Maintenance of meiotic arrest by increasing [cAMP]_i may have physiological relevance in bovine oocytes

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Invasive adenylate cyclase (iAC) reversibly inhibits spontaneous maturation of cumulus-enclosed bovine oocytes by increasing the intracellular concentration of cAMP, [cAMP]_i. In this study, physiological aspects of maintaining meiotic arrest in bovine oocytes by iAC were investigated. The maintenance of germinal vesicle arrest by iAC in both cumulus-enclosed and denuded bovine oocytes was concentration dependent ($r^2 = 0.857$). Denuded bovine oocytes were more sensitive to maintenance of meiotic arrest by iAC than were cumulus-enclosed oocytes. At the highest concentration, 70% of the cumulus-enclosed and 90% of the denuded bovine oocytes were maintained in meiotic arrest. The iAC increased [cAMP]_i in both intact cumulus–oocyte complexes and enclosed oocytes in a concentration-dependent manner ($r^2 = 0.795$). Cumulus-enclosed oocytes maintained in meiotic arrest by iAC retained developmental competence when subsequently cultured in iAC-free medium and then fertilized. The [cAMP]_i in bovine complexes decreased precipitously upon release from follicles and remained low for the next 125 min. However, the [cAMP]_i of the enclosed oocytes did not change. Bovine oocytes commit to undergo meiosis in a progressive manner. Approximately 10% of the oocytes were already committed when aspirated. This proportion increased to 40% at 2 h and 70% at 5 h. Use of two inhibitors of cAMP-dependent protein kinase A provided further evidence that cAMP functions in mediating meiotic arrest in bovine oocytes. Bovine oocytes, therefore, are sensitive to different cAMP concentrations, and are developmentally competent after iAC-induced arrest, and complexes containing oocytes exhibit a decrease in [cAMP]_i before spontaneous maturation. These results suggest that maintenance of meiotic arrest by iAC is accomplished through modulation of cellular machinery, and regulation of oocyte maturation by [cAMP]_i may be physiologically relevant.

Introduction

The oocytes of most mammals begin meiosis during fetal development and arrest at prophase I (germinal vesicle stage). Oocytes overcome this arrest either after hormonal stimulation or after removal from antral follicles. Progesterone-induced meiotic maturation in *Xenopus* oocytes (Maller and Krebs, 1977) as well as spontaneous maturation of mouse (Cho *et al.*, 1974), rat (Magnusson and Hillensjo, 1977) and hamster (Racowsky, 1985) oocytes is inhibited by increasing intracellular cAMP concentrations. Testosterone and dibutyryl cAMP (db-cAMP) inhibit pig oocyte maturation cooperatively (Rice and McGaughey, 1981). Steroid hormones (Smith and Tenney, 1980) and hypoxanthine (Eppig *et al.*, 1983; Downs *et al.*, 1985) inhibit mouse oocyte maturation. Effects of these

compounds have been shown to be at least partially due to their inhibitory effect on cyclic nucleotide phosphodiesterase (Kaji *et al.*, 1987; Downs *et al.*, 1989).

Further studies correlate progesterone-induced *Xenopus* oocyte maturation (Cicirelly and Smith, 1985) and spontaneous maturation in mouse (Schultz *et al.*, 1983a; Vivarelli *et al.*, 1983) and rat (Aberdam *et al.*, 1987) oocytes with decreasing [cAMP]_i. However, this is not the case with hamster oocyte maturation (Racowsky, 1985). Microinjection of catalytic subunits of cAMP-dependent protein kinase A inhibits oocyte maturation, whereas microinjection of a specific inhibitor of protein kinase A overrides the inhibition exerted by either dibutyryl cAMP or 3-isobutyl 1-methyl xanthine (IBMX) in *Xenopus* (Maller and Krebs, 1977) and mice (Bornslaeger *et al.*, 1986; Downs *et al.*, 1989). The physiological relevance of this inhibition is further demonstrated by the observation that mouse oocytes cultured in an inhibitor-supplemented medium for 16 h not only undergo nuclear maturation when transferred to inhibitor-free maturation medium, but also give rise to live

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offspring after fertilization and transfer to pseudopregnant females (Downs *et al.*, 1986). Mammalian oocytes possess a commitment period after which they may not be kept in meiotic arrest by increasing $[cAMP]_i$. This cAMP-sensitive period ends either abruptly, as observed with mouse oocytes (Schultz *et al.*, 1983a), or progressively, as observed with hamster oocytes (Racowsky, 1985). Taken together, these studies strongly support the hypothesis that $[cAMP]_i$ is the primary intracellular factor that maintains meiotic arrest in rodent and amphibian oocytes.

