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Effect of adenylate cyclase stimulation on meiotic resumption and cyclic AMP content of zona-free and cumulus-enclosed bovine oocytes *in vitro*

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The effect of adenylate cyclase stimulation via the components of the enzyme on nuclear maturation in bovine cumulus-enclosed and zona-free oocytes was examined. The stimulating agents were cholera toxin, pertussis toxin, forskolin, sodium fluoride and prostaglandin E_2. Cyclic AMP contents were measured in cumulus–oocyte complexes, cumulus-enclosed oocytes and in zona-free oocytes after stimulation, to establish the relationship between cumulus cell and oocyte cAMP concentrations and the meiotic status of the oocyte. In cumulus-enclosed oocytes, forskolin alone and 3-isobutyl-1-methylxanthine (IBMX), at 0.5 mmol l^{-1}, inhibited the resumption of meiosis after 8 h of culture; the other agents were without effect. After 24 h of culture, IBMX at 0.5 mmol l^{-1} was without effect, but at 2 mmol l^{-1} reduced the percentage of oocytes at the mature stage (51 versus 82% in control medium). Forskolin alone reduced the proportion of oocytes at the mature stage from 82 to 58%. Forskolin plus IBMX at 2 mmol l^{-1} and sodium fluoride plus IBMX at 2 mmol l^{-1} significantly diminished the maturation rate (6 and 17% mature oocytes, respectively). Cholera toxin (with IBMX) and forskolin (alone or with IBMX) stimulated the synthesis of high amounts of cAMP in complexes, but only forskolin had a significant effect on the cAMP contents of oocytes derived from complexes. Forskolin was more effective in zona-free oocytes than in cumulus-enclosed oocytes in inhibiting nuclear maturation (24% mature oocytes versus 73% in control medium) even after 24 h of culture; its effect was potentiated by IBMX; forskolin also stimulated cAMP synthesis. IBMX was as effective as forskolin in delaying nuclear maturation, but did not cause an accumulation of cAMP above the control value. The other agents were without effect on meiosis and cAMP concentrations in zona-free oocytes. These results suggest that increases in cAMP concentration in denuded oocytes inhibit maturation; but, when cAMP concentrations are high in cumulus cells, a maturation signal can be generated that bypasses the inhibitory effect of cAMP in the oocyte. Bovine oocytes can synthesize high amounts of cAMP, but its adenylate cyclase may not be coupled to G proteins sensitive to cholera or pertussis toxin.

Introduction

Mammalian oocytes are arrested at the diplotene stage of the first meiotic division from the time of birth to the preovulatory luteinizing hormone surge leading to ovulation. After the luteinizing hormone discharge, or in response to follicular atresia, some oocytes complete the first meiotic division that progresses to the metaphase II stage where oocytes are arrested again until fertilization. As early as 1935, Pincus and Enzmann demonstrated that mammalian oocytes removed from their follicular environment and cultured in standard media resume meiosis spontaneously, even in the absence of hormonal stimulation. This observation led to the conclusion that the follicle contains an arresting factor. In laboratory animals, cAMP has been proposed to mediate the maintenance of meiotic arrest, since analogues of cAMP and phosphodiesterase inhibitors can prevent nuclear maturation in rat, mouse and hamster oocytes (Cho *et al.*, 1974; Magnusson and Hillensjö, 1977; Dekel and Beers, 1980; Racowsky, 1986; Eppig, 1989).

In *Xenopus laevis*, cAMP can be synthesized by the oocyte (Schorderet-Slatkine *et al.*, 1978). In mammals, the extent of the contribution of cumulus cells and of the oocyte to the synthesis of cAMP is not known. Forskolin, a direct stimulator of the catalytic subunit of adenylate cyclase, inhibits meiotic resumption in rat cumulus-enclosed oocytes, but is without effect in denuded oocytes (Dekel *et al.*, 1984), even though it stimulates the synthesis of cAMP in denuded rat oocytes (Olsiewski and Beers, 1983). Cholera toxin delays nuclear maturation in cumulus-enclosed rat and mouse oocytes, but is not effective in denuded oocytes (Dekel and Beers, 1980; Schultz *et al.*, 1983b). These results led to the hypothesis that mammalian oocytes

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possess an atypical adenylate cyclase consisting of a single catalytic subunit. Arresting cAMP would be synthesized by cumulus cells and transferred to the oocyte via the gap junctions, ensuring metabolic coupling between cumulus cells and the oocyte (Dekel and Beers, 1980; Dekel *et al.*, 1988). Transfer of labelled cAMP from cumulus cells to the oocyte has not been demonstrated (Schultz *et al.*, 1983a, b; Eppig and Downs, 1984); but Bornslaeger and Schultz (1985) and Racowsky (1984, 1985) demonstrated that, when cAMP contents were high in cumulus–oocyte complexes, cAMP contents were also high in oocytes derived from these complexes.

