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Rat oocyte maturation *in vitro*: Relief of cyclic AMP inhibition by gonadotropins

(dictyate/meiosis/cumulus-oocyte complex/inhibitors of cyclic nucleotide phosphodiesterase)

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ABSTRACT The hormone-independent, spontaneous maturation that rat oocytes undergo *in vitro* can be inhibited by derivatives of cyclic AMP and inhibitors of cyclic nucleotide phosphodiesterase. In this study, we have shown that this inhibition of maturation can be partially relieved by preparations of ovine and rat luteinizing hormone or follicle-stimulating hormone. The ability of gonadotropins to foster the resumption of maturation in cultures of cyclic AMP-inhibited oocytes suggests that this system is suitable for studies of the hormonal control of oocyte development. The dose and time dependency of the response to gonadotropins has been examined in order to study the role of these hormones in oocyte maturation and to compare this effect to other known responses of the cumulus–oocyte complex. These studies show that highly purified preparations of rat gonadotropins are less effective inducers of maturation than the more commonly used, but considerably less purified, preparations of ovine gonadotropins. Almost complete relief of inhibition is observed, however, when the oocytes are exposed to a combination of rat luteinizing hormone and follicle-stimulating hormone. Oocyte maturation was not influenced by the sex steroids progesterone or 17β-estradiol. Our results suggest that: (*i*) cyclic AMP is involved in the intrafollicular inhibition of oocyte maturation; (*ii*) both gonadotropins are required for maximal stimulation of the resumption of oocyte meiosis; and (*iii*) steroids are not involved in this response to gonadotropins.

During its development, the mammalian follicular oocyte is arrested at the dictyate stage of meiosis (1). Prior to ovulation, but after the preovulatory surge of gonadotropins, meiosis is resumed (2). That gonadotropins are responsible for the resumption of maturation is suggested by the finding that, under tissue culture conditions, oocytes enclosed in their follicles remain arrested until they are exposed to luteinizing hormone (LH, lutropin) or follicle-stimulating hormone (FSH, follitropin) (3, 4). It appears, however, that meiotic arrest is caused by a follicular product, since isolated oocytes undergo spontaneous maturation in the absence of hormones (5, 6).

It has been reported that pig follicular fluid contains an inhibitor of meiosis and that the effect of this substance can be overcome by LH (7, 8). Other studies, conducted with rat and mouse oocytes, have revealed that $N^6,O^{2'}$-dibutyryladenosine 3′:5′-cyclic monophosphate (Bt_2cAMP) and cyclic nucleotide phosphodiesterase inhibitors can prevent the spontaneous maturation observed *in vitro* (9, 10).

In the study described below, we have examined the ability of gonadotropins to overcome the cyclic AMP (cAMP)-induced inhibition of meiosis. Particular emphasis has been placed on the temporal and dose-dependent aspects of this effect of the gonadotropins so that an evaluation can be made of the possible physiologic role of these hormones and cAMP in the intrafollicular regulation of oocyte maturation.

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MATERIALS AND METHODS

Immature Sprague–Dawley female rats (26 days old), obtained from Taconic Farms (Germantown, NY), were injected subcutaneously with 6 international units of pregnant mare serum gonadotropin (PMSG, Sigma Chemical Co., St. Louis, MO) in 0.1 ml of 0.9% NaCl. The rats were killed by cervical dislocation 48 hr after the injection. The ovaries were removed from the animals and placed in Leibovitz's L-15 tissue culture medium (GIBCO, Grand Island, NY) supplemented with 15% fetal bovine serum (Reheis Chemical Co., Phoenix, AR), penicillin (100 units/ml), streptomycin (100 μg/ml) (GIBCO), and cAMP, Bt_2cAMP, 8-Br-cAMP (Sigma Chemical Co.), or 3-isobutyl-1-methylxanthine (iBuMeXan) (Aldrich Chemical Co., Milwaukee, WI). As indicated in the text, incubations were conducted in the presence or absence of the following gonadotropins: ovine LH (oLH), NIH LH S-19; ovine FSH (oFSH), NIH FSH S-12; rat LH (rLH), NIH I-4; or rat FSH (rFSH), NIH I-3; all were generously provided by the National Institute of Arthritis, Metabolism, and Digestive Diseases. Progesterone or 17β-estradiol (Calbiochem, San Diego, CA) was added to culture medium with or without iBuMeXan, in the absence of gonadotropins.

