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Isoenzyme Inhibitors on Bovine Oocyte Meiotic Maturation

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The differential regulation of cAMP levels within the oocyte and somatic (cumulus) cell compartments of the bovine follicle, and the subsequent regulation of oocyte meiotic maturation was examined through specific cell-type localisation of phosphodiesterases (PDEs). Selective PDE inhibitors were used to modulate cAMP levels in each of the two follicular compartments and to examine their effects on oocyte meiotic maturation. Ovaries were obtained from an abattoir and cumulus-oocyte complexes (COC) were aspirated from antral follicles into culture medium supplemented with 4 mg/ml BSA and 2mM 3-isobutyl-1-methylxanthine (IBMX). COC, denuded oocytes (DO), or mural granulosa cells (MGC) were cultured either with or without forskolin or FSH, in the presence of specific PDE inhibitors; either milrinone (PDE3 inhibitor), cilostamide (PDE3 inhibitor), or rolipram (PDE4 inhibitor). COC/DO cultures were assessed for meiotic progression and cAMP content, and MGC for cAMP production. The type 3 PDE inhibitor, but not the type 4, prevented spontaneous meiotic maturation and elevated intraoocyte cAMP in cultured denuded oocytes. In contrast, the type 4 PDE inhibitor had no effect on the oocyte, but elevated mural granulosa and cumulus cell cAMP production. The results of this study indicate that specific PDE subtypes are differentially localised within the two compartments of the bovine follicle—the type 3 PDE in the oocyte and the type 4 PDE in the granulosa cells. In addition, oocyte cAMP levels are primarily regulated in bovine oocytes by its degradation by PDE, whereas granulosa cell cAMP levels are controlled mainly by active adenylate cyclase, with both sources able to participate in oocyte meiotic regulation. © 2002 Elsevier Science (USA)

Key Words: **oocyte; meiotic maturation; meiotic inhibition; cAMP; phosphodiesterase; granulosa cell.**

INTRODUCTION

The exact mechanisms and signals that regulate mammalian oocyte meiotic maturation have not been fully elucidated. In nonatretic follicles, oocytes remain at the germinal vesicle (GV) stage unless exposed to the preovulatory surge of luteinising hormone (LH) during each ovarian cycle. The oocyte in the dominant follicle(s) then resumes meiosis, undergoing germinal vesicle breakdown (GVBD) and proceeds through metaphase I (MI), only to be subsequently rearrested at the metaphase II (MII) stage of meiosis. Alternately, mechanical isolation of the oocyte from an antral follicle induces spontaneous meiotic resumption *in vitro* without the requirement for gonadotrophic hormonal stimulation. Experimental results such as these implicate the follicular environment in maintaining the oocyte in meiotic arrest, probably via inhibitory factors produced by the follicle, the suppression of a stimulatory molecule, or by a combination of both (Downs, 1996).

There is abundant evidence that the second messenger cyclic adenosine mono-phosphate (cAMP) plays an important role in maintaining meiotic arrest in rodent oocytes. The role of cAMP in the control of meiosis is less clearly demonstrated in bovine oocytes. Several agents that experimentally elevate oocyte cAMP transiently inhibit the spontaneous maturation of bovine oocytes isolated from their follicles *in vitro*. These include membrane permeable derivatives of cAMP such as dibutyryl-cAMP (dbcAMP) and 8-bromo-cAMP (Aktas *et al.,* 1995b; Homa, 1988; Sirard and First, 1988), phosphodiesterase (PDE) inhibitors (Bilodeau *et al.,* 1993; Mayes, 2000; Sirard and First, 1988), forskolin—a stimulator of adenylate cyclase (Homa, 1988; Sirard, 1990), and invasive adenylate cyclase (iAC) (Aktas *et al.,* 1995a,b).

