Maintenance of bovine oocytes in prophase of meiosis I by high [cAMP],

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The effects of high intracellular cAMP concentrations ([cAMP],) on germinal vesicle maintenance of bovine cumulus-oocyte complexes were investigated, using 8-bromo-3',5'-cAMP (8-Br-cAMP) or an invasive adenylate cyclase from Bordetella pertussis to increase the [cAMP],. The effects of interactions of these agents with macromolecular supplements in culture medium (fetal calf serum, FCS; polyvinylpyrrolidone, PVP; BSA), and different methods of processing complexes before culture, on subsequent germinal vesicle maintenance by invasive adenylate cyclase were studied. While 8-Br-cAMP was unable to maintain germinal vesicle arrest in the majority of oocytes for 20 h (36% with FCS, 24% with BSA, 18% with PVP), it maintained germinal vesicle arrest in a high proportion of cumulus-enclosed oocytes when BSA or PVP was used (37% with FCS, 52% with BSA, 53% with PVP). The difference in frequency of germinal vesicle maintenance between macromolecular supplements was not related to [cAMP], when assayed after culture for 2 h with invasive adenylate cyclase. Complexes processed in whole follicular fluid were not maintained in meiotic arrest (26%) when cultured with invasive adenylate cyclase and PVP. Complexes processed in follicular fluid with 3-isobutyl 1-methylxanthine (IBMX) plus invasive adenylate cyclase were arrested at the germinal vesicle stage at high frequencies (65%), while those processed in IBMX or IBMX plus 8-Br-cAMP-supplemented follicular fluid had intermediate (43% and 49%, respectively) frequencies of intact germinal vesicles. Oocyte complexes processed in follicular fluid supplemented with IBMX and invasive adenylate cyclase formed morulae and blastocysts (27.2%), as did oocytes processed in follicular fluid alone (26%). Phosphoprotein profiles showed that control oocytes and 8-Br-cAMP-treated oocytes share a profile that is different from that of oocytes treated with invasive adenylate cyclase. These results show that increased [cAMP], reversibly maintains bovine oocytes in meiotic arrest for an extended period without the occurrence of the post-translational protein modifications observed during meiotic resumption or transient arrest.

Introduction

Oocytes of most mammals begin meiosis during fetal development and arrest at prophase I (germinal vesicle, GV, stage). Oocytes overcome this arrest after either hormonal induction in situ or spontaneously after removal from antral follicles. The mechanism by which oocytes are kept in meiotic arrest in antral follicles has been the subject of many investigations. Cho et al. (1974) first implicated intracellular cAMP ([cAMP],) in maintaining meiotic arrest in mammals. In both mice (Schultz et al., 1983; Vivarelli et al., 1983) and rats (Aberdam et al., 1987), agents that maintain high intracellular concentrations of cAMP prevent spontaneous oocyte maturation. In mice (Schultz et al., 1983; Vivarelli et al., 1983) and rats (Aberdam et al., 1987), spontaneous maturation of oocytes is correlated with decreased [cAMP], in both cumulus-oocyte complexes and oocytes. In amphibians (Maller and Krebs, 1977), where hormonal induction of meiotic maturation is required, agents that maintain high [cAMP], also interfere with the induction of meiotic maturation.

Studies of domestic species with long oestrous cycles, compared with laboratory species with short cycles, have failed to produce such clear-cut results and increases in [cAMP], elicit only a transient delay in meiotic resumption, as evaluated by germinal vesicle breakdown. Whereas testosterone and a cAMP analogue maintain meiotic arrest cooperatively in pig oocytes, cAMP alone gives only a small decrease in maturation frequency (Rice and McGaughy, 1981). Bovine oocytes respond
to agents with only a transient maintenance of meiotic arrest (Homa, 1988; Sirard and First, 1988), with the exception of NaF (Sirard, 1990) or pharmacological doses of 3-isobutyl-1-methyl xanthine (IBMX; Homa, 1988). The effects of NaF are irreversible, while the reversibility of IBMX has not been reported.

Agents such as choler toxin and forskolin that directly stimulate adenylyl cyclase by affecting regulatory G-proteins do cause an increase in [cAMP] in pig (Racowsky, 1985a) and sheep (Crosby et al., 1985) oocytes, but either do not maintain (Crosby et al., 1985) or only transiently maintain (Racowsky, 1985a; Sirard, 1990) meiotic arrest. Adenylyl cyclase is found in cattle oocytes (Kuyt et al., 1988), but it is unclear whether it contributes significantly to [cAMP] in domestic species or whether cumulus cells are the main mediators for cAMP concentrations in oocytes, as has been proposed for laboratory species (Racowsky, 1984; Bornslaeger and Schultz, 1985). It is possible that, although meiotic arrest is dependent on high [cAMP], resumption of meiosis I in oocyte complexes is not dependent on decreasing [cAMP] in the face of gonadotrophin stimulation. As has been suggested in mice (Downs et al., 1988) and cows (Aktas et al., 1991a; Aktas, 1994), a positive signal originating from the follicle cells surrounding the oocyte may cause meiotic resumption despite unaltered [cAMP].

A series of studies to determine the effects of agents that increase [cAMP] on the maintenance of meiotic arrest in vitro was initiated to clarify the role of [cAMP] in mediating meiotic arrest in one species with long cycles, i.e. cattle. We report here that an invasive adenylyl cyclase (iAC) increases [cAMP], both in bovine cumulus cells and the oocyte, and keeps the oocyte arrested at the germinal vesicle stage for an extended period. The arrest is reversible and success of arrest is affected by both macromolecular supplement in the medium and procedures for recovering the oocyte-cumulus complexes from the ovary. Parts of this study have been reported as an abstract (Aktas et al., 1990).