An invasive extracellular adenylate cyclase (iAC) from *Bordetella pertussis* inhibits spontaneous bovine oocyte maturation, and this inhibition can be reversed (Aktas *et al.*, 1990; Aktas, 1994). In this study, we investigated physiological aspects of this inhibition, and report that iAC maintains both cumulus-enclosed and denuded bovine oocytes in meiotic arrest in a dose-dependent fashion. Maintenance of meiotic arrest is correlated with increased $[cAMP]_i$ in oocytes and cumulus cell components. Cumulus-enclosed bovine oocytes maintained in meiotic arrest by increased $[cAMP]_i$ retain developmental competence. The $[cAMP]_i$ of cumulus-enclosed oocytes declines precipitously in the course of spontaneous maturation, and bovine oocytes commit to undergo meiosis progressively, that is in an asynchronous fashion. Inhibitors of protein kinase A were used to demonstrate that cAMP is most likely working via regulation of protein kinase A activity. Parts of this study have been reported in abstract form (Aktas *et al.*, 1991a).

Materials and Methods

Oocyte collection 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. The thermos contents averaged 31°C upon arrival at the laboratory, which was 4–6 h after the first ovaries were collected. Follicular contents were aspirated from follicles 2–4 mm in diameter. When follicular fluid (FF) from the experimental groups reached 5 ml volume, it was supplemented with meiotic inhibitors, as described for each experiment. We have shown that meiotic inhibitors added to FF are necessary to prevent premature commitment during collection of oocytes, but after removal of inhibitors, the frequency of maturation and development are unchanged (Aktas *et al.*, 1990; Aktas, 1994). The FF from control groups was not altered unless described otherwise in each experiment. All chemicals and reagents were purchased from Sigma (St Louis, MO) unless described otherwise. Oocytes with intact, compact cumulus investments were selected (Leibfried and First, 1979) and diluted twice in a modified Tyrodes' solution without glucose (TL-Hepes; Bavister *et al.*, 1983), having the same concentration of meiotic inhibitors as in FF and supplemented with polyvinylpyrrolidone (PVP, 40 000 molecular mass, 3 mg ml⁻¹). The final dilution was carried out in TL-Hepes in the absence of meiotic inhibitors.

The control culture medium was TC-199 (with Earle's salts, Gibco, Grand Island, NY) supplemented with pyruvate

(0.2 mmol l⁻¹), gentamicin (25 µg ml⁻¹) and PVP (3 mg ml⁻¹). Other meiotic inhibitors were supplemented as described for each experiment. Oocytes were cultured in 96-well culture dishes in 125 µl medium, with the number of oocytes specified in each experiment and incubated in 5% CO₂ in air with high humidity at 39°C for 20 h. An iAC from *Bordetella pertussis* was kindly provided by E. Hewlett (University of Virginia Medical School). The activity of iAC, which arrested about 50% of denuded bovine oocytes at the germinal vesicle stage, was considered to be 10 iu. The concentration of crude protein is not given, since preparations of different purities were used throughout the experiments.

Measurement of cAMP

The processing of samples and the measuring of cAMP concentrations were carried out at 4°C. Oocytes were stripped of their cumulus cells or left intact, as explained for each experiment. Denuded oocytes or complexes were diluted three times in TL-Hepes supplemented with 0.5 mmol IBMX l⁻¹. A final dilution in TL-Hepes was carried out in the absence of IBMX. Samples were transferred to 130 µl extraction buffer (sodium acetate buffer) in 10 µl TL-Hepes (also used as a blank for each group). Trichloroacetic acid (10 µl 100%) was then added to the samples. The samples were vortexed for 30 s and centrifuged at 16 000 g for 8 min at 4°C. Two times the volume of a 4:6 mixture of tri-*n*-octylamine and 1,1,2-trichlorotrifluoroethane (Aldrich, Milwaukee, WI) was added to neutralize the trichloroacetic acid (Chen *et al.*, 1977; Schoff *et al.*, 1989). Organic and aqueous phases were separated by centrifugation and the aqueous phase (containing cAMP) was frozen in liquid nitrogen and stored at -70°C until assayed. A radioimmunoassay kit from Biomedical Technologies, Inc. (Stoughton, MA) was used to assay cAMP. The sensitivity of the assay was 5 fmol, and it used antibodies raised against succinyl cAMP-tyrosine methyl ester. An ¹²⁵I-labelled succinyl cAMP-tyrosine methyl ester was used as a tracer.

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

Experimental designs

The first experiment examined the correlation between maintenance of meiotic arrest in oocyte complexes and the concentration of iAC in culture medium. The complexes were recovered from FF supplemented with iAC (2 iu ml⁻¹) + IBMX (0.5 mmol l⁻¹). Previous work shows this is necessary to prevent premature commitment, but after removal does not impair subsequent events (Aktas *et al.*, 1990; Aktas, 1994). The complexes were then cultured with increasing concentrations of iAC (2.5, 5, 10, 20 and 40 iu ml⁻¹), together with a control group derived from unsupplemented FF and cultured in the absence of iAC. Twenty-four complexes were cultured in 125 µl maturation medium for 20 h, as described. The

experiment was repeated four times with each treatment represented in each replicate.