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In contrast, meiotic resumption is induced in follicle-enclosed rodent oocytes following injection of cAMP analogues into the follicular antrum (Tsafriri *et al.,* 1972), or exposure of the follicles to dbcAMP (Hillensjo *et al.,* 1978), phosphodiesterase inhibitors (Dekel *et al.,* 1981), or forskolin (Yoshimura *et al.,* 1992). These studies indicate that LH-induced oocyte maturation is a cAMP-mediated response, however the continuous presence of cAMP, cAMP analogues, and PDE inhibitors in the culture medium of isolated oocytes prevent their spontaneous maturation in culture.

These apparently opposing actions of cAMP present a contradiction: a rise in follicular cAMP mediates LH-induced meiotic maturation, but a rise in intraoocyte cAMP maintains oocyte meiotic arrest. A hypothesis to explain this paradox proposes that cAMP is compartmentalised within the follicle and is differentially regulated within the two compartments—the germ cell (oocyte) and the somatic (cumulus) cells due to the differential localisation of distinct phosphodiesterase (PDE) subtypes (Tsafriri *et al.,* 1996).

Mammalian PDEs currently constitute 11 distinct families of isoenzymes (PDE1–11), which consist of at least 20 gene types (Conti and Jin, 2000; Fawcett *et al.,* 2000). PDE isoenzymes are differentially expressed and regulated in different cellular and tissue locations (Nicholson *et al.,* 1991) and have a similar structural layout, with a conserved catalytic domain and a distinct regulatory domain, which confers the specific regulatory characteristics of the different PDE families (Kenan *et al.,* 2000). Compartmentalisation of two PDE types has been demonstrated in rat follicles, where PDE3A has been localised only to the oocyte and PDE4D and 4B to mural granulosa cells, theca and interstitial tissue only (Reinhardt *et al.,* 1995; Tsafriri *et al.,* 1996).

Nonspecific PDE inhibitors such as 3-isobutyl-1-methylxanthine (IBMX) and hypoxanthine have been used extensively to investigate the mechanisms underlying the regulation of meiotic maturation (for a review see (Downs, 1996; Eppig, 1993). However, the availability of subtype-specific PDE inhibitors provide a new opportunity for more extensive examination of oocyte maturation and represent new and powerful experimental tools for investigating oocyte-follicular cell interactions. Selective regulation of the activities of follicular PDE subtypes may account for the apparently paradoxical role of cAMP, and may provide further insight into the mechanisms of oocyte meiotic control. To date there are only a limited number of studies using type-specific PDE inhibitors to study the regulation of oocyte and follicular cell function, firstly in *Xenopus* (Sadler, 1991), and recently in rodent (Gilchrist *et al.,* 2001; Tsafriri *et al.,* 1996; Wiersma *et al.,* 1998) and bovine (Mayes, 2000) oocytes.

Induction of oocyte maturation is associated with increased follicular cAMP levels although intraoocyte cAMP has been observed to decrease before germinal vesicle breakdown. The aim of this study was to examine whether the differential regulation of cAMP levels within the oocyte and somatic (cumulus) cell compartments of the follicle controls bovine oocyte meiotic maturation through specific cell-type localisation of phosphodiesterases (PDEs). Specific PDE isoenzyme inhibitors were used to modulate cAMP levels in one of the two follicular compartments and to examine their effects on oocyte meiotic maturation.