**Materials and Methods**

**Oocyte recovery and culture**

Bovine ovaries were obtained at an abattoir (Pecks, Milwaukee, WI) and transported to the laboratory in thermos bottles containing saline (0.9% w/v NaCl) at 35–36°C (4–6 h for collection and transport). Temperature on arrival at the laboratory averaged 31°C. Contents were aspirated from antral follicles 2–4 mm in diameter. When 5 ml of pooled follicular fluid (FF) from the experimental groups had been collected, it was supplemented with one of the agents that increase intracellular [cAMP], depending on the individual experiments. Follicular fluid from control groups was not supplemented unless stated otherwise. Complete aspiration, washing and selection of the oocytes took an average of 2–3 h until all treatment groups were filled for a replicate. Oocytes with an intact, compact cumulus investment were selected and washed twice in CO2-equilibrated TC-199 with Earle’s salts (Gibco, Grand Island, NY), using a stereomicroscope and were then transferred to culture (Leibfried and First, 1979).

In Expts 1 and 2, the interaction between agents increasing oocyte [cAMP], and different macromolecular supplements in the culture medium was investigated. All reagents were purchased from Sigma (St Louis, MO), unless otherwise stated. Either 10 mmol 8-bromo-3’5’ cAMP l−1 (8-Br-cAMP), shown by Homa (1988) to be the most effective analogue and the most effective concentration for bovine oocytes, or 20 U iAC ml−1 from Bordetella pertussis (kindly provided by E. Hewlett, University of Virginia Medical School); activity of the enzyme that maintained 50% bovine denuded oocytes in meiotic arrest was considered 10 U, arbitrarily) were used to increase [cAMP], heat-treated fetal calf serum (FCS; 10% v/v, Gibco), BSA (3 mg ml−1, Fraction V) or polyvinylpyrrolidone (PVP; 40 kDa molecular mass, 3 mg ml−1) were used as macromolecular supplements. Oocytes were cultured in TC-199 medium (with Earle’s salts) supplemented with pyruvate (0.2 mmol l−1), gentamicin (25 µg ml−1) and meiotic inhibitors, as required for a particular experiment. Treatment groups were supplemented with 10 mmol 8-Br-cAMP l−1 for Expt 1, and 20 U invasive adenylyl cyclase ml−1 for Expt 2. Oocytes were cultured in 96-well culture dishes, with 25 complexes in 125 µl medium in 5% CO2 in air with high humidity at 39°C for 20 h. Follicular fluid aspirated from antral follicles was supplemented with 0.5 mmol IBMX l−1 + 0.5 mmol 8-Br cAMP l−1 for Expt 1 and 0.5 mmol IBMX l−1 + 2 U invasive adenylyl cyclase ml−1 for Expt 2. In these and all experiments, all treatments were represented in each replicate.

Experiment 3 was designed to compare different preinculture treatments that might prevent commitment before culture (i.e., during aspiration, collection and washing). Follicular fluid was supplemented with either IBMX (0.5 mmol l−1), IBMX + 8-Br-cAMP (0.5 mmol l−1), IBMX + invasive adenylyl cyclase (2 U ml−1) or there was no supplement. Again, each treatment was represented in every replicate. Oocytes collected from each preinculture treatment were randomly assigned for subsequent culture in TC-199 with PVP, with or without invasive adenylyl cyclase, for 20 h. Intracellular cAMP in whole complexes and cumulus-free oocytes was measured to determine whether differences in maintenance of germinal vesicle arrest among different treatments in Expts 2 and 3 were related to differences in initial cAMP concentrations when invasive adenylyl cyclase was present. In Expt 2, cAMP was measured after oocytes had been in culture for 2 h. In Expt 3, [cAMP] was measured after processing, at the time when oocytes would normally be put in to culture.

Experiment 4 was designed to study the reversibility of inhibition by invasive adenylyl cyclase. Cumulus-oocyte complexes derived from FF treated with IBMX (0.5 mmol l−1) + invasive adenylyl cyclase (2 U ml−1) and subsequently cultured in culture medium supplemented with PVP and invasive adenylyl cyclase (20 U ml−1) for 20 h were washed twice in pre-equilibrated TC-199, transferred to TC-199 + PVP without invasive adenylyl cyclase and incubated for an additional 20 h. A group of cumulus-oocyte complexes recovered from non-supplemented FF and cultured without invasive adenylyl cyclase served as the control.

**Preparation of oocytes for fertilization and embryo culture**

In Expt 5, oocytes (ten in a drop) recovered from FF without supplements or FF supplemented with IBMX + invasive
adenylate cyclase were subsequently cultured in TC-199 with LH (5 μg ml⁻¹), FSH (0.5 μg ml⁻¹) (both gonadotrophins were ovine derivatives provided by NIH), oestradiol (1 μg ml⁻¹), gentamicin (25 μg ml⁻¹) and pyruvate (0.2 mmol l⁻¹) in 50 μl droplets under paraffin oil in 5% CO₂ with high humidity at 39°C (Lenz et al., 1983) for 22 h (Sirard et al., 1988). Frozen-thawed semen (American Breeders Service, DeForest, WI) was used for fertilization using a swim-up procedure (Parrish et al., 1986). Fertilization medium was a modified Tyrode’s solution supplemented with fatty-acid-free BSA (6 mg ml⁻¹), pyruvate, gentamicin, hypotaurine, penicillamine, adrenaline and heparin (Leibfried and Bavister, 1982; Parrish et al., 1986). Glucose was eliminated from the formulation. The final concentration of spermatozoa was 10⁶ ml⁻¹. Embryos were stripped of cumulus cells and transferred onto oviductal cell monolayers (Eyestone and First, 1989) 48 h after insemination, cultured for an additional 4 days and then scored for morulae and blastocyst formation.

