Inhibition of gonadotrophin-induced ovulation in rabbits by perfusion with dibutyryl cAMP via reduction of ovarian prostaglandin production

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The role of cAMP in ovulation, oocyte maturation and prostaglandin production was assessed using a rabbit ovary preparation perfused in vitro. Dibutyryl cAMP (10⁻³, 10⁻⁴ or 10⁻⁵ mol l⁻¹) was added to the perfusate of one ovary. The contralateral, control ovary was perfused with medium alone. Thirty minutes after the onset of perfusion, 50 IU hCG was added to the perfusate of all ovaries. Dibutyryl cAMP inhibited hCG-induced ovulation in a dose-related fashion. No difference in ovum maturity or degeneration was found between control ovaries and ovaries treated with dibutyryl cAMP. Ovarian progesterone production was not affected by exposure to dibutyryl cAMP. The concentrations of 6-keto PGF₁α (the stable metabolite of prostacyclin) and PGF₂α in the perfusate of ovaries treated with dibutyryl cAMP were 49.6% and 32.0% of the control values, respectively, 12 h after hCG administration. Inhibition of 6-keto PGF₁α production by dibutyryl cAMP was dose related. Production of PGE₂ was unaffected by dibutyryl cAMP. These data raise the possibility that continuous exposure to dibutyryl cAMP may inhibit hCG-induced ovulation in the perfused rabbit ovary via a reduction in PGF₂α and prostacyclin production.

Introduction

During the growth of the mammalian ovarian follicle, gonadotrophin stimulation initiates a cascade of events including steroidogenesis and resumption of oocyte meiosis, leading to rupture of the follicle and release of a mature ovum (for reviews, see Thibault, 1977; Richards, 1980; Espey, 1980; Yoshimura and Wallach, 1987). Gonadotrophic stimulation affects steroidogenesis, receptor development and mitosis in target cells, as well as meiotic maturation of oocytes. cAMP acts as an intracellular second messenger in many of the affected processes (Richards, 1980; Dekel and Beers, 1980; Amsterdam et al., 1981; Knecht and Catt, 1982; Boynton and Whitfield, 1983). Forskolin, an activator of adenylyl cyclase, induces ovulation in vitro in the absence of gonadotrophin (Holmes et al., 1984), implying that cAMP production by the preovulatory follicle may be sufficient to initiate the ovulatory process. Ryan and Coronel (1969) observed that daily administration of cAMP prevented ovulation and pregnancy in mice. Intrafollicular injection of cAMP inhibits LH-induced luteinization of rabbit follicles (LeMaire et al., 1972), supporting the concept of an inhibitory effect of cAMP on reproductive function.

In the rabbit ovary that is perfused in vitro, ovulation occurs consistently in response to the addition of hCG to the perfusate (Yoshimura and Wallach, 1987; Yoshimura et al., 1987). Furthermore, ova that ovulate in vitro can be fertilized in vitro and after transfer to the Fallopian tube can establish normal pregnancies in the adequately prepared host rabbit (Kobayashi et al., 1983). Such observations establish the suitability of this model for studying ovum maturation as well as the ovulatory process under various experimental conditions. Since the direct involvement of cAMP during the periovulatory interval remains controversial, the present study was conducted to determine the effects of exogenous cAMP on hCG-induced ovulation, oocyte maturation and prostaglandin production using an isolated rabbit ovary preparation perfused in vitro.

Materials and Methods

Animals

Sexually mature New Zealand White rabbits were isolated for a minimum of 3 weeks under controlled light and temperature, and given free access to Purina rabbit chow (Ralston-Purina, St Louis, MO) and water. Rabbits weighing

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at least 3.5 kg were anaesthetized with pentobarbitone sodium i.v. (32 mg kg⁻¹), given heparin sulfate (120 U kg⁻¹ for anticoagulation) and subjected to laparotomy. Ovaries were excluded from further study if they appeared immature or if 50% or more of the surface follicles were haemorrhagic.

Ovarian perfusion

The ovarian artery was dissected free from surrounding adipose tissue, peritoneum and adventitia, and was cannulated in situ. Each cannulated ovary with supportive adipose tissue was then removed from the rabbit and immediately placed in a perfusion chamber. Ovaries were perfused with 150 ml of Medium 199 (MA Bioproducts, Walkersville, MD) supplemented with 200 U heparin sulfate 1⁻¹, 20 U insulin 1⁻¹, 75 mg penicillin G 1⁻¹, and 50 mg streptomycin 1⁻¹. The perfusion technique and surgical procedure, including arterial cannulation and ovarian removal, was described in detail by Lambertsen et al. (1976).