A dose-response curve for iAC with cumulus-free oocytes was then examined. Oocyte complexes recovered from FF supplemented with iAC (2 iu ml^{-1}) + IBMX (0.5 mmol l^{-1}) were stripped of their cumulus cells by repeated pipetting, and cultured with increasing concentrations of iAC (2.5 , 5 , 10 , 20 and 40 iu ml^{-1}), together with a control group derived from unsupplemented FF and cultured in the absence of iAC. Twenty-four denuded oocytes were cultured in $125 \mu\text{l}$ maturation medium for 20 h , as described. The experiment was repeated four times with every treatment represented in each replicate.

The third experiment investigated the correlation between the concentration of iAC in culture medium and the increase of [cAMP]_i in intact complexes and in the enclosed oocytes. The complexes were recovered from unsupplemented FF and cultured with increasing concentrations of iAC (0 , 2.5 , 5 , 10 , 20 and 40 iu ml^{-1}) for 2 h . The complexes were then chilled and processed for cAMP measurement. Five complexes were used to measure cAMP concentration in intact complexes, while 20 oocytes from complexes stripped of cumulus at 4°C in IBMX-supplemented medium were used to measure the [cAMP]_i in oocytes alone. Fifty cumulus-free oocytes were used for the control. Duplicate samples were obtained each day from complexes, cumulus-free oocytes and control oocytes, and this experimental design was replicated four times.

An evaluation of the developmental competence of bovine oocytes maintained in meiotic arrest *in vitro* by iAC was made in the fourth experiment. Oocytes were aspirated on two consecutive days. On the first day, FF from two-thirds of the ovaries was supplemented with iAC (2 iu ml^{-1}) + IBMX (0.5 mmol l^{-1}) and complexes obtained were cultured in medium with iAC (20 iu ml^{-1}), and with PVP as macromolecular supplement for 20 h . Complexes derived from unsupplemented FF were cultured without iAC (24 complexes in $125 \mu\text{l}$). On the second day, more complexes were recovered from unsupplemented FF from a new shipment of ovaries. The meiotically arrested complexes from the previous day were washed twice in TL-Hepes. Half the complexes cultured with iAC the previous day and complexes recovered from unsupplemented FF on both days were incubated in TC-199 supplemented with 10% FCS (v/v), oestradiol ($1 \mu\text{g ml}^{-1}$), FSH-P ($20 \mu\text{g ml}^{-1}$; Schering Corp., Kenilworth, NJ), sodium pyruvate (0.2 mmol l^{-1}) and gentamicin ($25 \mu\text{g ml}^{-1}$). The remaining iAC-treated complexes were incubated similarly, but PVP was substituted for FCS. After 24 h of further culture, all oocytes were stripped of their cumulus cells, washed, and transferred to fertilization medium.

Fertilization medium was a modified Tyrode's solution supplemented with fatty acid-free BSA (6 mg ml^{-1}), sodium pyruvate (0.2 mmol l^{-1}), gentamicin ($25 \mu\text{g ml}^{-1}$), hypotaurine ($10 \mu\text{mol l}^{-1}$), penicillamine ($20 \mu\text{mol l}^{-1}$), epinephrine ($1 \mu\text{mol l}^{-1}$) and heparin ($2 \mu\text{g ml}^{-1}$) and with glucose omitted (Leibfried and Bavister, 1982; Parrish *et al.*, 1986). Frozen-thawed semen (American Breeders Service, DeForest, WI) was used for fertilization after discontinuous Percoll gradient separation (J. J. Parrish, personal communication) at $1 \times 10^6 \text{ ml}^{-1}$ final concentration. After 18 – 20 h , some embryos were mounted on microscope slides and evaluated for

fertilization. The remaining embryos were cultured (20 embryos in $50 \mu\text{l}$) with bovine epithelial oviduct cell co-culture (Eyestone and First, 1989). On day 4, $50 \mu\text{l}$ fresh culture medium was added to each drop (TC-199 with 10% FCS v/v). Embryos were evaluated for morulae and blastocyst formation after 5 days. Five replications were performed.

Changes in [cAMP]_i during spontaneous maturation both in intact complexes and oocytes were observed in Expt 5. Complexes aspirated from 5 – 6 ovaries were collected quickly without addition of supplements to FF or TL-Hepes and cultured in control medium for 15 , 30 , 60 and 125 min , and then processed at 4°C for assay of [cAMP]_i. To obtain measurements at time 0, FF from 5 – 6 ovaries was cooled to 4°C immediately after aspiration and processed without delay. Five complexes or 50 oocytes freed of investments in IBMX-supplemented medium were used for each sample. The experiment was replicated three times, using material from different shipments of ovaries each time. Duplicate samples comprising different groups of oocytes for each treatment were obtained for each replicate.