Radionuclide labelling and two-dimensional gel electrophoresis of oocyte phosphoproteins

Experiment 6 compared phosphoprotein profiles of oocytes recovered and cultured with 8-Br-cAMP, or invasive adenylate cyclase, or in control media for a total of 9 h. Cumulus–oocyte complexes from each treatment were cultured in phosphate-free TC-199 medium supplemented with 0.5 μCi [³²P]orthophosphate ml⁻¹ (Amersham, Arlington Heights, IL) during the last 2 h 15 min. At the end of the labelling period, the cumulus cells were removed in phosphate-free TC-199 and washed in phosphate-free TALP–Hepes solution (Bavister et al., 1983). Seventy-five oocytes were pooled in 4 μl flash-frozen in liquid nitrogen and stored at −20°C until electrophoresis.

Before electrophoresis, the samples were frozen and thawed several times and 1 μl RNAse–DNase (20 μmol Tris–HCl 1⁻¹, 10 mmol MgCl₂ 1⁻², 60 mmol NaF 1⁻⁻, 2.2 mmol PMSF 1⁻⁻, 0.5 mg RNAse ml⁻¹ and 0.1 mg DNase ml⁻¹) was added. Samples were incubated on ice for 30 min. A lysis buffer (9.5 mol urea 1⁻⁻, 10% w/v CHAPS, 1.5% pH 5–7, 1.5% pH 6–8 and 1% pH 3–10 ampholytes; Bio-Rad, Hercules CA) with a trace of fast green was added. The samples were centrifuged at 16 000 g for 10 min, and the supernatant was loaded onto tube gels (7 cm in length). The gel mixture was 9.15 mol urea 1⁻⁻, 10% (w/v) CHAPS, 4% acrylamide and ammonium (1.5% pH 5–7, 1.5% pH 6–8, 1% pH 3–10). The upper chamber buffer was 20 mmol NaOH 1⁻⁻ and the lower chamber buffer was 10 mmol H₃PO₄ 1⁻⁻. The gels were initially run for 10 min at 200 V, 15 min at 300 V and 15 min at 400 V before the samples were loaded. Loaded gels were run at 750 V for 3.5 h. The first dimension gels were excised, incubated in solubilization buffer and loaded onto 8–15% (w/v) SDS slab gels with molecular weight markers (Laemmli, 1970). Silver-stained gels were dried between two pieces of cellophane paper under a vacuum. Preflashed X-ray film (X-AR-QMAT) was exposed for 3 days with intensifying screens, developed and the pixel intensities normalized using Collage (Photodynne, Hartland, WI) software. The data were analysed by completely randomized design to eliminate errors associated with replicates.

Measurements of cAMP

Oocytes were either stripped of cumulus cells by repeatedly aspirating complexes through a glass pipette (200 μm i.d.), or left intact and washed three times at 4°C in the presence of 0.5 mmol IBMX 1⁻⁻ in a modified Tyrode’s solution without glucose (TL-Hepes; Bavister et al., 1983). A fourth wash was carried out under the same conditions in the absence of IBMX. Each experiment was performed three times and duplicate samples were obtained for each replicate. Samples of oocyte–cumulus complexes or 50 denuded oocytes were transferred to 150 μl extraction medium (TC-199) in 10 μl of the last wash. Similar amounts of the last wash were used as blanks for each group and the cAMP concentration (background noise) in this blank was subtracted from the cAMP concentration measured from corresponding samples. Trichloroacetic acid (10 μl of 100% w/v, TCA) was added to the samples, and they were then vortexed for 30 s and centrifuged at 16 000 g for 8 min at 4°C. Twice the volume of a 4:6 mixture of tri-n-octylamine (Sigma) and 1,1,2-trichlorofluorocethane (Aldrich, Milwaukee, WI) were added to extract the TCA (Chen et al., 1977; Schoff et al., 1989). Samples were frozen in liquid nitrogen and stored at −70°C until assayed. A radioimmunoassay kit from Biomedical Technologies, Inc. (Stoughton, MA) was used to measure cAMP; the sensitivity of the assay was 5 fmol and highly specific antibodies raised against succinyl cAMP-tyrosine methyl ester were used. [³²P]succinyl cAMP-tyrosine methyl ester was used as a tracer.

The assay was validated by adding increasing numbers (20, 40, 60, 80) of oocytes to a constant volume of extraction medium, as well as by adding standard amounts of cAMP to samples and recovering proportional amounts of cAMP. No allowance was made for extraction losses which were presumed to be 5–10% (Chen et al., 1977).

Data evaluation and statistical analysis

For evaluation of meiotic stage after culture, oocytes were mounted on slides beneath a coverslip supported by 3:1 petroleum jelly:paraffin, fixed and cleared in acid alcohol (3:1 ethanol:acetic acid) and examined × 400 magnification using Nomarski optics. Oocytes were classified as: germinal vesicle stage, with intact nuclear membranes; intermediate, if after germinal vesicle breakdown to metaphase I; or mature, if at anaphase I to metaphase II.

Percentage data were analysed by one-way analysis of variance (ANOVA) with or without arcsine transformation, and the same results were obtained in each case. Means were compared using the Newman–Keuls method, if ANOVA revealed significant differences between treatments. The percentage of oocytes remaining at the germinal vesicle stage was used as the end point for comparison of treatments with inhibitors. The percentage of oocytes matured was the end point for comparison of control groups and reversal of invasive adenylate cyclase maintained arrest. The percentage of morulae and blastocysts was used as the end point for embryo development. The content of cAMP (fmol) per oocyte or per oocyte–cumulus complex was used as the end point for comparison of cAMP measurements. The cAMP data (obtained
in conjunction with Expt 3) were analysed both by including and excluding samples derived from follicular fluid supplemented with IBMX + 8-Br-cAMP. Correlations between cAMP concentration and the frequency of oocytes remaining at the germinal vesicle stage were calculated by simple linear regression after excluding the treatment group derived from FF supplemented with IBMX + 8-Br-cAMP.