Experimental design

Ovaries from six rabbits were used to study the effects of each of three concentrations of dibutyryl cAMP. One ovary from each rabbit was perfused with 10⁻⁵, 10⁻⁴ or 10⁻⁵ mol dibutyryl cAMP 1⁻¹ (Sigma Chemical Co, St Louis, MO). In each case the contralateral ovary, which served as a control, was simultaneously perfused with medium alone. Thirty minutes after the onset of perfusion, 50 U of hCG (Organon Inc, West Orange, NJ) was added (time 0), and both ovaries were perfused for 12 h thereafter. Perfusate samples (2 ml) were withdrawn at zero and at 1, 2, 4, 6, 8 and 12 h after administration of hCG, and were replaced immediately by an equal volume of fresh medium with or without dibutyryl cAMP. The samples were stored at −20°C until measurement of progesterone and prostaglandin concentrations by radioimmunoassay.

Time of ovulation, ovulatory efficiency and ovum characteristics

Ovaries were observed every 15 min for evidence of follicle growth and rupture. The ovulated ovum surrounded by its cumulus mass was carefully recovered from the ovarian surface at the time of follicle rupture. The interval from hCG administration to follicle rupture was recorded. The mean time of ovulation was calculated for each group. After 12 h exposure to hCG, follicular oocytes were recovered by aspiration from mature follicles (≥ 1.5 mm in diameter) and the experiment was concluded. Ovulatory efficiency, the percentage of mature follicles (≥ 1.5 mm) that proceed to rupture during ovarian perfusion, was calculated for each group. Both ovulated ova and follicular oocytes were assessed for stage of maturity and signs of degeneration as described by Yoshimura et al. (1990). The degree of ovum maturity was expressed as the percentage of ovum achieving germinal vesicle breakdown. Degenerative changes include vacuolation, cytolysis, necrosis and loss of spherical shape.

Radioimmunoassay

Progesterone was measured using a commercial radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, CA), in which the progesterone antibody is bound covalently to the inner surface of polypropylene assay tubes (Kubasik et al., 1984). The interassay and intra-assay coefficients of variation were 6.8% and 2.3%, respectively.

The concentrations of PGF₂α, PGE₂, prostacyclin (PGI₂) and thromboxane B₂ (TXB₂) released into the perfusate were measured by the methods described by Dubin et al. (1979, 1982). The amount of PGI₂ was determined by measurement of the stable endproduct, 6-keto PGF₁α. Standard prostanooid solutions were prepared in perfusion medium containing hCG, dibutyryl cAMP or both at concentrations used in the experiments. The curves generated were superimposable on curves generated in medium alone. However, for PGE₂ determination there was a significant increase in nonspecific radioactivity attributable to the presence of dibutyryl cAMP at a concentration of 10⁻³ mol 1⁻¹. Thus, PGE₂ concentration in the perfusate treated with dibutyryl cAMP at 10⁻³ mol 1⁻¹ was calculated using the appropriate nonspecific radioactivity. PGF₂α and PGE₂ antibodies each crossreact significantly with one double-bonded series of their classes, but less than 2% with other closely related prostaglandins. The 6-keto PGF₁α or TXB₂ antibody crossreacts less than 0.1% with the primary prosta glandin. For each prostanooid measured the intra-assay coefficient of variation was less than 9% and samples from a given experiment were assayed together to preclude error due to interassay variation.

Table 1. The effects of dibutyryl cAMP on hCG-induced ovulation in the perfused rabbit ovary

<table>
<thead>
<tr>
<th>Parameter</th>
<th>10⁻³</th>
<th>10⁻⁴</th>
<th>10⁻⁵</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of ovaries</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>18</td>
</tr>
<tr>
<td>Number of ovulating ovaries</td>
<td>2</td>
<td>6</td>
<td>6</td>
<td>18</td>
</tr>
<tr>
<td>Ovulations per ovulating ovary*</td>
<td>1.50 ± 0.50*</td>
<td>3.00 ± 0.86*</td>
<td>5.00 ± 0.58</td>
<td>6.39 ± 0.39</td>
</tr>
<tr>
<td>Time of ovulation (h)</td>
<td>7.61 ± 0.70</td>
<td>6.98 ± 0.62</td>
<td>7.53 ± 0.39</td>
<td>7.26 ± 0.18</td>
</tr>
</tbody>
</table>

*Values given are means ± SEM.

*Significantly different from control (P < 0.001).
Fig. 1. Ovulatory efficiency (mean ± SEM) in perfused rabbit ovaries treated with 50 iu hCG alone (□) or with 50 iu hCG plus dibutyryl cAMP (■). Ovulatory efficiency is the percentage of mature follicles (≥ 15 mm) that ruptured during perfusion. Asterisks indicate values that differ significantly from hCG-treated control ovaries (*P < 0.05, **P < 0.001).