The purpose of Expt 6 was to determine the time period during which bovine oocytes irreversibly commit to undergo meiosis. Oocyte complexes aspirated from 5 – 6 ovaries were processed rapidly and stripped of cumulus. The denuded oocytes were cultured in maturation medium for 0 , 2 , 3 , 4 and 5 h , and then cultured in the presence of iAC for an additional 20 , 18 , 17 , 16 and 15 h , respectively. Another group of oocytes was cultured for the full 20 h without iAC.

Experiments 7 and 8 were designed to determine whether the meiotic inhibitory effect of iAC was mediated by protein kinase A. Ovaries were aspirated, somatic cell investments were stripped of oocytes, and denuded oocytes were cultured with or without 1 mmol Rp-isomer of adenosine-3',5'-cyclic monophosphorothioate l^{-1} (Rp-cAMPS; BioLog, La Jolla, CA; Rothermel and Botelho, 1988) for 30 min . iAC (10 or 20 iu ml^{-1}) or an equal volume of TC199 was added to cultures, 1 h after the start of aspiration. In a separate experiment, oocyte complexes to be treated with iAC were recovered in the presence or absence of meiotic inhibitors, as described for other experiments, denuded and cultured with or without $20 \text{ iu iAC ml}^{-1}$ and with or without 25 or $100 \mu\text{mol}$ *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide $\cdot 2\text{HCl}$ l^{-1} (H-89; CalBiochem, San Diego, CA; Chijiwa *et al.*, 1990). The meiotic state of the oocytes was evaluated after 20 – 21 h in both experiments. These experiments were replicated three times.

Data evaluation and statistical analysis

For evaluation of meiotic stage or fertilization, oocytes or zygotes were compressed beneath a coverslip supported by 3:1 petroleum jelly and paraffin, fixed and cleaned in acid alcohol (3:1 ethanol:acetic acid) and examined at $\times 500$ magnification using Nomarski optics. Oocytes were classified as: germinal vesicle stage, with intact nuclear membranes; intermediate, if germinal vesicle breakdown through metaphase I; or mature, if at anaphase I to metaphase II. Zygotes with two pronuclei were considered normally fertilized.

Data were analysed by one-way analysis of variance (ANOVA) after arcsine transformation. Means were compared

by Duncan's multiple range test if ANOVA revealed significant main effects. Main effects and differences between treatments were considered significant at $P < 0.05$. The percentage of oocytes at the germinal vesicle stage was used as the end point for comparison of treatments in the presence of meiotic inhibitors. The endpoint for embryo development was percentage of morulae and blastocysts. The concentration of cAMP per oocyte (fmol) was used as the endpoint for comparison of cAMP measurements. Correlations between $[cAMP]_i$ and the maintenance of meiotic arrest, as well as the dose-response curve for iAC, were analysed by linear regression. Selective endpoints of interest (percentage germinal vesicle or mature) and $[cAMP]_i$ were used as the dependent variable and regressed on increasing doses of iAC, the independent variable.

Results

Experiment 1

To study the specificity of germinal vesicle maintenance by iAC, a dose-response curve was constructed, whereby maintenance of germinal vesicle and progression beyond anaphase I (termed maturation) were plotted against increasing concentrations of iAC (Fig. 1). The effect of iAC on bovine oocyte meiosis was concentration dependent. With increasing concentrations of iAC, the frequency of cumulus-enclosed oocytes remaining in germinal vesicle increased ($P < 0.05$; $r^2 = 0.857$), while the frequency of progression to anaphase I or beyond decreased ($r^2 = 0.582$). The frequency of maturation was inhibited by a lower concentration of iAC than was germinal vesicle breakdown. This was due to a transient delay in germinal vesicle breakdown as described by Sirard and First (1988). Even the lowest concentration of iAC delayed the progression to anaphase I or beyond ($P < 0.05$). This delay was nevertheless concentration dependent, since higher concentrations of iAC inhibited this process to a greater degree (that is, the difference between inhibition at 2.5 iu ml^{-1} , the lowest concentration, and that obtained at 10 iu ml^{-1} , was significant, $P < 0.05$).

Experiment 2

Maintenance of meiotic arrest in denuded bovine oocytes by iAC was dose dependent, as expected (Fig. 2). At lower doses of iAC (2.5 and 5 iu), progression beyond metaphase I was inhibited in a dose-dependent manner ($P < 0.05$). At higher concentrations ($> 5 \text{ iu}$) germinal vesicle breakdown was inhibited in a dose-dependent manner ($P < 0.05$; $r^2 = 0.795$). Denuded bovine oocytes were more sensitive to increasing doses of iAC than were random complexes. At the highest dose, meiotic maturation was blocked in about 90% of the oocytes. In denuded bovine oocytes, progression beyond metaphase I appeared more sensitive to iAC than was germinal vesicle breakdown.