Results

Interaction of macromolecular supplements with 8-Br-cAMP

The results from Expt 1, in which cumulus–oocyte complexes recovered from FF supplemented with IBMX + 8-Br-cAMP were cultured for 20 h with different macromolecular supplements (BSA, PVP, FCS) with or without 8-Br-cAMP (10 mmol l⁻¹), are given in Table 1. Regardless of macromolecular supplement, only a small proportion of the oocytes remained at the germinal vesicle stage after 20 h of culture when incubated with 8-Br-cAMP. The frequency of germinal vesicle arrest did not differ statistically between treatments containing 8-Br-cAMP. The majority of the oocytes were at metaphase I, indicating a transient delay in meiotic resumption. No further attempt was made to use lower or higher doses, since this has been done by others (Homa, 1988). Oocytes in control groups reached metaphase II at reasonably high frequencies regardless of the macromolecular supplement present.

Interaction of invasive adenylate cyclase with different protein supplements

In Expt 2, oocytes were recovered from FF supplemented with IBMX + invasive adenylate cyclase and cultured for 20 h with different macromolecular supplements (FCS, PVP, BSA), with or without 20 U invasive adenylate cyclase ml⁻¹ (Table 2). Oocytes cultured in medium containing FCS had a lower frequency (P < 0.05) of germinal vesicle maintenance (36%) than did those cultured with either PVP (53%) or BSA (52%). Approximately half of the oocytes cultured with either BSA or PVP in the presence of invasive adenylate cyclase maintained an intact germinal vesicle for 20 h. Most of the remaining oocytes in these three treatments also showed a transient delay in germinal vesicle breakdown, since few were classified as meiotically mature at 20 h. Again, oocytes matured equally well in all three macromolecular supplements.

In conjunction with this experiment, cAMP concentrations in whole complexes and in oocytes alone were measured after the complexes had been cultured in invasive adenylate cyclase (20 U ml⁻¹) for 2 h with different macromolecular supplements to determine whether differences in frequency of germinal vesicle maintenance could be correlated with differences in cAMP concentrations (Table 3). Neither complexes nor

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**Table 1.** Effect of 8-bromo cAMP (8-Br-cAMP) on germinal vesicle (GV) maintenance in bovine cumulus–oocyte complexes cultured with different macromolecular supplements

<table>
<thead>
<tr>
<th>Supplement</th>
<th>8-Br-cAMP (mmol l⁻¹)</th>
<th>GV (%) ± SE</th>
<th>Intermediate (%) ± SE</th>
<th>Mature (%) ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCS</td>
<td>0</td>
<td>36 ± 7.4</td>
<td>58 ± 6.9</td>
<td>6 ± 2.4</td>
</tr>
<tr>
<td>FCS</td>
<td>0</td>
<td>7 ± 2.1</td>
<td>23 ± 3.8</td>
<td>70 ± 3.7</td>
</tr>
<tr>
<td>PVP</td>
<td>10</td>
<td>18 ± 10.7</td>
<td>60 ± 10.2</td>
<td>16 ± 2.4</td>
</tr>
<tr>
<td>BSA</td>
<td>10</td>
<td>26 ± 5.1</td>
<td>55 ± 4.1</td>
<td>19 ± 4.5</td>
</tr>
<tr>
<td>BSA</td>
<td>0</td>
<td>2 ± 2.2</td>
<td>28 ± 2.8</td>
<td>70 ± 2.8</td>
</tr>
</tbody>
</table>

FCS: fetal calf serum; PVP: polyvinylpyrrolidone.

*Cumulus–oocyte complexes were collected in follicular fluid with 3-isobutyl 1-methylxanthine (IBMX 0.5 mmol l⁻¹) + 8 bromo-cAMP (0.5 mmol l⁻¹) added at 3 ml volume. After three washes in control medium, complexes were placed in TC-199 plus indicated additives for 20 h.

Total number of oocytes within each treatment ranged from 71 to 100 over five replications. No difference between treatments was observed.

**Table 2.** Effect of adenylate cyclase on germinal vesicle (GV) maintenance in bovine cumulus–oocyte complexes cultured with different macromolecular supplements

<table>
<thead>
<tr>
<th>Supplement</th>
<th>iAC (U ml⁻¹)</th>
<th>GV (%) ± SE</th>
<th>Intermediate (%) ± SE</th>
<th>Mature (%) ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCS</td>
<td>20</td>
<td>36 ± 3.8</td>
<td>60 ± 3.7</td>
<td>4 ± 2.2</td>
</tr>
<tr>
<td>FCS</td>
<td>0</td>
<td>8 ± 5.4</td>
<td>37 ± 3.9</td>
<td>55 ± 4.1</td>
</tr>
<tr>
<td>BSA</td>
<td>20</td>
<td>52 ± 3.7</td>
<td>46 ± 4.3</td>
<td>2 ± 1.9</td>
</tr>
<tr>
<td>BSA</td>
<td>0</td>
<td>7 ± 3.0</td>
<td>32 ± 4.6</td>
<td>61 ± 2.2</td>
</tr>
<tr>
<td>PVP</td>
<td>20</td>
<td>53 ± 4.7</td>
<td>43 ± 5.1</td>
<td>3 ± 1.8</td>
</tr>
<tr>
<td>PVP</td>
<td>0</td>
<td>16 ± 3.4</td>
<td>37 ± 2.7</td>
<td>47 ± 4.3</td>
</tr>
</tbody>
</table>

FCS: fetal calf serum; PVP: polyvinylpyrrolidone; iAC: invasive adenylate cyclase.

*Cumulus–oocyte complexes were collected in follicular fluid with invasive cyclase (2 U ml⁻¹) + 3-isobutyl 1-methylxanthine (IBMX 0.5 mmol l⁻¹) added at 3 ml volume. After three washes in control medium, complexes were placed in TC-199 medium plus the indicated additives for 20 h.

The total number of oocytes within each treatment ranged from 82 to 120 over five replications. Maintenance of germinal vesicle (GV) arrest with iAC presents within treatments with different superscripts is significantly different (P < 0.05). The percentage intermediate was not analysed.