MATERIALS AND METHODS

Collection and Preparation of Bovine Oocytes

Bovine ovaries were obtained at an abattoir and transported to the laboratory in a thermos bottle containing warm (29–32°C) saline (0.9% w/v NaCl supplemented with penicillin G (40 IU/ml; Sigma, St Louis, MO, USA) and streptomycin sulphate (40 μg/ml; Sigma), requiring 3–4 h for collection and transport. All ovaries collected on a day were pooled and used at random. Follicle aspiration was performed with an 18-gauge needle and a 10 ml syringe. Follicular contents were aspirated from follicles ~2–8 mm in diameter of unknown atresia status and transferred to 15 ml conical tubes (Falcon, Franklin Lakes, NJ, USA) containing 4 ml 25mM HEPES-buffered tissue culture medium-199 (H-TCM; ICN Biomedicals, CA, USA) supplemented with sodium pyruvate (2mM; Sigma), penicillin G (100IU/ml), streptomycin sulfate (100 μg/ml), bovine serum albumin (BSA, fraction V; 4 mg/ml; Sigma), and 3-isobutyl-1-methyl-xanthine (IBMX; 5mM; Sigma). The aspirate was left to sediment when total volume reached 10 ml. Follicular fluid was supplemented with the meiotic inhibitor IBMX to prevent premature commitment to meiotic progression during the collection of oocytes, and subsequent removal of inhibitors does not change the frequency of maturation and development (Aktas *et al.,* 1990).

Cumulus-oocyte complexes (COCs) with an intact, compact cumulus investment were selected under a dissecting microscope and transferred to 35 mm Petri dishes (Falcon) containing H-TCM + 2mM IBMX for counting. Where specified, COCs were mechanically denuded in 0.5 ml H-TCM + IBMX (2 mM) before or after culture using vigorous pipetting to remove all cumulus cells surrounding the oocytes. COCs were washed twice in H-TCM, and then transferred to dishes containing bicarbonate-buffered TCM-199 (B-TCM) supplemented with sodium pyruvate (0.23 mM), antibiotics and BSA (4 mg/ml) before being transferred to respective treatments.

Oocyte Culture

Groups of up to 60 COCs or denuded oocytes (DOs) were transferred in 50 μl of B-TCM to individual wells of 4-well multi dishes (Nunc, Denmark). Inhibitors and other agents, diluted in B-TCM, were then added to give a final volume of 500 μl. Replicates of each experiment utilised an average of 90 COCs or DOs per treatment group. Putative meiotic inhibitors were added alone or in combination, these being the nonspecific phosphodiesterase (PDE) inhibitor IBMX, type 3 PDE specific inhibitors milrinone (MR; Sigma) and cilostamide (CM; ICN Biomedicals), the type 4 PDE specific inhibitor rolipram (RP; Sigma), and the adenylate cyclase stimulator forskolin (FK; Sigma). Because FSH induces cumulus cell expansion, accompanied by breakdown of gap junctional communication with the oocyte, disrupting possible passage of cAMP from the cumulus cells into the oocyte (Eppig, 1991),

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forskolin was used as an alternative to rhFSH because of its ability to stimulate the catalytic subunit of oocyte adenylate cyclase directly. Millimolar stock concentrations of PDE inhibitors were stored at −20°C dissolved in dimethyl-sulphoxide (DMSO; Sigma) and solutions containing inhibitor were diluted fresh for each experiment. The final concentration of DMSO in any treatment well was <0.1%. DMSO concentration of 3% or greater per treatment well inhibited spontaneous maturation of bovine COC and DO in a dose dependant manner (3%: ~20%GV; 15%: ~100%GV; unpublished results). All COCs and DOs were cultured in B-TCM + BSA for either 5 or 16h at 39°C, 96% humidity in an atmosphere of 5% CO_2 in air.

Oocyte Meiotic Assessment

At the end of the incubation, COCs were denuded as described above. Oocytes to be assessed for meiotic status were transferred to a fixing solution (3:1 ethanol: acetic acid) for >24 h before staining with 1% Orcein (Sigma). Fixed oocytes were then mounted on slides and compressed beneath a cover slip supported by petroleum jelly and retained with glue. Oocytes were examined with a phase contrast microscope at 400× and classified as being at either germinal vesicle (GV), diakinesis I, metaphase I (MI), anaphase I, telophase I or metaphase II (MII) stages (Homa, 1988; Motlik *et al.,* 1978). For graph simplicity, those oocytes at diakinesis I were pooled with and classified as MI, and those at anaphase I and telophase I were pooled with and classified as MII. Approximately 10% of oocytes that were denuded before culture were at the GV stage and of a degenerative appearance upon meiotic assessment and were consequently not included in the final assessment.