Statistical analysis

Data for ovulations per ovulating ovary, time of ovulation, and the concentrations of progesterone and prostaglandin are presented as means ± SEM. Statistical analysis was performed by Student's t test for two categories and by analysis of variance for three categories followed by the Scheffé test. The χ² test with Yates' correction was used to assess the significance of differences in degeneration and maturation of follicular oocytes and ovulated ova. Differences with P < 0.05 were considered significant.

Results

Effects of dibutyryl cAMP on hCG-induced oocyte maturation and ovulation

All ovaries treated with hCG alone or with hCG plus dibutyryl cAMP at a concentration of 10⁻⁴ or 10⁻⁵ mol l⁻¹ ovulated (Table 1). The mean number of ovulations per ovulating ovary was significantly lower in ovaries perfused with dibutyryl cAMP at 10⁻³ or 10⁻⁴ mol l⁻¹, but time of ovulation was not affected. Ovulatory efficiency was significantly reduced by dibutyryl cAMP (Fig. 1), and inhibition of ovulation was related to dose.

Stage of maturation and degeneration of ovulated ova and follicular oocytes are summarized in Table 2. There was no significant difference in the percentage of ovulated ova demonstrating germinal vesicle breakdown. However, the percentage of follicular oocytes achieving germinal vesicle breakdown was significantly reduced by 10⁻³ mol dibutyryl cAMP l⁻¹. The percentage of ovulated ova and follicular oocytes that showed evidence of degeneration was comparable.

Fig. 2. Concentration of progesterone in the perfusate during perfusion of rabbit ovaries with medium alone (○), 50 iu hCG (●), 10⁻³ mol dibutyryl cAMP l⁻¹ (□), or 50 iu hCG plus 10⁻³ mol dibutyryl cAMP l⁻¹ (■).

Progesterone production by the perfused rabbit ovary

Progesterone concentrations in the perfusate increased within 1 h of hCG administration (Fig. 2), reached a maximum by 4 h, and thereafter declined. The presence of dibutyryl cAMP had no effect on progesterone production by hCG-treated ovaries.

Prostaglandin production by the perfused rabbit ovary

After exposure to hCG, the concentration of 6-keto PGF₁α in the perfusate increased steadily throughout the entire perfusion period (Fig. 3). Six hours after hCG administration, the production of 6-keto PGF₁α was significantly lower in ovaries treated with dibutyryl cAMP. The concentration of 6-keto PGF₁α in the perfusate of ovaries treated with dibutyryl cAMP was 49.6% of the control value 12 h after hCG administration (P < 0.001). In contrast, during the first 2 h after hCG exposure, PGE₂ and PGF₂α concentrations in the perfusate did not change in control ovaries or in those treated with dibutyryl cAMP (Fig. 3). Thereafter, PGE₂ and PGF₂α increased steadily in response to hCG administration. PGF₂α concentration in the perfusate of ovaries treated with dibutyryl cAMP was 32.0% of the control value 12 h after hCG administration. This difference was significant (P < 0.05). However, the production of both PGE₂ and TBX₂ following hCG administration was not affected by continuous exposure to dibutyryl cAMP (Fig. 3).

The concentration of 6-keto PGF₂α in the perfusate of ovaries treated with the three concentrations of dibutyryl cAMP was determined 12 h after hCG administration (Fig. 4). The addition of dibutyryl cAMP to the perfusate resulted in a dose-related inhibition in hCG-stimulated 6-keto PGF₁α production. However, the concentrations of PGE₂ and PGF₂α in the perfusate of ovaries treated with dibutyryl cAMP at 10⁻⁴ or 10⁻⁵ mol l⁻¹ did not differ significantly from those observed in the contralateral control ovaries.
Table 2. Effect of dibutyryl cAMP on maturation and degeneration of ovulated ova and follicular oocytes in the perfused rabbit ovary

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dibutyryl cAMP (mol 1⁻¹)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10⁻³</td>
<td>10⁻⁴</td>
<td>10⁻⁵</td>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>Ovulated ova</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total number</td>
<td>3</td>
<td>18</td>
<td>30</td>
<td>115</td>
<td></td>
</tr>
<tr>
<td>% GVBD</td>
<td>100</td>
<td>72.2</td>
<td>96.7</td>
<td>90.4</td>
<td></td>
</tr>
<tr>
<td>% Degeneration</td>
<td>33.3</td>
<td>27.8</td>
<td>10.0</td>
<td>14.8</td>
<td></td>
</tr>
<tr>
<td>Follicular oocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total number</td>
<td>35</td>
<td>28</td>
<td>10</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>% GVBD</td>
<td>45.7*</td>
<td>82.1</td>
<td>100</td>
<td>87</td>
<td></td>
</tr>
<tr>
<td>% Degeneration</td>
<td>25.7</td>
<td>35.7</td>
<td>10.0</td>
<td>26.1</td>
<td></td>
</tr>
</tbody>
</table>

GVBD: germinal vesicle breakdown.
*Significantly different from control (P < 0.005).