Cumulus–oocyte complexes (approximately 20 per pair of ovaries) were collected by the following procedure: Large follicles were punctured under a dissecting microscope by using an extra fine dissecting scalpel (Clay Adams, Parsippany, NJ) to release their contents into the culture medium. The cumulus–oocyte complexes were transferred to a fresh drop of medium with a fine polyethylene micropipette. The complexes were then incubated in Terasaki tissue culture plates (Falcon, Oxnard, CA) in 0.1 ml of medium (1–10 complexes per well), at 37°C in air at a relative humidity of 100%.

Resumption of meiosis, as indicated by disappearance of germinal vesicles (GVs) in individual oocytes, was monitored 0, 4, 8, and 24 hr after the complexes were isolated, using an inverted microscope equipped with Hoffman modulation contrast optics (×320). For each study, the data of several individual experiments were combined, and the results are reported as the fraction of oocytes containing GVs. In studies of the effects of gonadotropins, the term "maximal effect" is defined as the proportion of oocytes in which maturation was initiated after 24 hr in culture at saturating levels of hormone. Half-maximal effects were calculated as ½ of the difference between the maximal effect and the proportion of oocytes containing GVs in the absence of gonadotropins.

Abbreviations: cAMP, adenosine 3′:5′-cyclic monophosphate; Bt_2cAMP, $N^6,O^{2'}$-dibutyryladenosine 3′:5′-cyclic monophosphate; 8-Br-cAMP, 8-bromoadenosine 3′:5′-cyclic monophosphate; iBuMeXan, 3-isobutyl-1-methylxanthine; GV, germinal vesicle; LH, luteinizing hormone (lutropin); FSH, follicle stimulating hormone (follitropin); r, rat hormone; o, ovine hormone; ED_{50}, half-maximally effective dose.

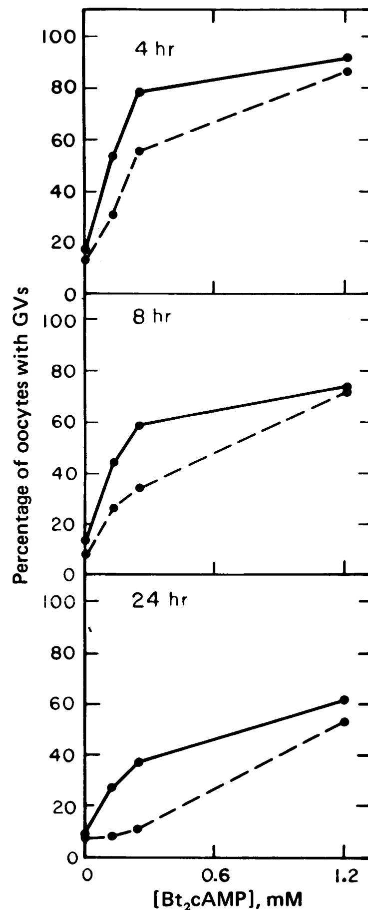

FIG. 1. Effect of oLH on GV breakdown *in vitro* in oocytes maintained in meiotic arrest by Bt$_2$cAMP. Isolated cumulus–oocyte complexes were incubated in the presence of the indicated concentrations of Bt$_2$cAMP with (●——●) or without (●— —●) oLH at 10 μg/ml. The individual oocytes were examined after 4, 8, and 24 hr of incubation. The data of nine different experiments are represented in this figure. The number of oocytes examined under each experimental condition is listed below. Four hours, in order of increasing Bt$_2$cAMP concentrations, without LH: 560, 176, 279, 248; with LH: 310, 142, 99, 90. Eight hours, increasing Bt$_2$cAMP, without LH: 189, 128, 227, 205; with LH: 207, 79, 45, 60. Twenty-four hours, increasing Bt$_2$cAMP, without LH: 449, 128, 236, 230; with LH: 310, 142, 99, 90.