Granulosa Cell Preparation and Culture

Ovary collection and follicular fluid aspiration was performed as described above. All debris (stromal and thecal tissue, preantral follicles, and oocytes) was removed from the follicular fluid and the remaining aggregated mural granulosa cells (MGCs) transferred to a separate tube. MGCs were collected by centrifugation for 5 min and the supernatant discarded. MGCs were washed once in H-TCM + BSA and twice in B-TCM + BSA. A 100 μl aliquot of the washed cells was removed and cells dissociated (Luciano *et al.,* 2000) to permit accurate counting by haemocytometer to determine MGC concentration. Aggregated undissociated MGCs were cultured at a density of approximately 1×10^5 cells/well and a final well volume of 250 μl gave a final cell density of 4×10^5 cells/ml.

MGCs were treated in duplicate with or without recombinant human follicle-stimulating hormone (rhFSH, Gonal-F, Serono, French's Forest, NSW, Australia) or forskolin (FK), and the type 3 specific PDE milrinone (MR), type 4 specific PDE inhibitor rolipram (RP), and nonspecific PDE inhibitor (IBMX). Depending on treatments of individual experiments, hormones, inhibitors, and B-TCM were added to wells of a 96-well flat bottomed plate (Falcon) to make up final volume of 250 μl. Cells were cultured in an atmosphere of 39°C, 96% humidity with 5% CO_2 in air for 24 h. At the completion of culture, 200 μl of supernatant was removed and frozen (−20°C) for cAMP analysis.

Measurement of cAMP

Cyclic AMP content of COC, complex-derived oocytes (CDO), DO and supernatants of granulosa cell cultures were measured using a radioimmunoassay method described and validated previously (Reddoch *et al.,* 1986). CDO (COCs cultured with their cumulus mass intact and then denuded before assay) were used to evaluate the effect of the surrounding cumulus cells on intraoocyte cAMP concentrations. After 5 h of culture, 6–10 COC, 10–16 CDO, and 16–24 DO were washed in H-TCM + 2mM IBMX, transferred to 0.5 ml of ethanol (100%) and stored at −20°C. Before cAMP measurements, samples were vortex agitated for 30 s and then centrifuged at 3000*g* for 15 min at 4°C. Briefly, supernatants were removed, evaporated, resuspended in assay buffer (50mM sodium acetate, pH 5.5) and assayed following acetylation [the addition of triethylamine (AJAX Chemicals, Sydney, Australia) and acetic anhydride (BDH Laboratory Supplies, Poole, England) 2:1 v/v] and appropriate dilution. For assay of cAMP produced by MGCs after culture, media was removed from wells and stored at −20°C until diluted, acetylated, and assayed. ^{125}I-labelled cAMP (specific activity of 2175 Ci/mM, prepared by iodinating 2′-0-monosuccinyladenosine-3′:5′-cyclic monophosphate tyrosyl methyl ester (Sigma) using the chloramine T method (Hunter, 1970) and cAMP antibody (as prepared by Reddoch *et al.,* (Reddoch *et al.,* 1986) were added and duplicate samples covered and left overnight at 4°C. The following day, 1 ml cold 100% ethanol was added and the samples were centrifuged at 3000*g*. The supernatant was removed and the pellet dried and counted using a gamma counter. Triplicate samples were used to produce a standard curve (0–800 fmol cAMP).

Statistical Analysis

Differences in the proportion of oocytes within each preparation (COC and DO) at each of the meiotic stages (GV, MI, MII) were examined using Chi-squared analysis. Differences in cAMP production by MGCs were assessed using multifactorial ANOVA analysis (SAS package) and a Waller-Duncan K-ratio t-test to determine individual differences between treatments. Differences between intracomplex or intraoocyte cAMP measurements were determined following natural logarithmic transformation of data using a one-way ANOVA and differences between treatment means tested using Student-Newman-Keuls post hoc comparisons. Probabilities of less than 0.05 were considered statistically significant.