Discussion

The present study demonstrates that continuous exposure to dibutyryl cAMP reduces hCG-induced ovulation in the rabbit ovary perfused in vitro in a dose-dependent manner, without affecting steroidogenesis. The effects of hCG and dibutyryl cAMP on progesterone production are not additive. These results are in accord with the data of Mills (1975), which indicated that incubation of follicles with LH and cAMP yields steroid concentrations similar to those obtained following incubation with either agent alone. The present data are consistent with the earlier observation of Ryan and Coronel (1969) that the daily systemic injection of mice with cAMP for prolonged periods results in a marked reduction in the size of ovaries and prevents ovulation. Furthermore, the injection of cAMP directly into the Graafian follicles of rabbits in oestrus inhibits the morphological luteinization induced by LH (LeMaire et al., 1972). Marsh et al. (1973) have also reported that injection of hCG in vivo results in decreased follicular synthesis of cAMP in response to LH in vitro. This decline is observed 30 min after hCG administration in vivo and continues up to the time of ovulation. We found that addition of hCG to the rabbit ovary perfusate significantly increased the intrafollicular cAMP concentration within 30 min (Yoshimura et al., 1992b). Follicular cAMP concentration reached a maximum after 1 h and then decreased abruptly. These data, in conjunction with the present observations of the direct inhibitory effects of dibutyryl cAMP in the process of ovulation, suggest that a decline in cAMP accumulation is causally related to the process of ovulation.

Forskolin has been reported to induce ovulation in the absence of gonadotrophin by others using a similar rabbit ovary system perfused in vitro (Holmes et al., 1986). Observations supporting this finding have also been made in a rat ovary perfusion system (Brannström et al., 1987). Since forskolin activates the catalytic subunit of adenyl cyclase and thereby increases intracellular cAMP concentrations (Seamon et al., 1981), these data suggest that cAMP may induce a metabolic pathway leading to ovulation. Although the release of cAMP was greater after treatment with forskolin than with LH, ovulatory efficiency was lower in the forskolin-treated

Fig. 3. Concentrations of the prostaglandins 6-keto PGF₁α, PGF₂α, PGE₁, and thromboxane B₂ (TXB₂) in the perfusate during perfusion of rabbit ovaries in the presence of hCG and medium alone (○) or 10⁻³mol dibutyryl cAMP 1⁻¹ (●) over time (mean ± se). Asterisks indicate a significant difference from control ovaries (*P < 0.05, **P < 0.001).
ovaries. These observations suggest that LH may induce ovulation by mechanisms other than those mediated by cAMP. In contrast, we found that continuous or transient exposure to dibutyryl cAMP failed to induce ovulation in the absence of gonadotrophin (Hosoi et al., 1989). Furthermore, we could not induce ovulation by ovarian perfusion with forskolin at $10^{-6}$–$10^{-4}$ mol l$^{-1}$ (Yoshimura et al., 1992b). Although forskolin treatment stimulated a greater increase in cAMP than did hCG treatment, forskolin did not induce ovulation. This finding suggests either that there is cell compartmental specificity for these treatments or that forskolin stimulates different types of cell from hCG. The discrepancy between the present data and those of others remains to be resolved. Although it is possible that cAMP is not an essential component of the ovulatory process, it is also possible, as suggested by Holmes et al. (1986), that the location of the release of cAMP within the cell is critical. Binding of gonadotrophin to its receptor may release cAMP at a location that stimulates specific protein kinases that are critical for follicle rupture. In contrast, perfusion with dibutyryl cAMP or forskolin may not achieve the specific compartmental concentration of cAMP needed to activate these kinases.