RESULTS

GVs were present in 90–100% of the oocytes [total number examined (n) = 282] at the time of isolation. Upon subsequent incubation in control medium (L-15, serum, and antibiotics), the nuclear structure disappeared, indicating the resumption of meiosis in all but 17% of the oocytes (n = 560) within 4 hr. By 8 hr, 13% of the oocytes (n = 189) contained germinal vesicles and by 24 hr the proportion was reduced to 9% (n = 449). When oocytes were isolated and incubated in the presence of Bt$_2$cAMP, the rate of disappearance of the GVs was significantly diminished in a manner similar to that described by Magnusson and Hillensjö (10). The inhibitory effect of Bt$_2$cAMP was dose dependent (Fig. 1) with a half-maximally effective dose (ED_{50}) at 0.12 mM, when measured after 4 hr of incubation. As seen in Table 1, a slight inhibitory effect on oocyte maturation was obtained with underivatized cAMP. 8-Br-cAMP had no inhibitory effect at 1.2 mM; however, at a concentration of 5 mM this nucleotide was moderately effective (Table 1).

Table 1. Inhibitory effect of cAMP and its derivatives on GV breakdown *in vitro*

Addition to medium	Concentration, mM	Oocytes with GVs, %	Total oocytes examined (n)
None	—	13	169
Bt$_2$cAMP	1.2	90	248
Butyrate	2.4	12	49
cAMP	1.0	28	39
	2.0	23	168
	5.0	27	78
8-Br-cAMP	1.2	13	77
	5.0	67	43

Cumulus–oocyte complexes were isolated and incubated in medium containing the indicated drugs. The individual oocytes were examined after 4 hr of incubation for the presence or absence of GVs.

To test whether gonadotropins might influence oocyte maturation in the inhibited cultures, oocytes were isolated in medium containing both Bt$_2$cAMP and oLH at 10 μg/ml. This hormone preparation fostered the breakdown of GVs in oocytes cultured in low concentrations of Bt$_2$cAMP (0.12 and 0.24 mM). At higher concentrations of the nucleotide, few, if any, of the oocytes responded to LH (Fig. 1). Failure of gonadotropins to induce maturation in oocytes treated with maximally effective doses of Bt$_2$cAMP has also been described in the mouse (11).

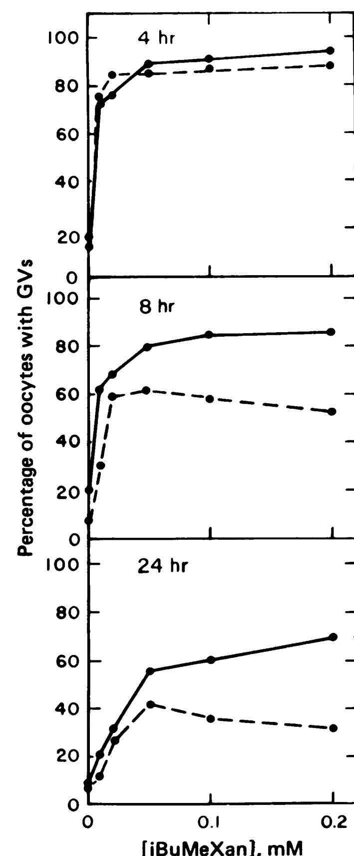

FIG. 2. Effect of oLH on GV breakdown *in vitro* in oocytes maintained in meiotic arrest by iBuMeXan. Isolated cumulus–oocyte complexes were incubated in the presence of the indicated concentrations of iBuMeXan with (●——●) or without (●— —●) oLH at 10 μg/ml. The same oocytes were examined after 4, 8, and 24 hr of incubation. The data of six experiments are represented in this figure. The number of oocytes examined under each experimental condition is listed, in order of increasing iBuMeXan concentrations; without LH: 71, 63, 70, 47, 256; with LH: 97, 147, 140, 85, 251.