**Table 3.** [cAMP], in bovine oocyte complexes and in oocytes cultured with adenylate cyclase and different macromolecular supplements

<table>
<thead>
<tr>
<th>Macromolecular supplement</th>
<th>cAMP (fmol per complex)</th>
<th>cAMP (fmol per oocyte)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCS</td>
<td>119.4 ± 32.7</td>
<td>7.7 ± 0.7</td>
</tr>
<tr>
<td>PVP</td>
<td>152.8 ± 11.4</td>
<td>7.5 ± 1.6</td>
</tr>
<tr>
<td>BSA</td>
<td>177.0 ± 21.8</td>
<td>9.0 ± 1.7</td>
</tr>
</tbody>
</table>

FCS: fetal calf serum; PVP: polyvinylpyrrolidone.

*Complexes were incubated for 2 h in invasive adenylate cyclase (20 U ml⁻¹), and then processed for cAMP assay. Some were stripped of cumulus (50 oocytes in a sample), while others were measured as whole complexes (5 complexes in a sample). Duplicate samples were obtained for every treatment within each replicate. Values are means for three replications.
cumulus-free oocytes had statistically different cAMP concentrations, suggesting that the lower frequency of germinal vesicle maintenance, when cultured with FCS, was due to interaction of yet unknown factors in FCS with cAMP. The use of FCS as a supplement in culture medium when studying meiotic arrest was discontinued from this point. In this experiment no control measurements were made of untreated oocytes or complexes; however, such observations were made in a later experiment (Table 5). Comparison of the estimates of cAMP concentrations show that they were about ten times higher in the presence of invasive adenylate cyclase.

Effect of supplementing follicular fluid during collection on subsequent maintenance of meiotic arrest

Sirard and First (1988) suggested that bovine oocytes commit to undergo meiosis soon after removal from the follicle. Our preliminary studies also suggested that care should be taken to prevent premature meiotic commitment during aspiration, collection and wash procedures. For that reason, it was necessary to develop a practical system to prevent commitment while collecting and washing the bovine material. This was done in Exp3. Follicular fluid was supplemented with IBMX (0.5 mmol l⁻¹), IBMX plus 8-Br-cAMP (0.5 mmol l⁻¹), IBMX plus invasive adenylate cyclase (2 U ml⁻¹), or for the controls there was no supplement. Oocytes recovered from these four different preculture collection treatments were allocated randomly for culture with or without invasive adenylate cyclase (20 U ml⁻¹) for 20 h (Table 4). Concentrations of cAMP in complexes or oocytes were measured at the time when they would have been transferred to culture, to determine whether preculture procedures affected [cAMP], at the onset of culture (Table 5).

Oocytes from FF supplemented with IBMX + invasive adenylate cyclase had the highest frequency of germinal vesicles (65%), whereas arrested oocytes from FF supplemented with IBMX alone (43%) or IBMX plus 8-Br-cAMP (49%) were intermediate (Table 4). The oocytes treated with invasive adenylate cyclase recovered from unsupplemented FF (26%) were similar to controls (P > 0.05). These data suggest that bovine oocytes begin commitment to undergo meiotic maturation if they are kept in FF for ≥ 2 h. None of the pretreatments affected the frequency of maturation in control groups.

The [cAMP]ᵢ of both complexes (28 fmol) and oocytes (0.97 fmol) recovered from IBMX + iAC FF supplement was higher than that of complexes (10.9 fmol) or oocytes (0.5 fmol) derived from unsupplemented FF (P < 0.05, Table 5) when measured after preculture treatments. The complexes and the oocytes recovered from FF with IBMX added had intermediate [cAMP]ᵢ (15.15 and 0.54 fmol in complexes and oocytes, respectively), which correlates with their degree of retention of the germinal vesicle. Oocytes recovered from FF supplemented with IBMX plus 8-Br-cAMP had very high contents of [cAMP]ᵢ mostly attributable to the cAMP analogue, yet maintenance of meiotic arrest was only comparable with oocytes recovered from FF supplemented with IBMX alone. The role of cAMP in meiotic arrest was assessed by estimating the correlation between the maintenance of the germinal vesicle and the concentration of cAMP after the different pretreatments. The correlation was 0.93 for cumulus–oocyte complexes and 0.78 for denuded oocytes, when oocytes derived from IBMX + 8-Br-cAMP supplemented FF were not considered. This group was excluded from further analysis as representing an outlier.

Reversibility of inhibition

Cumulus–oocyte complexes recovered from IBMX + invasive adenylate cyclase supplemented FF and cultured in the presence of iAC for 20 h were washed twice and cultured in the same medium without invasive adenylate cyclase for an additional 20 h to test whether the effect of iAC on oocytes was reversible. The results (Table 6) show that inhibition of germinal vesicle breakdown by invasive adenylate cyclase was reversible. Although not statistically different, the slightly lower frequency for completion of meiotic maturation may be

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### Table 4. Effect of preculture collection and wash procedures on germinal vesicle (GV) maintenance in bovine oocyte complexes subsequently cultured with invasive adenylate cyclase (iAC)

<table>
<thead>
<tr>
<th>Inhibitor in follicular fluid</th>
<th>iAC (U ml⁻¹)</th>
<th>GV (%) ± SEM</th>
<th>Intermediate (%) ± SEM</th>
<th>Mature (%) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBMX + iAC</td>
<td>20</td>
<td>66 ± 2.8ᵇ</td>
<td>31 ± 4.7</td>
<td>4 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>10 ± 3.1</td>
<td>33 ± 5.2</td>
<td>57 ± 5.0</td>
</tr>
<tr>
<td>IBMX + 8-Br-cAMP</td>
<td>20</td>
<td>49 ± 4.9ᵇ</td>
<td>43 ± 5.1</td>
<td>9 ± 2.7</td>
</tr>
<tr>
<td>IBMX</td>
<td>0</td>
<td>10 ± 2.9</td>
<td>37 ± 3.7</td>
<td>53 ± 4.4</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>43 ± 4.9ᵇ</td>
<td>52 ± 5.4</td>
<td>5 ± 2.1</td>
</tr>
<tr>
<td>None</td>
<td>20</td>
<td>26 ± 5.0ᵃ</td>
<td>62 ± 3.8</td>
<td>13 ± 3.4</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>9 ± 2.7</td>
<td>24 ± 4.4</td>
<td>67 ± 3.5</td>
</tr>
</tbody>
</table>

ᵇValues for treatments having iAC present with different superscripts are significantly different (P < 0.05). The percentage of intermediates was not analysed.