These experiments demonstrate that the addition of hCG to the perfusate significantly increases prostaglandin production by the perfused rabbit ovary. PGF$\text{1}_\alpha$, as reflected by 6-keto PGE$\text{2}$, production, was the major prostaglandin produced by rabbit ovaries in response to gonadotrophin administration. Various ovarian tissues including rat granulosa cells (Koo and Clark, 1982), rabbit preovulatory follicles (Yamada et al., 1991), porcine preovulatory follicles (Evans et al., 1983), and bovine corpus luteum (Milvae and Hansel, 1983) have also been shown to synthesize PGF$\text{1}_\alpha$ as the predominant prostaglandin. Production of PGE$\text{2}$ and PGF$\text{2}_\alpha$ is less than half that of 6-keto PGE$\text{2}$ and PGF$\text{1}_\alpha$ production. Previous studies have emphasized the importance of prostaglandins in the process of ovulation (for reviews, see Armstrong, 1981; Yoshimura and Wallach, 1987). The observation that continuous exposure to dibutyryl cAMP significantly inhibits both hCG-induced ovulation and prostaglandin production in rabbit ovaries could therefore be explained by a reduction in prostaglandin production. Alternatively, the decline in cAMP concentrations within the preovulatory follicle may be an important prerequisite for prostaglandin accumulation in the process of ovulation.

Kita et al. (1985) described alterations in the microvasculature of the rabbit ovarian follicle during the process of ovulation. Characteristic changes include vasodilation, extravasation caused by hyperpermeability, and filling defects at the perilisal region of the preovulatory follicle. These observations indicate that follicle rupture may be achieved through vasodilation and increased vascular permeability within the wall of the preovulatory follicle, regardless of the stimulus. PGF$\text{2}_\alpha$ is a more potent vasodilator than are other prostaglandins, and also acts as a chemical inflammatory mediator, as does PGE$\text{2}$ (Ford-Hutchinson et al., 1980; Moncada and Vane, 1977). Data derived from rat granulosa cell culture suggest that PGF$\text{2}_\alpha$ may serve as a mediator of preovulatory ovarian function (Koo and Clark, 1982). Although circumstantial evidence has been provided for the involvement of PGF$\text{2}_\alpha$ in the process of ovulation, the instability of this compound has limited any experimentation designed to investigate its role in ovarian physiology (Dusting et al., 1978). We demonstrated that PGF$\text{2}_\alpha$ methyl ester, with its longer half-life (2.7 h at pH 7.4), induces ovulation in vitro in the absence of gonadotrophin (Yoshimura et al., 1989). Furthermore, the vascular changes in PGF$\text{2}_\alpha$-treated ovaries parallel those observed when ovaries are exposed to gonadotrophin both in situ and in vitro (Kita et al., 1985; Yoshimura et al., 1988). These data suggest that PGF$\text{2}_\alpha$ may be significant in provoking perifollicular vascular changes during the process of ovulation.

The present data provide additional support for a significant role for PGF$\text{2}_\alpha$ in the ovulatory process. First, the amount of PGF$\text{2}_\alpha$ produced by rabbit ovaries in response to hCG stimulation is greater than that of either PGE$\text{2}$ or PGF$\text{2}_\alpha$. Second, the reduction of PGF$\text{2}_\alpha$ production in ovaries treated with dibutyryl cAMP can be observed 6 h after hCG exposure, which corresponds to the time of the first ovulation in vitro. Finally, dibutyryl cAMP inhibits both hCG-stimulated PGF$\text{2}_\alpha$ production to a greater extent than PGE$\text{2}_\alpha$ or PGE$\text{2}$ and hCG-induced ovulation in a dose-dependent manner. These findings raise the possibility that synthesis of PGF$\text{2}_\alpha$ in the preovulatory follicle in response to gonadotrophin exposure may be essential for the process of follicle rupture.

Cyclic AMP plays a significant role in the intrafollicular arrest of meiotic resumption (Dekel and Beers, 1978, 1980; Hosoi et al., 1989; Yoshimura et al., 1992a, b). Treatment of cumulus-enclosed oocytes with dibutyryl cAMP results in a reversible block of spontaneous maturation (Dekel and Beers, 1978, 1980). In the rabbit ovary perfused in vitro, continuous exposure to dibutyryl cAMP did not affect maturation of ova ovulated in response to hCG. This finding is consistent with the data reported by Dekel and Beers (1978) demonstrating that the inhibitory effect of cAMP on maturation of cumulus-enclosed oocytes can be overcome by addition of gonadotrophins.
In conclusion, continuous exposure to dibutyryl cAMP significantly inhibits hCG-induced ovulation in the perfused rabbit ovary. The inhibitory effect of cAMP on hCG-induced ovulation appears to be mediated via a reduction in 6-keto PGF$_{1\alpha}$ or PGF$_{2\alpha}$ production or both by rabbit ovaries. The present study raises the possibility that PGF$_2\alpha$ as well as PGF$_{2\alpha}$ may be involved in the process of ovulation.

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