nature of the oil stimulus producing the DCR, more chemically defined oils were tested. Intraluminal injection of triolein produced a DCR comparable to that observed with sesame oil injection. Intraluminal injection of mineral oil produced a DCR and cyclic nucleotide response similar to that obtained with sesame oil (Table 1). Subsequent experiments, however, have demonstrated that the extent of the mineral oil stimulated DCR is more variable than the sesame oil stimulated DCR. Nevertheless, the involvement of undefined substances of plant origin (mitogens, estrogens, etc.) has been eliminated by these experiments.

DISCUSSION

A very early event in the uterus following sesame oil stimulation of the DCR is a 10-fold increase in the level of cAMP in the stimulated horn. This increase in cAMP is accompanied by a smaller increase in cGMP levels; however, increased levels of cGMP are observed in both the stimulated and unstimulated uterine horns. Leroy et al. have demonstrated a 1.6-fold increase in cAMP following a trauma induced DCR in the rat (1974). It was suggested that this small increase resulted from highly localized effects which were obscured when averaged over the entire uterus. Direct comparison of cyclic nucleotide levels following sesame oil and trauma stimulation of the DCR shows (Table 1) a smaller increase in cAMP associated

with trauma stimulation. It should also be noted that a less extensive DCR is achieved with trauma stimulation.

The coupling of the cAMP response with the subsequent DCR is suggested by establishing a correlation between deciduogenic stimuli and increased cAMP levels. As shown in Table 1 treatments producing a DCR are sesame oil, mineral oil, trauma, cholera toxin and glycerol (ligated). All of these stimuli also cause an increase in uterine cAMP levels. Conversely, neither glycerol nor sham operation produces a DCR or an alteration in cAMP levels. Saline is ineffective as a stimulus of the DCR, but does result in a slight increase in cAMP levels. This increase is probably not localized and is not sufficient to trigger a DCR.

If cAMP is involved in triggering the DCR, it should be possible to produce a DCR by intraluminal injection of cAMP or a cAMP effector. As seen in Table 1, intraluminal injection of dibutyryl cAMP does not produce a DCR. As in the case of saline, cAMP levels in responsive cells may not have been sufficiently elevated to trigger a DCR. Injection of cholera toxin, however, increases cAMP levels in the injected horn and produces a massive DCR involving both uterine horns. These latter data suggest a possible dissociation of the DCR and the cAMP response. Levels of cAMP were measured 15 min after injection of cholera toxin, and the DCR was scored 72 h later. Twenty-four hours after injection of trypan blue (in saline) into the right uterine horn, dye was evident in the left uterine horn (data not shown). Thus, the bilateral DCR observed following cholera toxin injection probably resulted from diffusion of the toxin into the left uterine horn, which might have caused a delayed increase in cAMP levels.

Increased uterine levels of cGMP observed following stimulation of the DCR with oils appear to be unique to this mode of stimulation. No other means of stimulating the DCR affected uterine cGMP levels. Additionally, the oil stimulation of cGMP levels occurred in both the stimulated and the unstimulated horns. These data suggest that cGMP is not involved in triggering the DCR. The mechanism by which the stimulus is transmitted to the contralateral horn is still unclear. Ligation of the ovarian end of the stimulated horn eliminated diffusion of oil through the peritoneal cavity as an explanation for increased cGMP levels in the unstimulated horn. Untested possibilities include transmission via the nervous system, the circulatory system, or diffusion of the oil through the cervical end of the stimulated horn.

An attempt has been made to understand the nature of the oil stimulus triggering the DCR. Physical manipulation and simple distention of the uterus do not result in a DCR since neither saline nor glycerol was effective as a stimulus. However, ligation of the uterus following unilateral injection of glycerol resulted in a bilateral DCR. This phenomenon is perhaps a consequence of extreme distention of the uterus. The distention is due to hypertonicity of the glycerol and accompanied by a bilateral increase in cAMP levels. Oil stimulation on the other hand appears to be a hydrophobic effect since pure oils such as triolein are effective stimuli. The effect of a hydrophobic stimulus on the luminal epithelium is to activate adenylate cyclase and guanylate cyclase directly or indirectly. Sufficient activation of adenylate cyclase then results in levels of cAMP which trigger a DCR. Cyclic AMP is known to alter cell metabolism by the activation of protein kinases which phosphorylate cellular proteins (Sutherland, 1972). The phosphorylation of chromatin proteins may be responsible for the transformation of the uterine stromal cells to decidual cells.

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REFERENCES

DeFeo, V. J. (1967). Decidualization. *In* "Cellular Biology of the Uterus" (R. M. Wynn, ed.), pp. 191-290, North Holland Publishing Company, Amsterdam.

Finn, C. A. (1966). Endocrine control of endometrial sensitivity during the induction of the decidual cell reaction in the mouse. J. Endocr. 36, 239-248.

Finn, C. A. and Martin, L. (1972). Endocrine control of the timing of endometrial sensitivity to a decidual stimulus. Biol. Reprod. 1, 82-86.

Gilman, A. E. (1970). A protein binding assay for adenosine 3′5′-cyclic monophosphate. Proc. Natl. Acad. Sci. 67, 305-312.

Glasser, S. R. and Clark, J. H. (1975). A determinant role for progesterone in the development of uterine sensitivity and ovo-implantation. *In* "The Developmental Biology of Reproduction" (C. L. Markert and J. Papaconstantinou, eds.), pp. 311-345, Academic Press, Inc., New York.

Goldberg, N. D., Haddox, M. K., Dunham, E., Lopez, C. and Hadden, J. W. (1974). The Yin-Yang hypothesis of biological control: opposing influences of cyclic GMP and cyclic AMP in the

regulation of cell proliferation and other biological processes. *In* "Control of Proliferation in Animal Cells" (B. Clarkson and R. Baserga, eds.), pp. 609-626, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Ledford, B. E., Rankin, J. C., Markward, R. R. and Baggett, B. (1976). Biochemical and morphological changes following artificially stimulated decidualization in the mouse uterus. Biol. Reprod. 15, 529-535.

Leroy, F., Vansande, J., Shetgen, G. and Brasseur, D. (1974). Cyclic AMP and the triggering of the decidual reaction. J. Repro. Fert. 39, 207-211.

Miller, B. G. and Emmens, C. W. (1969). The effects of oestradiol and progesterone on the incorporation of tritiated uridine into the genital tract of the mouse. J. Endocr. 43, 427-436.

Shelesnyak, M. C. (1957). Some experimental studies on the mechanism of ova-implantation in the rat. Recent Prog. Hormone Res. 13, 269-322.

Steiner, A. L., Parker, C. W. and Kipnis, P. W. (1972). Radioimmunoassay for cyclic nucleotides. I. Preparation of antibodies and iodinated cyclic nucleotides. J. Biol. Chem. 247, 1106-1113.

Sutherland, E. W. (1972). Studies on the mechanism of hormone action. Science 177, 401-408.

RECOMMENDED REVIEWS

DeFeo, V. J. (1967). Decidualization. *In* "Cellular Biology of the Uterus" (R. M. Wynn, ed.), pp. 191-290, North Holland Publishing Company, Amsterdam.

Glasser, S. R. and Clark, J. H. (1975). A determinant role for progesterone in the development of uterine sensitivity to decidualization and ovo-implantation. *In* "The Developmental Biology of Reproduction" (C. L. Markert and J. Papconstantinou, eds.), pp. 311-345, Academic Press, Inc., New York.