In bovine cumulus–oocyte complexes and denuded oocytes, cAMP analogues, phosphodiesterase inhibitors and forskolin can delay nuclear maturation *in vitro* (Homa, 1988; Sirard and First, 1988; Sirard, 1990). This indicates that cAMP can play a role in the control of meiosis in this species and that bovine oocytes can synthesize cAMP. The present study examined the effects of the stimulation of adenylate cyclase via its different components on the meiotic maturation in cumulus-enclosed and zona-free oocytes. Cyclic AMP concentrations were also measured in cumulus–oocyte complexes, in oocytes derived from complexes and in zona-free oocytes after stimulation of adenylate cyclase to establish the relationship between cumulus cell and oocyte cAMP concentrations and oocyte meiotic status. Zona-pellucida-free oocytes were used instead of cumulus-free oocytes to ensure that the effects observed were on the adenylate cyclase enzyme of oocytes rather than on the adenylate cyclase of cumulus cell processes embedded in zonae pellucidae (Kuyt *et al.*, 1988). The stimulating agents used were cholera toxin, pertussis toxin, sodium fluoride, forskolin and prostaglandin E_2. Cholera toxin and pertussis toxin modify G protein function by using NAD to ADP-ribosylate Gs and Gi, respectively; these modifications lead to the irreversible stimulation of adenylate cyclase by Gs or to the inability of Gi to turn off the enzyme (Birnbaumer *et al.*, 1987; Gilman, 1987). Sodium fluoride can alter the function of the enzyme complex by irreversibly coupling the α subunit of Gs to the catalytic component (Ross and Gilman, 1980), and the catalytic component can be directly activated by forskolin (Seamon *et al.*, 1981). Prostaglandin E_2 was chosen as the agent to activate the adenylate cyclase system via a hormone receptor because its effect on the meiotic resumption of bovine oocyte is not known, and, in other systems, prostaglandin E_2 modifies adenylate cyclase activity via a receptor that has a hydrophobic-binding domain inside the plasma membrane (Ringe and Petsko, 1990).

Materials and Methods

Recovery and preparation of oocytes

Oocytes were collected by aspiration of small follicles (1–5 mm) from ovaries obtained at a slaughterhouse. Ovaries were transported (1 h) in a saline solution, maintained between 25 and 35°C, which contained 10 000 iu penicillin, 10 mg streptomycin and 25 μg amphotericin B l^{-1} (Sigma Chemical Co., St Louis, MO). Aspiration was performed with a 10 ml syringe and an 18 gauge needle; follicular fluid was pooled in 50 ml conical tubes. Oocytes with an intact, unexpanded, tightly adherent mass of cumulus cells were recovered under a stereomicroscope. About half of the cumulus-enclosed oocytes recovered were vortex-agitated for 5–10 min in aspirated fluid to remove cumulus cells. In this fluid, bovine oocytes do not become committed to resume meiosis: in a previous study, most oocytes were still at the germinal vesicle stage after 24 h of culture in aspirated fluid (Sirard and Bilodeau, 1990). The cumulus-free oocytes were rapidly washed twice in a solution of 4-(hydroxyethyl)-1-piperazine-ethanesulfonic acid, Hepes-buffered (TLH, pH 7.4) Tyrode's medium (Bavister *et al.*, 1983) containing 10% fetal calf serum (FCS; Flow Laboratories, McLean, VA) and gentamicin sulfate (50 μg ml^{-1}; Sigma). The oocytes were then transferred to a TLH solution containing 1% FCS and 3 mg protease ml^{-1} (Sigma); they were kept in this solution until the zonae pellucidae were completely digested (1–4 min). After the digestion, the zona-free oocytes were transferred to a neutralizing solution of 50% TLH and 50% FCS and were kept in this solution for < 5 min. Cumulus-enclosed oocytes that were not denuded were washed three times in TLH solution.