Spontaneous maturation of oocytes *in vitro* was also retarded by the cyclic nucleotide phosphodiesterase inhibitor iBuMeXan. The inhibitory influence of iBuMeXan exhibited an ED_{50} of 0.01 mM when measured after 4 hr of incubation (Fig. 2).

After 4 hr of incubation in the presence of iBuMeXan, the inhibition of GV breakdown could be reversed by transferring the oocytes to iBuMeXan-free control medium (Fig. 3). This treatment resulted in the disappearance of GV in 71% of the oocytes during the following 4 hr. In contrast, only 11% of the oocytes incubated in the presence of iBuMeXan continuously for 8 hr lost their nuclear structure.

oLH was able to overcome the inhibitory influence of iBuMeXan when added either at the beginning of the incubation (Fig. 2) or 4 hr later (Fig. 3). Under both conditions the rate of GV breakdown was similar. The dose dependency of the effect of oLH is shown in Fig. 4. The ED_{50} of this gonadotropin preparation was 1.2 μg/ml when measured after 24 hr of incubation. These experiments were conducted in medium containing 0.2 mM iBuMeXan; however, the stimulatory effect of oLH on GV breakdown was similar to that shown in the figures at all concentrations of iBuMeXan greater than 0.05 mM (Fig. 2). In the absence of iBuMeXan or Bt_2cAMP, LH was not found to influence the extent of oocyte maturation as compared to control cultures (Fig. 3).

oFSH was also effective in relieving the inhibition caused by iBuMeXan (Fig. 4). Half-maximal effects with this gonadotropin preparation were obtained at a concentration of 0.25 μg/ml. The magnitude of the response to FSH was approximately half that seen with LH after 24 hr in culture.

The effect of highly purified preparations of rat gonadotropins of iBuMeXan-inhibited oocytes was also examined. The maximal effect of rLH was greater than that obtained by rFSH (Fig. 5), but the ED_{50} for LH was 62 ng/ml compared to 1 ng/ml for FSH. Moreover, the magnitude of the effect of the purified rat gonadotropins was less than that obtained with the ovine preparations. The smaller effect of these gonadotropins was difficult to interpret because the rat preparations are approximately 30-fold higher in purity than the ovine preparations. If the effects observed with the ovine hormones were due simply to the actions of either LH or FSH alone, the magnitudes of the responses to the rat hormones might have been expected to be the same or greater than those caused by preparations from a different species.

The ovine hormone preparations are known to contain considerable contaminating gonadotropin activity (e.g., oLH S-19 exhibits a ratio of LH to FSH activity of approximately 30:1). In order to test whether the greater effectiveness of these preparations was due to a mixture of hormones and to better understand the specificity of this response, we examined the effect of the combinations of the rat gonadotropins on oocyte maturation. As seen in Fig. 6, the combined effect of the two gonadotropins was greater than that of either alone. At 100 ng of gonadotropin (LH:FSH, 30:1) per ml, 43% of the oocytes lost their GV relative to cultures that were not exposed to gonadotropin; at 100 ng of LH alone per ml the corresponding value was 15%; with 100 ng of FSH per ml it was 21%. These studies indicated that the maximal effective ratio of gonadotropins varied with the overall gonadotropin concentration; however, at optimal gonadotropin ratios, half-maximal effects were observed with approximately 1 ng of gonadotropins (LH:FSH, 6:1) per ml. This value is well below the levels of circulating gonadotropins in the rat during the hours preceding ovulation (12).