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### Table 5. [cAMP]ᵢ after preculture procedures for collecting bovine cumulus–oocyte complexes

<table>
<thead>
<tr>
<th>Inhibitor added to follicular fluid</th>
<th>cAMP (fmol per complex) ± SEM</th>
<th>cAMP (fmol oocyte per) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBMX + iAC</td>
<td>27.9 ± 4.5ᵇ</td>
<td>0.97 ± 0.16ᵇ</td>
</tr>
<tr>
<td>IBMX + 8-Br-cAMP</td>
<td>240.4 ± 94.7ᵇ</td>
<td>13.47 ± 5.90ᵇ</td>
</tr>
<tr>
<td>IBMX</td>
<td>15.2 ± 2.9ᵇ</td>
<td>0.54 ± 0.05ᵇ</td>
</tr>
<tr>
<td>None</td>
<td>10.9 ± 1.5ᶜ</td>
<td>0.50 ± 0.04ᶜ</td>
</tr>
</tbody>
</table>

ᵇWithin a column, values for treatments with different superscripts are significantly different (P < 0.05). Means are presented for three replications. Fifty denuded oocytes and five complexes were assayed per sample. Duplicate samples of each treatment were taken at every replication.
Table 6. Reversibility of meiotic arrest in vitro of bovine oocytes maintained by invasive adenylyl cyclase (iAC)

<table>
<thead>
<tr>
<th>Inhibitor in culture</th>
<th>GV (%) ± SEM</th>
<th>Intermediate (%) ± SEM</th>
<th>Mature (%) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>iAC</td>
<td>58 ± 4.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>41 ± 3.3</td>
<td>3 ± 1.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>11 ± 5.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>29 ± 5.8</td>
<td>60 ± 6.7&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Reversal&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>15 ± 3.8&lt;sup&gt;d&lt;/sup&gt;</td>
<td>36 ± 3.6</td>
<td>49 ± 7.0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

GV: germinal vesicle.
<sup>a</sup>Reversal: complexes cultured with iAC for 20 h, and then cultured for a further 20 h without iAC. Total number of oocytes within each treatment ranged from 73 to 99 over five replications.
<sup>b</sup>Within a column, values for treatments with different superscripts are significantly different (P < 0.05). The percentage of intermediates was not analysed.

Table 7. Development of bovine oocytes after suppressing meiotic resumption during collection procedures

<table>
<thead>
<tr>
<th>Inhibitor added to follicular fluid</th>
<th>n</th>
<th>&gt; 2 cell (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Morulae and blastocysts (%)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBMX + iAC</td>
<td>283</td>
<td>54</td>
<td>27</td>
</tr>
<tr>
<td>None</td>
<td>342</td>
<td>55</td>
<td>20</td>
</tr>
</tbody>
</table>

<sup>a</sup>The number of fertilized oocytes cleaving at least once 48 h after adding spermatozoa/total number of oocytes cultured (n) averaged over three replicates.
<sup>b</sup>Number of morulae and blastocysts observed 6 days after fertilization/total oocytes (n) cultured averaged over three replicates.

due to the time necessary for neutralization or clearance of invasive adenylyl cyclase from the cells.

Developmental capacity of oocytes recovered from follicular fluid supplemented with IBMX + invasive adenylyl cyclase

Experiment 5 was designed to evaluate possible detrimental effects of supplementing FF with IBMX plus invasive adenylyl cyclase during the 2–3 h processing period on the subsequent developmental potential of oocytes. Treated oocytes were cultured in standard maturation medium for 24 h, and were then fertilized and evaluated for development with a control group recovered from FF that was not supplemented. Oocytes in both groups cleaved and progressed to morula and blastocyst stages in vitro at equal frequencies (Table 7), indicating that supplementation of FF with IBMX plus invasive adenylyl cyclase during collection was not detrimental.

Phosphoprotein profiles of bovine oocytes

In Expt 6, phosphoprotein profiles of oocytes maintained in meiotic arrest permanently by treatment with invasive adenylyl cyclase or transiently by 8-Br-cAMP were analysed. Cumulus-enclosed oocytes were cultured for 9 h with either 20 U iAC ml<sup>−1</sup>, 10 mmol 8-Br-cAMP l<sup>−1</sup> or in control medium. The treatment groups were transferred to similar culture medium (phosphate-free TC-199 medium with added [<sup>32</sup>P]orthophosphate) for the last 2 h 15 min of culture.