Culture of oocytes

Oocytes were distributed into multiwell (24) plates (Corning, NY) containing the culture medium TCM-199 (1 ml per well; with 10 to 20 oocytes per well) with Hank's salts, glutamine, bicarbonate, supplemented with 10% FCS and gentamicin sulfate (50 μg ml^{-1}) and either pertussis toxin (10 μg ml^{-1}), forskolin (0.1 mmol l^{-1}), sodium fluoride (1 mmol l^{-1}) prostaglandin E_2 (1 μmol l^{-1}) or cholera toxin. Cholera toxin was used at 10 μg ml^{-1} with zona-free oocytes, but at 0.1 μg ml^{-1} with cumulus-enclosed oocytes, since higher doses resulted in the degeneration of the oocytes. For determination of the effects of these agents on nuclear maturation, the agents were used alone or in combination with IBMX at 0.5 and 2 mmol l^{-1}. Oocytes that were to be cultured in the presence of IBMX were also washed and digested in solutions containing IBMX. For the study of cAMP synthesis, the agents were used only in combination with IBMX at 0.5 mmol l^{-1}; forskolin was also assayed alone. Cholera toxin, pertussis toxin and sodium fluoride were prepared in distilled water; forskolin and prostaglandin E_2 were prepared in 95% ethanol. No more than 10 μl of water or 5 μl of ethanol were added per ml of culture medium; these dissolving agents used alone were without effect. Oocytes were cultured for 8 or 24 h for the study of nuclear maturation or for 6 h for the measurements of cAMP; they were cultured at 38.5°C in a moisture-saturated atmosphere of 5% CO_2 in air. Each treatment was repeated at least three times and replicates were made on different days. All products were purchased from Sigma.

Fixation of oocytes for the evaluation of nuclear maturation

At the end of the culture period, cumulus-enclosed oocytes were transferred to small tubes containing 400 μl of a trypsin solution (1 mg ml^{-1} in Hank's balanced salts solution) and vortex-agitated for 2 min. Denuded zona-free oocytes were then recovered under a stereomicroscope and transferred on glass slides. Vaseline and paraffin wax were used to maintain a coverslip in contact with the oocytes, the coverslips were secured with epoxy glue and the slides immersed in a fixative solution (ethanol: acetic acid, 3:1, v/v) for a minimum of 24 h. The slides were

stained with 1% aceto-orcein and examined at × 100 and × 400 magnification. Oocytes were classified as being at the germinal vesicle stage, or at an intermediate stage (including germinal vesicle breakdown, condensation of chromosomes and metaphase I) or as being mature (including anaphase I, telophase I and metaphase II). Oocytes with an abnormal chromatin configuration were classified as degenerated. The significance of individual comparisons was evaluated by χ^2 test (Snedecor and Cochran, 1980).

Radioimmunoassay of cAMP

After 6 h of culture, zona-free oocytes were rapidly washed in Hank's balanced salt solution containing 2 mmol IBMX l^{-1}. The oocytes were then transferred in 1 ml ethanol (95%) and stored at −80°C. Cumulus-enclosed oocytes were transferred to small tubes containing 200 µl of Hank's balanced salt solution with 2 mmol IBMX l^{-1}; they were vortex-agitated for 2 min and then the denuded oocytes were recovered, washed until no cumulus cells were visible and stored as described for zona-free oocytes. Cumulus-enclosed oocytes were cultured with the various agents for 6 h and then transferred directly to ethanol without separation of cumulus cells from oocytes, to evaluate the effect of the various agents on the accumulation of cAMP by the cumulus cells. Before cAMP measurements, samples were vortexed for 1 min and centrifuged at 12 000 *g* for 15 min at 4°C; the supernatants were removed, evaporated, resuspended in assay buffer and assayed after acetylation (Harper and Brooker, 1975) and the proper dilution. The labelled antigen was a succinyl tyrosine-^{125}I-methyl ester derivative of cAMP. Separation of bound from free antigen was achieved by the use of a primary and secondary antibody complex generously provided by S. Heisler (Heisler, 1985). The inter- and intra-assay coefficients of variation were 5.3 and 8.2%, respectively. Blank samples composed of the same volume of Hank's solution with 2 mmol IBMX l^{-1} contained no significant amounts of cAMP. The results were analysed by Waller–Duncan variance analysis.

Results

Effects of adenylate cyclase stimulation on the meiotic maturation of cumulus-enclosed oocytes

When cholera toxin, pertussis toxin, forskolin, sodium fluoride and prostaglandin E_2 were used alone, only forskolin had a significant effect on meiosis after 24 h of culture (82 and 58% of mature oocytes for control medium and forskolin, respectively, $P < 0.005$) (Fig. 1a). IBMX at 0.5 mmol l^{-1} did not diminish the maturation rate, and the combinations of 0.5 mmol IBMX l^{-1} with cholera toxin, pertussis toxin, sodium fluoride or prostaglandin E_2 were without effect when compared with IBMX alone or with the agents alone. The combination of forskolin and IBMX (0.5 mmol l^{-1}) resulted in a slight increase in the maturation rate compared with forskolin alone, but this increase was not significant (58 and 69% mature oocytes for forskolin and forskolin + 0.5 mmol IBMX l^{-1}, respectively. In control medium, IBMX at 2 mmol l^{-1} significantly reduced the maturation rate observed after 24 h of culture (82 and 51% mature oocytes for control and 2 mmol IBMX l^{-1}, respectively, $P < 0.05$); moreover, the combination of IBMX at 2 mmol l^{-1} with sodium fluoride or forskolin resulted in a significant decrease in the maturation rate compared with 2 mmol IBMX l^{-1} alone (51% mature oocytes with IBMX at 2 mmol l^{-1}; 17 and 6% for sodium fluoride + IBMX and forskolin + IBMX, respectively, $P < 0.01$).