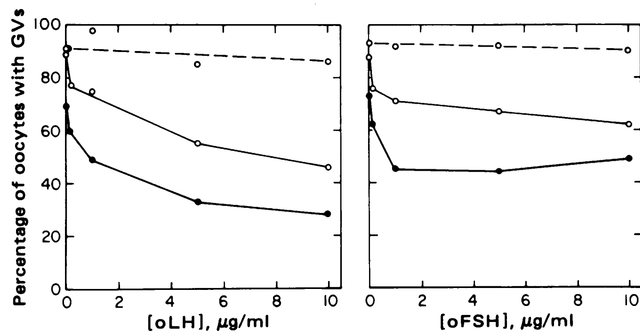

FIG. 4. Dose dependency of effect of ovine gonadotropins on GV breakdown *in vitro*. Cumulus–oocyte complexes were isolated in medium containing 0.2 mM iBuMeXan and the indicated concentrations of oLH or oFSH. The individual oocytes were examined after 4 (O- -O), 8 (O——O), and 24 (●——●) hr of incubation. The number of oocytes examined in each experimental group is listed, in increasing order of hormone concentration, LH: 35, 43, 55, 33, 50; FSH: 41, 152, 187, 121, 177.

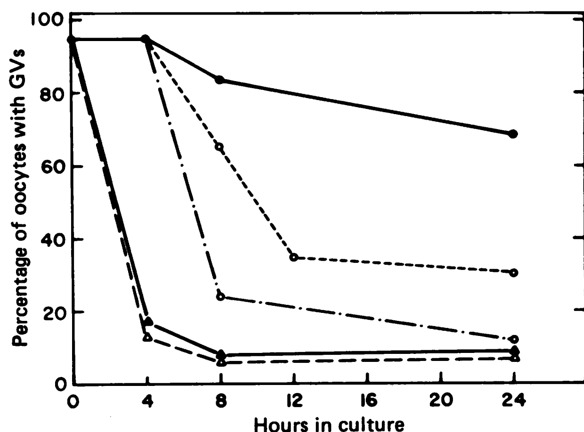

FIG. 3. Time course of GV breakdown *in vitro*. Cumulus–oocyte complexes were isolated under the following conditions. One group was isolated in medium to which no additions were made (control) (▲——▲, n = 560), a second group in medium containing 0.2 mM iBuMeXan (●——●), and a third in medium containing oLH at 10 μg/ml (Δ- -Δ, n = 310). After 4 hr, a subpopulation of cumulus–oocyte complexes that had been cultured in the presence of iBuMeXan was transferred to iBuMeXan-free medium (O·—·O, n = 83); another subpopulation was transferred to medium containing 0.2 mM iBuMeXan and oLH at 10 μg/ml (O- - -O, n = 45); a third subpopulation was incubated continuously in 0.2 mM iBuMeXan (●——●, n = 83). The fraction of oocytes containing GVs was monitored after 0, 4, 8, and 24 hr of incubation.

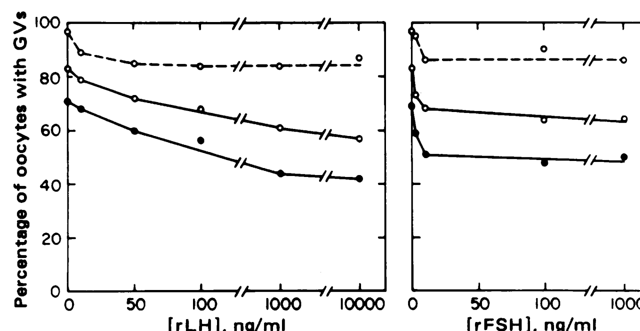

FIG. 5. Dose dependency of effect of rat gonadotropins on GV breakdown *in vitro*. Cumulus–oocyte complexes were isolated in medium containing 0.2 mM iBuMeXan and the indicated concentrations of rLH or rFSH. The individual oocytes were examined after 4 (O- -O), 8 (O——O), and 24 (●——●) hr of incubation. The number of oocytes examined in each experimental group is listed, in increasing order of hormone concentration, LH: 69, 96, 92, 118, 122, 71; FSH: 69, 37, 37, 50, 58.