Fig. 1. Phosphoprotein profiles of bovine oocytes maintained in meiotic arrest with (a) (iAC) invasive adenylyl cyclase, (b) transiently with 8-bromo-cAMP or (c) maturing spontaneously. Complexes were cultured in 20 IU iAC ml<sup>−1</sup>, 10 mmol 8-Br-cAMP l<sup>−1</sup> or control medium only for a total of 9 h. For the last 2 h 15 min, the complexes were transferred to phosphate-free medium supplemented with [<sup>32</sup>P]orthophosphate (0.5 µCi ml<sup>−1</sup>) and the appropriate inhibitor. At the end of culture, cumulus cells were removed and oocytes were washed in phosphate-free TL-Hepes several times. A gel is shown that used 75 oocytes per lane, selected from five replications, where each replicate used oocytes collected on separate days. Two 29 kDa proteins (circled) are shown which become minimally phosphorylated in transiently arrested and spontaneously maturing oocytes. Arrows indicate 24, 25, and 2 kDa proteins, which become more heavily phosphorylated in these two types of oocyte.
adenylate
found
post-translational
moreover, as 8-Br-cAMP
premature
aspects
the
during
24 protein
minimally
27,
(maintained
Oocytes
aPixel
25
27
29
Protein
adenylate
Table
8. Phosphoproteins in bovine oocytes maturing sponta-
neously, or maintained in germinal vesicle arrest with invasive
adenylate cyclase (iAC), or transiently with 8-bromo-cAMP
(8-Br-cAMP)

<table>
<thead>
<tr>
<th>Protein (kDa)</th>
<th>Intensity (pixels × 10⁻³) ± s.e.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>29</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>29</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>27</td>
<td>115 ± 8</td>
</tr>
<tr>
<td>25</td>
<td>47 ± 5</td>
</tr>
<tr>
<td>24</td>
<td>30 ± 1</td>
</tr>
</tbody>
</table>

*Pixel intensities from gels were averaged over five replications, each consisting of gel's run with oocytes recovered on different days.

Oocytes completely maintained in meiotic arrest (i.e., cultured in the presence of invasive adenylate cyclase, 90% at germinal vesicle stage at 9 h) showed phosphoprotein profiles dissimilar to those of mature oocytes (Fig. 1, Table 8). These oocytes contained two prominent 29 kDa phosphoproteins. Moreover, 27, 25 and 24 kDa proteins seen in mature oocytes were only minimally phosphorylated. Oocytes cultured with 8-Br-cAMP (maintained in meiotic arrest transiently) showed a phospho-
protein profile indistinguishable from control oocytes (10% at germinal vesicle stage), although they still had distinct germinal vesicles (85% intact germinal vesicles). Oocytes cultured with 8-Br-cAMP and those cultured in control medium showed maturation related phosphoproteins of 27, 25 and 24 kDa and two 29 kDa proteins that were minimally phosphorylated compared with oocytes treated with invasive adenylate cyclase. These differences were obviously and consistently present in all five replications and indicated that, during transient delay of meiotic arrest, post-translational modifications in proteins related to maturation still occur.

Discussion
These studies have demonstrated that bovine oocytes can be reversibly maintained in meiotic arrest for an extended period by increasing [cAMP]; therefore, cAMP may play a role in maintaining meiotic arrest in bovine oocytes as in other animals. Precautions to prevent premature commitment and choice of macromolecular media supplements were important aspects of this model. Use of a preculture treatment to prevent premature commitment to meiotic resumption did not impair the developmental potential of the oocytes. Bovine oocytes could not be kept in meiotic arrest with cAMP analogues such as 8-Br-cAMP or dibutyryl-cAMP (db-cAMP, data not shown); moreover, the phosphoprotein profiles of oocytes treated with 8-Br-cAMP clearly demonstrated that during transient arrest, post-translational modification of proteins, characteristic of maturing oocytes, continue to occur. These changes were not found in oocytes maintained in meiotic arrest with invasive adenylate cyclase.

Since the demonstration by Maller and Krebs (1977) that progesterone-induced oocyte maturation in Xenopus is inhibited by analogues of cAMP or phosphodiesterase inhibitors, numerous studies have been undertaken to elucidate possible functions of cAMP in maintaining mammalian oocytes in meiotic arrest (for review, see Schultz, 1988). It is now considered that intracellular concentrations of cAMP regulate the meiotic state of oocytes in many amphibians (Maller and Krebs, 1977) and mammals (Bornslaeger et al., 1986). Both progesterone-induced Xenopus oocyte maturation (Cicirelli and Smith, 1985) and spontaneous mouse oocyte maturation (Schultz et al., 1983) are accompanied by a decrease in intra-oocyte cAMP concentrations. Interference with this reduction prevents maturation (Schultz et al., 1983; Vivarelli et al., 1985).

Several studies (Rice and McGaughhey, 1981; Homa, 1988; Sirard and First, 1988) carried out with oocytes of mammals with long oestrous cycles have failed to produce unequivocal results. Studies with oocytes (Tormell et al., 1984) and other cells in vitro (for review, see Lohmann and Walter, 1984) have shown that cAMP analogues may differ and that their effects on cell growth may be different from those of cellular cAMP. As reviewed by Lohman and Walter (1984), the action of cAMP and its analogues may be modified by the presence of serum, growth factors and hormones in culture medium, as we observed in the bovine model for studying meiotic regulation. The conflicting results reported on the effect of cAMP on oocyte maturation in domestic animals may result from different means of increasing [cAMP], and different culture conditions (i.e., supplementation with serum or hormones). A further complication arises because bovine oocytes (Sirard and First, 1988), like those of other species (Dekel and Beers, 1980; Schultz et al., 1983), may commit to undergo meiosis during the collection process. These experiments were therefore conducted to describe the optimal culture conditions and means to increase [cAMP], for the study of effects of cAMP on bovine oocyte maturation.