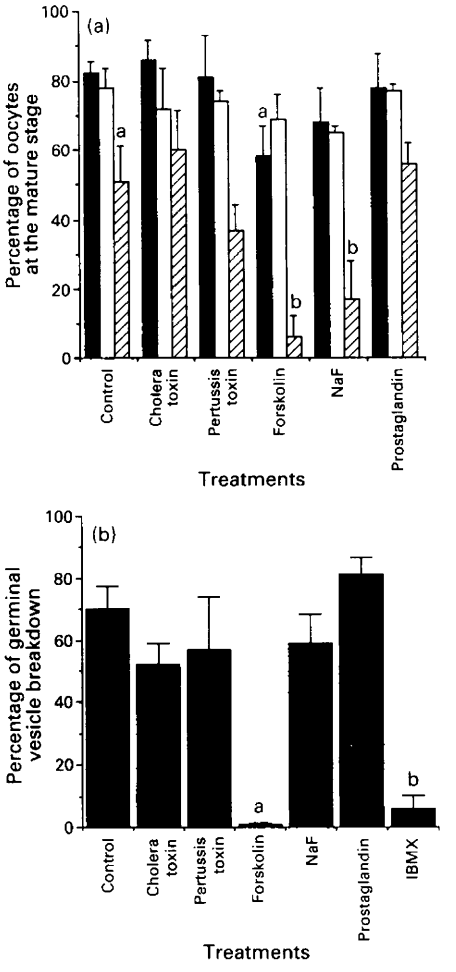

Fig. 1. Effect of cholera toxin (0.1 µg ml^{-1}), pertussis toxin (10 µg ml^{-1}), forskolin (0.1 mmol l^{-1}), sodium fluoride (1 mmol l^{-1}) and prostaglandin E_2 (1 µmol l^{-1}) alone (■) or in combination with 3-isobutyl-1-methylxanthine (IBMX) at 0.5 (□) or 2 (▨) mmol l^{-1} on the percentages of bovine cumulus-enclosed oocytes (a) at the mature stage, including anaphase I, telophase I and metaphase II after 24 h of culture, and (b) with germinal vesicle breakdown after 8 h of culture. [a]Significant difference from the control medium using χ^2 analysis ($P < 0.05$ in (a) and $P < 0.001$ in (b)); [b]significant difference with 2 mmol IBMX l^{-1} alone ($P < 0.01$). The mean number of oocytes used in each treatment was 48 in (a) and 30 in (b). Data are expressed as means ± SEM of three replicates.

In bovine oocytes, germinal vesicle breakdown usually occurs after 6–9 h of culture (Sirard *et al.*, 1989). To test the possibility that IBMX at 0.5 mmol l^{-1}, cholera toxin, pertussis toxin, sodium fluoride and prostaglandin E_2 could have a transient effect on nuclear maturation not detected after 24 h, we cultured cumulus-enclosed oocytes with these agents and fixed them after only 8 h, when most control oocytes should have resumed meiosis. A high

percentage of oocytes cultured in control medium, with cholera toxin, pertussis toxin, sodium fluoride and prostaglandin E_2 had resumed meiosis (70, 52, 57, 59 and 81% of germinal vesicle breakdown for these treatments, respectively) (Fig. 1b). Oocytes cultured with 0.5 mmol IBMX l^{-1} or forskolin were still at the germinal vesicle stage since only 6 and 0% of oocytes, respectively, had undergone germinal vesicle breakdown after 8 h of culture (significant difference from control medium, $P < 0.001$). IBMX at 0.5 mmol l^{-1} was without effect after 24 h of culture, showing that it is possible that a transient effect could be detected after 8 h of culture, but not after 24 h; this was not the case for cholera toxin, pertussis toxin, sodium fluoride and prostaglandin E_2.

Effects of adenylate cyclase stimulation on the meiotic maturation of zona-free oocytes

Preliminary experiments showed that zona-free and denuded oocytes reacted in a different way to cholera toxin (results not shown). Thus, to study the effect of adenylate cyclase stimulation on the oocyte itself, zonae pellucidae were removed to ensure that the effects observed were on the adenylate cyclase of oocytes rather than on the adenylate cyclase of cumulus cell processes embedded in zonae pellucidae (Kuyt *et al.*, 1988). In control medium, zona-free oocytes matured to the same extent as cumulus-enclosed or denuded oocytes, indicating that the enzymatic digestion did not affect the capacity of oocytes to mature spontaneously *in vitro* and to expel the first polar body.