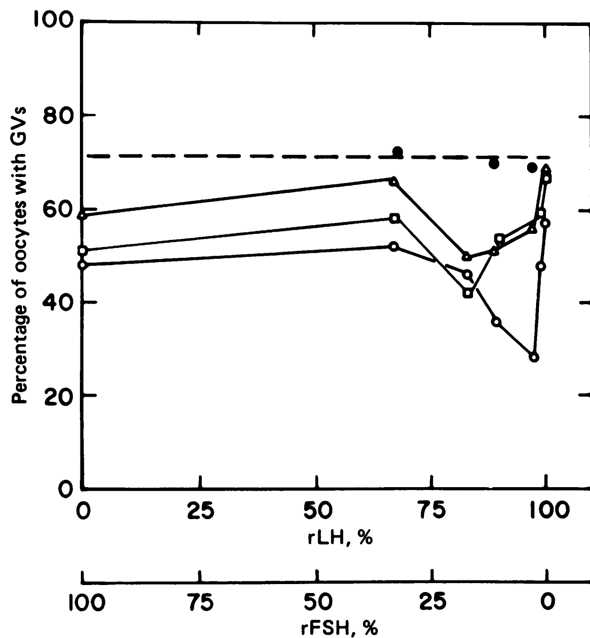

FIG. 6. Effect of combinations of rat gonadotropins on GV breakdown *in vitro*. Cumulus-oocyte complexes were isolated in medium containing 0.2 mM iBuMeXan and indicated amounts of rat gonadotropins. The individual oocytes were examined after 24 hr of incubation for the presence or absence of GVs. The curves represent the results obtained at a constant amount of total gonadotropin, given below, as the ratio of LH:FSH was varied. The number of oocytes in each group was as follows: (- - -) 0.2 mM iBuMeXan, 69; (●) iBuMeXan + gonadotropins at 0.1 ng/ml, in order of increasing LH: 68, 70, 81, 36; (△——△) iBuMeXan + gonadotropins at 1 ng/ml: 102, 125, 57, 99; (□——□) iBuMeXan + gonadotropins at 10 ng/ml: 58, 73, 92, 91; (○——○) iBuMeXan + gonadotropins at 100 ng/ml: 46, 150, 130, 160, 134.

In order to determine whether the effect of the gonadotropins might be mediated by sex steroids produced by the cumulus-oocyte complex, the effects of progesterone and 17β-estradiol on oocyte maturation were also examined. The fraction of iBuMeXan-inhibited oocytes containing GVs was not influenced by either of these steroids at 1 μM.

DISCUSSION

The intrafollicular environment inhibits mammalian oocyte maturation. By an unknown mechanism, LH overcomes this inhibition and stimulates oocyte maturation *in vivo* (13) and *in vitro*, in cultures of whole follicles (3, 4). However, when cumulus-oocyte complexes are isolated from the follicle, the oocytes mature spontaneously, independent of the hormonal environment (5, 6). Bt_2cAMP and cyclic nucleotide phosphodiesterase inhibitors prevent this spontaneous maturation (9, 10). In this study, we have shown that the inhibition of oocyte maturation in isolated cumulus-oocyte complexes, caused by Bt_2cAMP or iBuMeXan, can be relieved by gonadotropins. Thus, cultures of inhibited oocytes such as these represent an *in vitro* system for studies of the hormonal control of the resumption of oocyte maturation.

Oocytes removed from the inhibitory follicular environment lost their GVs within 4 hr (ref. 14 and Fig. 3). A similar time course of GV breakdown was found for iBuMeXan-inhibited oocytes after transfer to inhibitor-free medium. A longer time period is required for LH stimulation of oocyte maturation. When iBuMeXan-inhibited oocytes were exposed to gonadotropins *in vitro*, a maximal proportion of responding oocytes was observed within 8 hr of incubation. *In vivo*, the completion of GV breakdown occurs approximately 6 hr after the injection of an ovulatory dose of human chorionic gonadotropin (15).

In studies on the specificity of the gonadotropin-mediated effect, we found that relatively crude preparations of ovine gonadotropins were more effective in promoting oocyte maturation than were highly purified rat gonadotropins. The oLH preparation used in this study contains FSH:LH at approximately 1:30. This preparation effectively stimulated maturation of the oocytes cultured in the presence of Bt_2cAMP or iBuMeXan. However, the purified preparations of rat gonadotropins elicited a lesser response than that caused by the ovine hormones. An effect of the same or greater magnitude could be obtained only when a combination of both rat gonadotropins was used. The maximally effective ratios of gonadotropin varied with the overall hormone concentration in a complicated manner. The basis of this synergism is not understood. However, it should be mentioned that, *in vivo*, the levels of LH and FSH are both elevated prior to ovulation, and both may be required to allow the resumption of oocyte maturation.