Experiment 3

The results of Expts 1 and 2 show that maintenance of meiotic arrest in cumulus-enclosed and denuded oocytes by

Fig. 1. Response of cumulus-enclosed bovine oocytes to increasing concentrations of invasive adenylate cyclase (iAC). Bovine cumulus-oocyte complexes were collected and cultured with increasing doses of iAC for 20 h. The mean percentage of oocytes completing nuclear maturation (▲) or remaining in germinal vesicle arrest (○) is shown $\pm$ SEM. The total number of oocytes used over four replications ranged from 69 to 84 for the various concentrations of iAC.

Fig. 2. Response of cumulus-free bovine oocytes to increasing concentrations of invasive adenylate cyclase (iAC). Cumulus-oocyte complexes were collected, stripped of surrounding cells by aspirating through a micropipette and cultured with increasing concentrations of iAC for 20–21 h. The mean percentage of oocytes completing nuclear maturation (▲) or remaining in germinal vesicle arrest (○) is shown $\pm$ SEM. The total number of oocytes used over four replications ranged from 74 to 90 for the various concentrations of iAC.

iAC is concentration dependent. This finding is meaningful only if the increase in $[cAMP]_i$ is also dependent on the concentration of iAC in the culture medium. Increasing concentrations of iAC in culture result in a proportional increase in $[cAMP]_i$, both in intact complexes and oocytes derived from these complexes (Fig. 3). An increase in $[cAMP]_i$ was evident at concentrations of $> 5 \text{ iu iAC ml}^{-1}$ (as was the maintenance of meiotic arrest). The correlation between $[cAMP]_i$ in complexes and the maintenance of meiotic arrest ($r^2 = 0.705$) and also

Fig. 3. Effect of invasive adenylate cyclase (iAC) on concentration of intracellular cAMP ($[cAMP]_i$) of bovine cumulus-oocyte complexes and oocytes denuded of cumulus cells. Cumulus-oocyte complexes were collected and cultured with increasing concentrations of iAC for 2 h, and then processed for assay of cAMP in (a) intact complexes or (b) oocytes denuded of cumulus cells before assay of $[cAMP]_i$. Means $\pm$ SEM are given for four replications. Duplicate samples of five complexes and 20 oocytes were obtained for each treatment within each replication.

between $[cAMP]_i$ in oocytes and the maintenance of meiotic arrest ($r^2 = 0.734$) was very high and accounted for a significant proportion of the variation observed.

Experiment 4

Oocytes matured 24 h or 44 h before insemination and oocytes maintained in meiotic arrest with iAC and then released from meiotic arrest before insemination were all penetrated at high frequencies by spermatozoa (Table 1). Oocytes matured in the control medium for 44 h showed a lower frequency of normal fertilization. This was due to more polyspermy in this group ($P < 0.05$).

All treatment groups showed equal frequencies of zygotes cleaving one or more times (initial cleavage), except for the group cultured for 44 h in control medium before insemination

Table 1. Effect of maintaining meiotic arrest with invasive adenylate cyclase (iAC) on fertility of bovine oocytes

Treatment ^a	<i>n</i>	Total fertilized (%) $\pm$ SEM	Normal fertilized (%) $\pm$ SEM
24 h control	66	78 $\pm$ 5.9	61 $\pm$ 6.0 ^b
44 h control	52	78 $\pm$ 9.4	37 $\pm$ 3.6 ^c
iAC (PVP)	50	69 $\pm$ 8.0	52 $\pm$ 4.8 ^{bc}
iAC (FCS)	38	76 $\pm$ 5.9	58 $\pm$ 3.4 ^b

^aPolyvinylpyrrolidone (PVP) was used in medium with iAC for the first 24 h of culture, then either fetal calf serum (FCS) (10% v/v) or PVP (3 mg ml⁻¹) in hormone-supplemented medium during the reversal of arrest. The 24 h control oocytes were cultured in FCS and hormone supplemented medium, while the 44 h control group were in PVP medium for the first 24 h, then FCS and hormone supplemented medium for the last 24 h.

^{b,c}Values with different superscripts within the same column are significantly different ($P < 0.05$). Means were averaged over five replicates.