Among the agents used alone, after 24 h of culture only forskolin significantly reduced the maturation rate in comparison with the control treatment (73 and 24% mature oocytes for the control and forskolin, respectively, $P < 0.001$) (Fig. 2a). The small decrease in the proportion of mature oocytes seen when oocytes were cultured with cholera toxin alone was not significant (73 versus 58% for control medium and cholera toxin, respectively) nor was the slight increase caused by pertussis toxin and prostaglandin E_2 (82 and 80% mature oocytes for these two treatments, respectively). Sodium fluoride had no effect on the number of oocytes resuming meiosis (69% mature oocytes). It is known that mouse oocytes possess a very active phosphodiesterase (Bornslaeger *et al.*, 1984); the same agents were therefore combined with the phosphodiesterase inhibitor IBMX to see whether the absence of effects with cholera toxin, pertussis toxin, sodium fluoride and prostaglandin E_2 was due to very fast degradation of cAMP synthesized in response to these agents. It can be seen that the addition of IBMX (at 0.5 or 2 mmol l^{-1}) greatly reduced the percentage of mature zona-free oocytes in control medium and with all the agents studied (significant differences between control and IBMX (Fig. 2a); or between agents alone and agents plus IBMX, $P < 0.001$). Because percentages of mature oocytes were low in all treatments containing IBMX, to have a better idea of the effect of IBMX on the resumption of meiosis, it was necessary to look at the proportion of oocytes still at the germinal vesicle stage for each treatment (Fig. 2b). With 0.5 mmol IBMX l^{-1} in control medium, few oocytes remained at the germinal vesicle stage, so this concentration of IBMX was effective in delaying meiotic maturation, but not in preventing it; most oocytes were at the intermediate stage (not shown in the figures). With IBMX at 2 mmol l^{-1}, 44% of oocytes were still at the germinal vesicle stage after 24 h of culture. The inhibitory

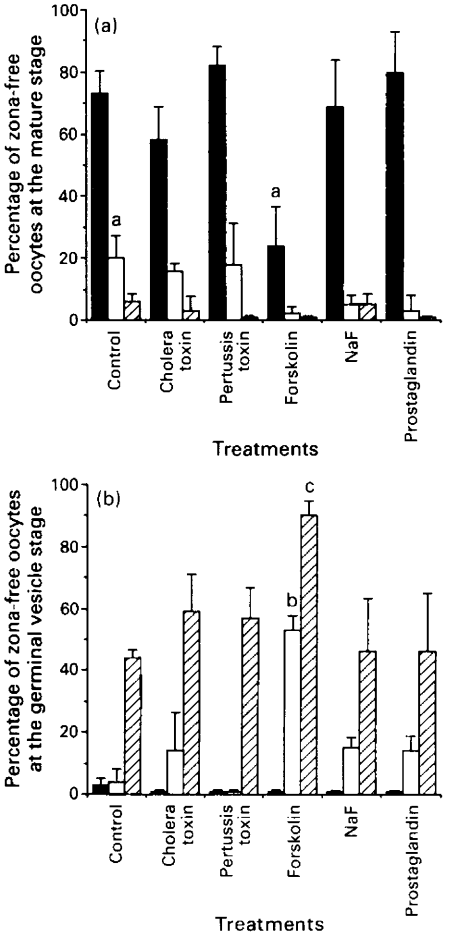

Fig. 2. Effect of cholera toxin (10 μg ml^{-1}), pertussis toxin (10 μg ml^{-1}), forskolin (0.1 mmol l^{-1}), sodium fluoride (1 mmol l^{-1}) and prostaglandin E_2 (1 μmol l^{-1}) alone (■) or in combination with 3-isobutyl-1-methylxanthine (IBMX) at 0.5 (□) or 2 (▨) mmol l^{-1} on (a) the percentages of bovine zona-free oocytes at the mature stage, including anaphase I, telophase I and metaphase II; and (b) the percentages of oocytes at the germinal vesicle stage after 24 h of culture. [a]Significant difference from control using χ^2 analysis ($P < 0.001$); [b]significant difference from IBMX at 0.5 mmol l^{-1} alone; [c] significant difference from IBMX at 2 mmol l^{-1} alone ($P < 0.001$). A mean number of 45 oocytes was used in each treatment. Data are expressed as means ± SEM of at least three replicates.

effect of forskolin was potentiated by both concentrations of the phosphodiesterase inhibitor (0% of oocytes at the germinal vesicle stage with forskolin alone; 4 and 44% for IBMX at 0.5 and 2 mmol l^{-1}; 53 and 92% for forskolin + IBMX at 0.5 and 2 mmol l^{-1}, respectively, $P < 0.001$), but the other agents were without effect on the resumption of meiosis even in combination with IBMX at 2 mmol l^{-1}.