The roles of each of the gonadotropins in follicular development, oocyte maturation, and ovulation have been extensively investigated (16). Some of these studies indicate that LH is the only gonadotropin involved in maturation, while others demonstrate identical potencies for both gonadotropins (3, 4, 16). The results presented in this study show that, *in vitro*, a combination of both gonadotropins is needed for maximal promotion of oocyte maturation. The extent to which a similar situation exists *in vivo* is not known; however, it appears from these studies that care must be taken in assigning specific roles to LH or FSH unless preparations of sufficient purity are available.

As a result of the preovulatory gonadotropin surge, ovarian steroidogenic activity is stimulated (17). In amphibians, steroids are known to promote oocyte maturation (6). The failure of progesterone and 17β-estradiol to induce maturation in iBuMeXan-inhibited oocytes in culture suggests that, in the *in vitro* system, these steroids are not involved in the gonadotropin-stimulated resumption of meiosis.

It has been reported that gonadotropins cause a significant increase in ovarian cAMP (18-20). Moreover, it would appear from previous work that gonadotropins do raise cAMP levels in the cumulus oophorus because cAMP and its derivatives can mimic the known effects of the hormones on these cells (21). In addition, it is known that follicular oocytes mature after the preovulatory LH surge (2). On the other hand, as seen in this study, and as described elsewhere, high cAMP levels inhibit oocyte maturation (9, 10). Taken together, these findings present an apparent paradox—i.e., if gonadotropins elevate cAMP levels in the cumulus-oocyte complex, and since cAMP inhibits oocyte maturation, what mechanism allows the oocyte to mature under the influence of gonadotropins?

One explanation for the stimulatory effect of gonadotropins on oocyte maturation is to assume that these hormones act directly on the oocyte via a cAMP-independent mechanism. Alternatively, it may be postulated that the inhibition of oocyte development prior to ovulation is mediated by the cells of the cumulus oophorus, and that the relief of inhibition is a result of the effect of gonadotropins on the cumulus and not the oocyte itself. It is not known whether gonadotropins exert a direct effect on the oocyte; however, it has been shown that cell-to-cell communication exists between the cells of the cumulus and the oocyte. Near the time of ovulation, cumulus-oocyte communication is terminated (22). Concomitant with termination of communication, the cumulus cell processes, which bridge the zona pellucida and are thought to be channels for communi-

cation, are distintegrated (22–24,*). These phenomena suggest the following model for the control of oocyte maturation.

Prior to ovulation, meiotic arrest may be maintained by increased concentrations of cAMP in the oocyte. According to this theory, the nucleotide concentration in the oocyte would be increased indirectly by increasing cumulus cell cAMP, which could be transferred to the oocyte via the transzona processes. This contention is based on studies using a model system to demonstrate cell contact-dependent communication of hormonal stimulation. In these studies, the most likely candidate for the substance that is communicated to signal hormonal stimulation is cAMP (25). A possible effector of the increased concentrations of the nucleotide may be the oocyte maturation inhibitory factor described by Tsafriri and Channing (7).

As mentioned above, near the time of ovulation, apparently as a result of gonadotropin stimulation, the transzona processes are disintegrated and communication is terminated in the cumulus–oocyte complex. Thus, provided the oocyte does not contain an adenylate cyclase that can be stimulated by gonadotropins, its supply of cyclic nucleotide would also be interrupted. In the absence of the inhibitory influence of cAMP, the oocyte would be free to resume meiosis.

While this model is consistent with known events relating to mammalian oocyte maturation, it is very hypothetical. It is testable, however, by using preparations of oocytes from which the cumulus has been removed and by an examination of the regulation of cumulus–oocyte ionic coupling by gonadotropins *in vitro*. We hope that through experiments such as these we will gain additional insight into this fundamental physiological process.

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