Table 2. Effect of maintaining meiotic arrest with invasive adenylate cyclase (iAC) on the development of bovine oocytes

Treatment	<i>n</i>	Initial cleavage (%) $\pm$ SEM	M + BL ^a /cleaved (%) $\pm$ SEM
24 h control	208	59 $\pm$ 3.8 ^c	35 $\pm$ 6.1 ^c
44 h control	221	38 $\pm$ 5.8 ^d	3 $\pm$ 1.4 ^e
iAC (PVP) ^b	221	54 $\pm$ 5.9 ^c	23 $\pm$ 6.6 ^{cd}
iAC (FCS) ^b	198	49 $\pm$ 4.7 ^{cd}	18 $\pm$ 4.7 ^d

^aNumber of morulae (M) and blastocysts (BL)/number of zygotes cleaved at least once $\times$ 100.

^bPolyvinylpyrrolidone (PVP) was used in medium with iAC for the first 24 h of culture, and then with either fetal calf serum (FCS) (10% v/v) or PVP (3 mg ml⁻¹) in hormone-supplemented medium during the reversal of arrest.

^{c,d}Values with different superscripts within the same column are significantly different ($P < 0.05$). Means were averaged over five replicates.

(Table 2). Development from oocyte complexes cultured with iAC during the initial 20 h, and then in PVP supplemented medium without iAC, was statistically equivalent to the 24 h control group. Oocytes cultured with FCS after release from arrest had a lower frequency of development compared with the 24 h control, but did not differ from its PVP counterpart (Table 2). The 44 h control group had little capacity for sustained development.

Experiment 5

If increased $[cAMP]_i$ was responsible for the maintenance of meiotic arrest, then either a decrease in $[cAMP]_i$ or a positive stimulus overriding inhibitory effects of high $[cAMP]_i$ would be required for the resumption of meiosis. To test whether $[cAMP]_i$ concentrations showed any consistent change, either in the oocyte or in somatic cell components, $[cAMP]_i$ was measured both in intact complexes and in oocytes isolated from complexes during the course of spontaneous maturation. The $[cAMP]_i$ in intact complexes showed a precipitous decline after incubation for 15 min and despite some recovery at

Fig. 4. Concentration of intracellular cAMP [cAMP]_i in (a) bovine complexes and (b) cumulus enclosed oocytes during the course of spontaneous maturation. Complexes were aspirated and cultured for 0, 15, 30 and 60 and 125 min and processed for [cAMP]_i assay. Means $\pm$ SEM are presented for three replications. Duplicate samples of five intact complexes or 50 oocytes were obtained at each replication.

60 min stayed low during 2 h of culture (Fig. 4). No consistent change in the [cAMP]_i of oocytes derived from complexes at these time points could be detected statistically, although an overall pattern similar to cAMP concentrations in the complexes did result.

Experiment 6

Meiotic commitment of oocytes during spontaneous nuclear maturation is defined as the period after which meiotic inhibitors can no longer prevent germinal vesicle breakdown. Bovine oocytes commit to undergo meiosis progressively (Fig. 5). Preliminary studies showed that no commitment occurred during the first 1 h (data not shown); therefore, 2 h was chosen as the shortest period in control medium before transfer to iAC supplemented medium. Approximately 40% of the oocytes were committed to undergo meiosis by 2 h, and this percentage was increased to 70% by 5 h in control medium. Compared with our unpublished data, as well as a previous study by Fulka

Fig. 5. Effect of delaying invasive adenylate cyclase (iAC) addition to culture medium on maintenance of meiotic arrest in bovine oocytes. Bovine oocyte complexes were stripped of cumulus and cultured in control medium for 0, 2, 3, 4 or 5 h, and then cultured with iAC for the remainder of a 20 h culture period. A group of oocytes was cultured for 20 h without iAC. The total number of oocytes within each treatment ranged from 104 to 118 over the five replicates.

et al. (1991) on the kinetics of germinal vesicle breakdown, it appears that increased [cAMP]_i can exert an effect on meiotic events in bovine oocytes up until 3 h before germinal vesicle breakdown takes place.

Experiments 7 and 8

Denuded oocytes cultured with Rp-cAMPS and iAC or iAC alone did not resume meiosis (Fig. 6a). Rp-cAMPS alone did not affect the rate of meiotic resumption; however, it affected the progression of meiosis negatively, as lower proportions of oocytes progressed beyond metaphase I in the presence of Rp-cAMPS compared with oocytes cultured in control medium (51% versus 77%, $P < 0.05$). The H89, however, antagonized the maintenance of meiotic arrest (Fig. 6b) by 20 iu iAC ml⁻¹, both at 25 and 100 μ mol l⁻¹ after 20–21 h culture (86%, 29% and 19% intact germinal vesicle with 0, 25 and 100 μ mol H89 l⁻¹ in the presence of iAC, respectively; $P < 0.05$). When present alone, H89 completely suppressed progression of meiosis beyond metaphase I, although it had no effect on germinal vesicle breakdown. Oocytes cultured in control medium underwent germinal vesicle breakdown and matured at a high frequency (100% germinal vesicle breakdown and 78% anaphase I to metaphase II).