Agents that stimulate the adenylate cyclase enzyme via the regulatory G proteins or a hormonal receptor could have a transitory effect on nuclear maturation of zona-free oocytes that is not detected after 24 h of culture. This possibility was tested by culturing oocytes in the presence of cholera toxin, pertussis toxin, sodium fluoride, forskolin or IBMX at 0.5 mmol l^{-1} for only 8 h (Fig. 3). At this time point, most oocytes cultured with

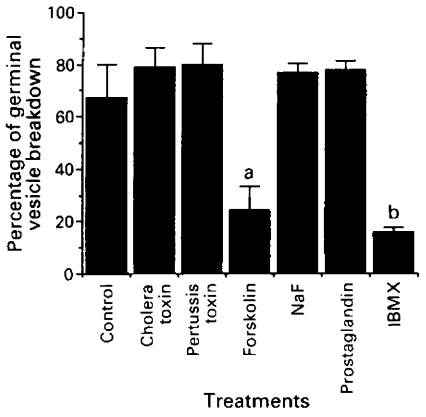

Fig. 3. Effect of cholera toxin (10 μg ml^{-1}), pertussis toxin (10 μg ml^{-1}), forskolin (0.1 mmol l^{-1}), sodium fluoride (1 mmol l^{-1}), prostaglandin E_2 (1 μmol l^{-1}) and 3-isobutyl-1-methylxanthine (IBMX) at 0.5 mmol l^{-1} on the percentages of bovine zona-free oocytes with germinal vesicle breakdown after 8 h of culture. [a,b]Significant difference from control ($P < 0.001$). A mean number of 35 oocytes was used in each treatment. Data are expressed as means ± SEM of three replicates.

0.5 mmol IBMX l^{-1} or forskolin were still at the germinal vesicle stage since only 16% and 24% of oocytes had resumed meiosis (significant difference from control medium, $P < 0.05$). Oocytes cultured with cholera toxin, pertussis toxin, sodium fluoride and prostaglandin E_2 resumed meiosis in the same proportion as oocytes cultured in control medium, indicating that these agents do not have a transient effect on nuclear maturation.

Effects of adenylate cyclase stimulation on cAMP content of cumulus–oocyte complexes, cumulus-enclosed oocytes and zona-free oocytes

Contents of cAMP were measured in cumulus–oocyte complexes, cumulus-enclosed oocytes and zona-free oocytes after 6 h of culture to determine whether the different effects of the agents studied on the nuclear maturation were due to their different potency to stimulate cAMP synthesis. Measurements were made after 6 h of culture to determine the conditions (cAMP content) in which germinal vesicle breakdown can occur. Whole complexes were used to evaluate the synthesizing capability of cumulus cells because, to achieve a good separation of cumulus cells from oocytes, complexes must be vortexed and this procedure results in the disruption of many cells and loss of cAMP in the medium (results not shown). In whole complexes, cholera toxin in combination with IBMX (0.5 mmol l^{-1}) and forskolin (alone and with IBMX) resulted in the accumulation of high amounts of cAMP while other treatments were not different from control; these three treatments were also different from each other (Waller-Duncan, $P < 0.05$) (Table 1). In oocytes cultured with their cumulus mass, forskolin (alone and with IBMX) significantly increased the amount of oocyte cAMP (5.3 ± 1.4, 90.8 ± 8.5 and 287.0 ± 63.3 fmol per oocyte for control medium, forskolin alone and forskolin + IBMX at 0.5 mmol l^{-1}, respectively); cholera toxin, pertussis toxin, sodium fluoride and prostaglandin E_2 were without effect.

Zona-free oocytes cultured in control medium alone, with IBMX at 0.5 mmol l^{-1} and with pertussis toxin + IBMX at 0.5 mmol l^{-1} contained the same amount of cAMP (2.5, 2.1 and 3.7 fmol per oocyte, respectively); IBMX at 2 mmol l^{-1}, cholera toxin, sodium fluoride and prostaglandin E_2 (+IBMX at 0.5 mmol l^{-1}) resulted in a very small (but not significant) increase in the cAMP content of oocytes (10.5, 6.4, 7.9 and 7.3 fmol per oocyte, respectively). Forskolin alone was significantly more potent than the previous combinations (15.1 fmol per oocyte) and forskolin + IBMX at 0.5 mmol l^{-1} resulted in the accumulation of high amounts of cAMP (52.6 fmol per oocyte).