The effects of increased [cAMP], were investigated by using cumulus-intact bovine oocytes to examine different agents that increase [cAMP], and determining how these substances interact with the macromolecular components of maturation medium. An attempt was also made to describe an optimal system for collecting these oocytes before culture. Homa (1988) demonstrated that 8-Br-cAMP was more potent than dibutyryl-cAMP in inhibiting bovine oocyte maturation. Preliminary studies showed that the same concentration of 8-Br-cAMP was more potent than db-cAMP when inhibition of progression beyond metaphase I was taken as an end point (data not shown). Our studies showed that 8-Br-cAMP, even when used at a concentration of 10 mmol l⁻¹ (reported by Homa, 1988, to be the most effective concentration in maintaining bovine oocytes in meiotic arrest transiently) was a weak inhibitor of germinal vesicle breakdown of bovine oocytes, and invasive adenylate cyclase could inhibit germinal vesicle breakdown in bovine oocytes at a high frequency with reproducible results, when PVP and BSA were used as macromolecular supplements in culture medium. The correlation between cAMP concentration in oocytes and complexes and inhibition of germinal vesicle breakdown suggests that the enzyme acts through cAMP. By contrast, analogues of cAMP were less effective at preventing germinal vesicle breakdown.
Fulka et al. (1993), by fusing mouse and bovine oocytes, showed that cAMP analogues fail to block germinal vesicle breakdown in both mouse and bovine oocyte nuclei within the same cytoplasm, even at doses as high as 5 mmol l⁻¹. However, both types of nuclei in the fused cell remained intact when cultured with IBMX (1 mmol l⁻¹). These data suggest that, although bovine oocytes are insensitive to cAMP analogues and that maturation promoting factor created in bovine oocyte cytoplasm in the presence of these analogues causes germinal vesicle breakdown in both types of nucleus, bovine oocytes are sensitive to changes in concentration of cellular cAMP. The demonstration by Floma (1988) that IBMX, a phosphodiesterase inhibitor that causes accumulation of cAMP, was far more effective than either dibutyryl-cAMP or 8-Br-cAMP in maintaining meiotic arrest is also in agreement with our results. The weak effect of 8-Br-cAMP may be due to its inability to cross bovine oocyte cell membranes. However, our measurements of the cAMP content of bovine oocytes showed that 8-Br-cAMP would accumulate in bovine oocytes very efficiently. Under the assay conditions used in the study reported here, similar concentrations of 8-Br-cAMP and standard cAMP displaced [¹²⁵I]-labelled tracer to the same extent. The difference between the ability of 8-Br-cAMP and cAMP produced by invasive adenylate cyclase to maintain meiotic arrest may be due either to a difference in their subcellular distribution or in their ability to bind regulatory subunits on the cAMP-dependent protein kinase.

Earlier work with sheep (Moor and Heslop, 1981; Crosby et al., 1985; Moor, 1988) suggested that increased [cAMP] would not inhibit oocyte maturation. Gonadotrophins included during the culture of ovine oocytes, rather than species difference, may account for the observed differences in patterns of [cAMP]. A positive stimulus from follicle cells induced by gonadotrophins may cause nuclear maturation without a concomitant fall in cAMP concentration (Downs et al., 1988) as we have observed in cattle.

An invasive adenylate cyclase has been used by Aberdam et al. (1987) at higher concentrations and in combination with IBMX and pertussis toxin on rat oocytes, and results comparable to those reported here were obtained. Aberdam et al. (1987) and others (Wheeler and Veldhuis, 1988; Gilboa-Ron et al., 1989; Gordon et al., 1989) showed that the invasive adenylate cyclase accumulated in the host cells, and was inactivated by the host cells in a time-dependent fashion if cells were transferred to a medium without invasive adenylate cyclase. The concentration of crude preparations of invasive adenylate cyclase used initially was inferred from a previous study using the same preparation (Wheeler and Veldhuis, 1988). This preparation and others were later characterized using maintenance of meiotic arrest in denuded bovine oocytes as a bioassay.

In rat cumulus-oocyte complexes, the inactivation and resulting recovery from the effect of invasive adenylate cyclase was detectable by 1 h and completed by 24 h. When invasive adenylate cyclase plus IBMX were added to FF during collection and processing of the complexes, no long lasting effects were noted, since both the frequency of meiotic maturation and the formation of late stage preimplantation embryos were similar to control values. Thus, pretreatment during collection prevents spontaneous commitment of bovine oocytes to meiotic resumption and allows study of the mechanisms for meiotic regulation of the oocyte. Our demonstration that inhibition of oocyte maturation for 20 h in culture by invasive adenylate cyclase is reversible is in agreement with other findings that inhibition of oocyte maturation by either cAMP analogues or agents that increase [cAMP], is reversible in mouse, rat, pig and hamster oocytes (Rice and McGaughy, 1981; Schultz et al., 1983; Aberdam et al., 1987; Racowsky, 1985b). Since reversal of the extended arrest was possible without compromising the developmental potential of most of the oocytes (Achts et al., 1991b; Aktas, 1994), we consider that [cAMP] has a physiologically relevant role in maintaining meiotic arrest.

Meiotically arrested bovine oocytes have different phosphoprotein profiles from transiently arrested or spontaneously maturing oocytes. Differences in the phosphoproteins of mature and immature oocytes have been demonstrated in mouse (Bornslaeger et al., 1986) and frog (Maller and Smith, 1985) oocytes. That the oocytes incubated with 8-Br-cAMP show a phosphoprotein profile similar to that of control oocytes, even while possessing intact germinal vesicles, points to a possible reason why invasive adenylate cyclase, but not 8-Br-cAMP, maintains bovine oocytes in meiotic arrest for an extended period. Whether these phosphorylation and dephosphorylation events are the cause or the result of meiotic resumption is not yet clear. Similarly, it is not known whether two 29 kDa phosphoproteins prominent in meiotically arrested oocytes are responsible for meiotic arrest or are the result of these events. Several proteins of 40–80 kDa, which were heavily phosphorylated in mature or maturing oocytes, are likely to be structural proteins (i.e. lamins) the phosphorylation of which is required for germinal vesicle breakdown to occur. The small gels used in this study do not facilitate further separation of these proteins.

In conclusion, the results of these studies suggest that [cAMP], has a physiological role in maintaining meiotic arrest in bovine primary oocytes. They also present a useful model for further study of meiotic regulation in mammals with long oestrous cycles. Although previous work in domestic species has indicated that [cAMP], plays a role in the maintenance of meiotic arrest, as in other animals, the work presented here provides a model for studying meiotic arrest when post-translational mechanisms regulating meiotic resumption are held in abeyance. This is in strong contrast to the situation in which only a transient delay in meiotic resumption is induced.

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