Discussion

Ever since the demonstration by Cho *et al.* (1974) and Maller and Krebs (1977) that mouse and frog oocyte maturation is

Fig. 6. Effect of protein kinase A inhibitors on invasive adenylate cyclase (iAC)-maintained meiotic arrest. (a) Denuded bovine oocytes were cultured with (+) or without (-) 1 mmol Rp-isomer of adenosine-3'-5'-cyclic monophosphorothioate (Rp-cAMPS) l^{-1} and 10 or 20 $iu\ ml^{-1}$ of iAC or in control medium for 20–21 h. The total number of oocytes within treatments summed over three replicates ranged from 46 to 62. (b) Denuded bovine oocytes were cultured with (+) or without (-) 20 $iu\ iAC\ ml^{-1}$ and with 25 or 100 $\mu mol\ N$ -[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide $\cdot\ 2HCl$ (H89) l^{-1} for 20–21 h. The total number of oocytes within treatments summed over three replicates ranged from 46 to 56. In both experiments, oocytes were classified as germinal vesicle intact ($\square$); intact germinal vesicle), intermediate ($\blacksquare$); germinal vesicle breakdown to metaphase I) or mature ($\hatched$); anaphase I to metaphase II) after culture. The frequencies of maturation or germinal vesicle maintenance with different superscripts are significantly different ($P < 0.05$).

inhibited by increased $[cAMP]_i$, numerous studies have been undertaken to elucidate the physiological relevance of cAMP-maintained meiotic arrest (Maller and Krebs, 1977; Schultz *et al.*, 1983a, b; Vivarelli *et al.*, 1983; Cicirelli and Smith, 1985; Bornslaeger *et al.*, 1986; Downs *et al.*, 1986). Inhibition of meiotic resumption not only by increased $[cAMP]_i$, but also by microinjection of catalytic subunits of cAMP-dependent protein kinase A and high $[cAMP]_i$ -maintained meiotic arrest by

specific inhibitor of this kinase in frogs (Maller and Krebs, 1977), mice (Schultz *et al.*, 1983a; Vivarelli *et al.*, 1983) and rats (Aberdam *et al.*, 1987) supports the physiological relevance of cAMP-mediated meiotic arrest. This argument is strengthened further by the observation that mouse oocytes cultured in inhibitor-supplemented medium for 16 h, not only underwent nuclear maturation when transferred to inhibitor-free maturation medium, but also gave rise to live offspring after fertilization and transfer to pseudopregnant females (Downs *et al.*, 1986). Although there is evidence to implicate $[cAMP]_i$ in mediating meiotic arrest in domestic species with long oestrous cycles, the evidence comes mostly from studies in which only transient delays in meiotic resumption were encountered when attempting to maintain meiotic arrest *in vitro* (Sirard and First, 1988; Homa, 1988). The work presented here and previously (Aktas, 1994), provides a definitive case for the physiological role of $[cAMP]_i$ in meiotic arrest in domestic species, and also a strong model for studying the molecular events of meiotic regulation.

The results presented here demonstrate that iAC maintains meiotic arrest in cumulus-enclosed and denuded bovine oocytes in a dose-dependent manner. Two different preparations of enzyme were used in these studies with estimated purities of $< 20\%$ and about 80% . Similar concentrations of both preparations maintained meiotic arrest in bovine oocytes to a similar degree when correction was made for the degree of purity. It is, therefore, unlikely that another contaminant protein may be responsible for maintenance of meiotic arrest. A nonprotein fraction is unlikely to be responsible for the observed effects, since the enzyme was dialysed on a membrane with a cut-off value of 12–14 kDa for four times in 100 times the volume of TC-199 each time before use. Moreover, not only maintenance of meiotic arrest in bovine oocytes, but also accumulation of cAMP in both intact complexes and oocytes isolated from complexes, was dependent on the concentration of iAC. The frequency of maintenance of meiotic arrest correlates highly both with $[cAMP]_i$ increased by iAC and concentrations of iAC in the culture medium. An iAC has also been used by Aberdam *et al.* (1987), in higher concentrations and in combination with IBMX and pertussis toxin, to inhibit rat oocyte maturation. Results of our study are in agreement with their findings in that iAC could pass the plasma membrane, increase $[cAMP]_i$ and inhibit resumption of meiosis in a dose-dependent manner.

Bovine complexes maintained in meiotic arrest *in vitro* by iAC retained their developmental competence. Oocytes maintained in meiotic arrest by iAC for the initial 20 h of culture closely resemble the oocytes matured for 24 h before fertilization, having similar rates of fertilization, cleavage and development to morulae and blastocysts. These results are in agreement with those reported by Downs *et al.* (1986), who showed that mouse oocytes maintained in meiotic arrest *in vitro* could give rise to preimplantation embryos. Moreover, when transferred to pseudopregnant recipients, these embryos gave rise to live offspring.