Discussion

In bovine cumulus–oocyte complexes, when cAMP synthesis was stimulated in cumulus cells only (with cholera toxin), the maturation rate was not diminished; and, when cAMP contents were increased to a great extent in cumulus cells and in the oocytes (with forskolin), the maturation rate was only slightly diminished or not affected. Forskolin plus IBMX increased cAMP in zona-free oocytes, but to a lesser extent than in cumulus-enclosed oocytes; but a higher proportion of the zona-free oocytes was still at the germinal vesicle stage after 24 h of culture with these agents. This suggests that increases of cAMP in cumulus cells could activate a stimulatory signal for maturation and that this signal could overcome the inhibitory effect of high contents of cAMP in the oocyte itself. This hypothesis is supported by the fact that IBMX is less potent in inhibiting the resumption of meiosis in cumulus-enclosed oocytes than in zona-free oocytes. It could be argued that increasing cAMP in cumulus cells induces maturation by promoting oocyte cAMP hydrolysis. The high contents of cAMP found in complex-derived oocytes of culture with forskolin indicate that cAMP hydrolysis does not prevent accumulation after 6 h. We did not measure cAMP after a longer culture period since the commitment to resume meiosis has already occurred at that time (Sirard *et al.*, 1989).

In mouse cumulus–oocyte complexes, FSH induced germinal vesicle breakdown in the presence of inhibitory concentrations of hypoxanthine, but the hormone had no effect on denuded oocytes. It was suggested that germinal vesicle breakdown was promoted by an FSH-induced stimulatory signal from cumulus cells that would act even in the presence of a meiosis-inhibiting factor (Downs *et al.*, 1988).

In zona-free oocytes, forskolin significantly delayed the resumption of meiosis and stimulated the synthesis of high amounts of cAMP, indicating that the bovine oocyte possesses adenylate cyclase activity and can generate cAMP in sufficient amounts to affect nuclear maturation *in vitro*. However, cholera toxin, pertussis toxin and sodium fluoride had no effect on the maturation rate or on the cAMP content of zona-free oocytes. When these agents were used in combination with the phosphodiesterase inhibitor IBMX, the maturation rate was the same as the maturation rate achieved with IBMX alone; by contrast, the effect of forskolin on nuclear maturation and on cAMP content was potentiated by IBMX.

An immunocytochemical study using various antisera to βγ or α subunits of G proteins showed the presence of G proteins in rat oocyte and bovine spermatozoa (Garty *et al.*, 1988). Moreover, pertussis toxin-catalysed ADP-ribosylation and immunoblotting demonstrated the presence of a pertussis toxin substratum in

Table 1. Effects of adenylate cyclase stimulation on cAMP content in bovine cumulus–oocyte complexes, complex-derived oocytes and zona-free oocytes[a]

Agents	Amount of cAMP (fmol per complex or oocyte)		
	Complexes	Complex-derived oocytes	Zona-free oocytes
Control	48.3 ± 18.9	5.3 ± 1.4	2.5 ± 0.9
IBMX (0.5 mmol l^{-1})	39.2 ± 10.8	9.2 ± 1.3	2.1 ± 0.2
IBMX (2 mmol l^{-1})	45.2 ± 11.0	11.1 ± 3.2	10.5 ± 5.6
Cholera toxin + IBMX (0.5 mmol l^{-1})	1094.9 ± 132.2[b]	18.2 ± 11.4	6.4 ± 2.1
Pertussis toxin + IBMX (0.5 mmol l^{-1})	54.5 ± 19.9	11.1 ± 2.5	3.7 ± 0.8
Forskolin	4876.1 ± 408.5[c]	90.8 ± 8·5[b]	15.1 ± 4.0[b]
Forskolin + IBMX (0.5 mmol l^{-1})	9235.2 ± 1272.6[d]	287.0 ± 63.3[c]	52.6 ± 8.5[c]
NaF + IBMX (0.5 mmol l^{-1})	41.8 ± 4.8	5.1 ± 1.2	7.9 ± 1.3
Prostaglandin E_2 + IBMX (0.5 mmol l^{-1})	37.1 ± 2.0	7.6 ± 2.5	7.3 ± 3.5

[a]Cumulus–oocyte complexes or zona-free oocytes were incubated with the various agents for 6 h. At the end of the incubation, cAMP was determined in intact complexes in oocytes derived from complexes and in zona-free oocytes. Data are expressed as means ± SEM of at least three replicates using 6 to 10 complexes, 8–16 complex-derived oocytes or 8–28 zona-free oocytes per assay.

[b,c,d]Values with different superscript letters indicate significant differences between amount of cAMP in individual treatments (Waller–Duncan variance analysis, $P < 0.05$).

IBMX: 3-isobutyl-1-methylxanthine.

mouse oocytes, eggs and embryos (Allworth *et al.*, 1990; Jones and Schultz, 1990). The demonstration of adenylate cyclase on the membrane of bovine oocytes (Kuyt *et al.*, 1988), and of G proteins in oocytes and the fact that pertussis toxin and cholera toxin were without effect in this study suggest that adenylate cyclase of the bovine oocyte may be uncoupled to G proteins or coupled to G proteins insensitive to cholera toxin and pertussis toxin. Similar conclusions were derived from studies with rat oocytes in which cholera toxin and pertussis toxin were not effective in preventing nuclear maturation in denuded oocytes (Dekel and Beers, 1980; Dekel *et al.*, 1984; Aberdam *et al.*, 1987). Moreover, only forskolin and FSH but not cholera toxin and luteinizing hormone stimulated cAMP synthesis in denuded oocytes (Olsiewski and Beers, 1983). In *Xenopus* oocytes, the action of progesterone on nuclear maturation, in spite of being nucleotide-dependent, is not mediated by Gi; and it was shown that spermatozoa activate *Xenopus* eggs at fertilization by way of a pertussis- and cholera-toxin-insensitive G protein (Sadler *et al.*, 1984; Kline *et al.*, 1991). The adenylate cyclase of mammalian spermatozoa is not activated by hormones, sodium fluoride or cholera toxin (Garbers and Kopf, 1980); it is possible that male and female gametes share an atypical adenylate cyclase.

Prostaglandin E_2 did not affect the maturation rate and the cAMP content of bovine zona-free oocytes. It is not known whether bovine oocytes possess prostaglandin E_2 receptors, but in the light of the results mentioned above, the absence of effect of prostaglandin E_2 is not surprising, since prostaglandin E_2 receptors are coupled to adenylate cyclase via the regulatory protein Gs which seems to be absent in the bovine oocyte.

IBMX at 0.5 mmol l^{-1} was as effective as forskolin in delaying nuclear maturation in zona-free oocytes and it delayed the resumption of meiosis in cumulus-enclosed oocytes, but did not affect the cAMP content of oocytes. Moor and Heslop (1981) also observed that IBMX (1 mmol l^{-1}) did not increase the cAMP content of ovine oocytes. Three possible hypotheses could explain the inhibitory effect of IBMX on meiotic maturation: (i) the effects of IBMX on oocyte maturation are due to their known side effects on adenosine receptors and on Ca^{2+} concentrations (Wells and Kramer, 1981); (ii) IBMX can prevent a drop in oocyte cAMP that occurs early during the culture period, thus preventing a critical variation in cAMP contents; and (iii) IBMX inhibits the degradation of cAMP that is compartmentalized within the oocyte, and this cAMP, even in low concentration, could affect nuclear maturation. In another system, cellular response to cAMP requires concentrations 10 to 100 times lower after activation with β-adrenergic agonists than with forskolin (Dokhac *et al.*, 1986).

IBMX alone, pertussis toxin, sodium fluoride and prostaglandin E_2 did not affect the nuclear maturation in cumulus-enclosed oocytes or the cAMP content of cumulus–oocyte complexes. These agents stimulated the expansion of the cumulus, so it is possible that they had an effect on cAMP synthesis, but this effect was not detected after 6 h of culture. Cumulus-enclosed oocytes cultured in the presence of IBMX, cholera toxin, pertussis toxin, sodium fluoride or prostaglandin E_2 (all with IBMX at 0.5 mmol l^{-1}) contained a mean value of about twice as much cAMP as in zona-free oocytes. The extra cAMP present in cumulus-enclosed oocytes may be due to cAMP trapped in cumulus cell processes embedded in the zona pellucida; but it is also possible that cumulus-enclosed oocytes synthesized more cAMP than zona-free oocytes because of the presence of cumulus cells and the absence of disturbance caused by the removal of cumulus cells and zona pellucida. When cumulus-enclosed oocytes were cultured in the presence of forskolin (alone and with IBMX at 0.5 mmol l^{-1}), oocytes contained 6 and 5.5 times more cAMP than zona-free oocytes cultured in the same conditions. Some cAMP synthesized by cumulus cells in response to forskolin could be transferred to the oocytes, but it does not seem

to equilibrate between cumulus cells and the oocyte, since cumulus-enclosed oocytes cultured with forskolin contained 17 and 54 times more cAMP than control oocytes, while whole complexes contained 101 and 191 times more cAMP than control complexes.

This study shows that, in bovine zona-free oocytes, meiosis is delayed by lower contents of cAMP than in cumulus-enclosed oocytes indicating that increases of cAMP in cumulus cells can result in a signal inducing maturation. The bovine oocyte can synthesize high amounts of cAMP, but adenylate cyclase may not be coupled to regulatory G proteins sensitive to cholera toxin and pertussis toxin.

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