The $[cAMP]_i$ in bovine oocyte complexes dropped precipitously in the course of spontaneous maturation. This contrasts strongly with work in sheep (Moor and Heslop, 1981) in which an increase in $[cAMP]_i$ was observed in cumulus-oocyte complexes during meiotic resumption *in vitro*. In this case,

gonadotrophins were added to the medium used for oocyte culture; the addition of this hormonal stimulus can increase $[cAMP]_i$ in complexes, while inducing germinal vesicle breakdown in oocytes (Eppig and Downs, 1988) *in vivo* and *in vitro* (Schultz *et al.*, 1983b). Therefore, it becomes clear that $[cAMP]_i$ profiles can be quite different during spontaneous maturation or when gonadotrophins are present, whether *in vivo* or *in vitro*.

However, a decline was not observed for $[cAMP]_i$ of bovine oocytes after removal from the complexes, in contrast to the situation in mice (Schultz *et al.*, 1983a; Vivarelli *et al.*, 1983) and rats (Aberdam *et al.*, 1987). This discrepancy may be explained by the observation that mice and rats have shorter commitment periods and are more synchronous in committing to undergo meiosis. Bovine oocytes are recovered from a heterogeneous population from different age groups, breeds, stage of follicle development and points in the oestrous cycle. All these variables may result in a heterogeneous population of bovine oocytes, which commit to undergo meiosis progressively over a fairly long period, as demonstrated by the results of this study. If a drop in $[cAMP]_i$ of oocytes occurs before commitment, it may not be detected with the present technique, which requires large numbers of oocytes to measure $[cAMP]_i$. It is also possible that while increased $[cAMP]_i$ is necessary for the maintenance of meiotic arrest, a decline may not be necessary for meiotic resumption, as suggested in several studies. It is possible that a positive signal can cause release from meiotic arrest in the face of increasing cAMP concentrations in cumulus cells, as shown for both mice (Eppig and Downs, 1988) and cattle (Aktas *et al.*, 1991b; Aktas, 1994). A system that uses living oocytes for continuous measurements of $[cAMP]_i$ (Adams *et al.*, 1991) would solve this problem.

It was surprising that Rp-cAMPS could not antagonize meiotic arrest maintained by iAC and suggests that iAC-maintained meiotic arrest was not mediated by protein kinase A. The Rp-cAMPS antagonizes cAMP activation of protein kinase A by binding the regulatory subunit and interfering with the dissociation of catalytic and regulatory subunits. Its effects are thought to be irreversible, as it is resistant to phosphodiesterases. However, it has no effect on the active catalytic subunit. Thus, Rp-cAMPS is a useful tool for preventing further activation of protein kinase A, but has no effect on previously active catalytic subunits. To circumvent this problem, we used H89, a potent and highly selective inhibitor of protein kinase A (Chijiwa *et al.*, 1990). The H89 not only antagonized the meiotic arresting action of iAC but also prevented progression of meiosis beyond meiosis I. In this respect, Rp-cAMPS and H89 were similar, except that H89 blocked progression of meiosis completely, whereas Rp-cAMPS had only a partial effect. These data suggest that follicular bovine oocytes possess sufficient protein kinase A activity to maintain meiotic arrest *in vivo*; however, the possibility cannot be excluded that Rp-cAMPS may partition to an irrelevant subcellular compartment and so be unable to prevent activation of protein kinase A in bovine oocytes. In pigs (Jung *et al.*, 1992), H89 used in conjunction with gonadotrophins during culture of oocyte complexes did not seriously impair the progression of germinal vesicle breakdown in this situation either.

These results demonstrate that the maintenance of meiotic arrest in bovine oocytes by iAC may have physiological relevance in that it is dose dependent, correlated with increases

of $[cAMP]_i$ both in oocyte complexes and oocytes and, after reversal of iAC-maintained meiotic arrest, oocytes are developmentally competent. The $[cAMP]_i$ in cumulus-oocyte complexes was shown to drop precipitously early in the course of spontaneous maturation. The $[cAMP]_i$ in oocytes showed a similar pattern of changes, although this was not found to be statistically significant. This is likely to be due to the highly asynchronous commitment to meiotic resumption observed in the population of bovine primary oocytes. Evidence is also presented that implicates protein kinase A as the likely mediator for the effects of cAMP in the meiotic regulation of bovine oocytes. The results reported here offer a system that may be used to study the molecular mechanisms used in maintaining meiotic arrest, as well as those involved in the initiation of maturation. We are currently undertaking experiments to study the molecular mechanisms by which an iAC-mediated increase of $[cAMP]_i$ can maintain the stage of germinal vesicle arrest in bovine oocytes and the ways in which oocytes may overcome this inhibition.

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