RESULTS

Effect of Type 3 and 4 PDE Isoenzyme Inhibitors on Spontaneous Maturation

The proportion of DO maintained at the immature GV stage of meiosis did not increase with increasing concentrations of the type 3 PDE inhibitor, however, the proportion of oocytes reaching MII decreased significantly with increasing doses of the inhibitor (Fig. 1). Rolipram treatment alone, even at the highest concentration of 500 μm, did not maintain any DO at the GV stage. Progression of rolipram-treated DO from MI to MII was not inhibited at concentrations of 50 or 250 μm, however, the proportion reaching MII after treatment with the very high concentration of 500 μm was significantly decreased. This is likely due to nonspecific effects of the PDE4 inhibitor on oocyte PDEs—an effect observed by a previous study where PDE type 4 and 5 specific inhibitors suppressed mouse oocyte maturation at concentrations 200–500 times higher than that of the type 3 inhibitor (Wiersma *et al.,* 1998).

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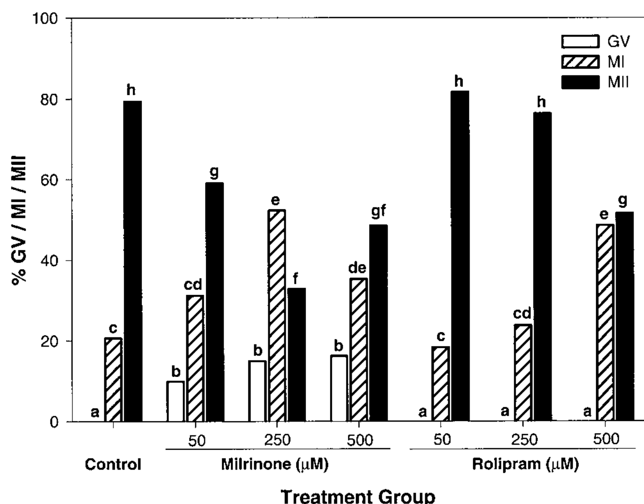

FIG. 1. Effect of increasing doses of the type 3 (milrinone; 50 μm) and type 4 (rolipram; 50 μm) PDE inhibitors on the spontaneous meiotic maturation of denuded oocytes following a 16 h culture period. Oocytes were cultured in milrinone- or rolipram-supplemented culture media and were then assessed for meiotic progression and classified as GV, MI, or MII stage. A mean number of 90 oocytes were used in each treatment group from three replicate experiments. Means within the same meiotic stage and with no common superscripts are significantly different (Chi squared analysis, $P < 0.05$).

The type 3 PDE inhibitor milrinone, but not the type 4 PDE inhibitor rolipram, inhibited GVBD in an increased proportion of COCs (13% GV) compared to control-treated COCs cultured for 16 h (0% GV; Fig. 2A). Similarly, DO GVBD was prevented by milrinone at a frequency comparable to that in COCs (18% GV; Fig. 2B), but was unaffected by treatment with rolipram. In addition to its effects on GVBD, treatment by milrinone, but not rolipram, significantly delayed the progress of spontaneous maturation, causing a decreased proportion of both COCs and DOs from reaching MII stage (44% and 45%, respectively) compared to controls (84 and 72% MII, respectively). The ability of milrinone to inhibit meiosis in both COCs and DOs suggests the absence of cumulus cells has no consequence on its meiotic inhibitory capacity.

Inclusion of the type 3 PDE inhibitor in the culture maintains a proportion—but not all oocytes—at the GV stage. This suggests PDE inhibition alone may not be sufficient to maintain a significantly elevated level of intraoocyte cAMP—possibly due to an insufficient level of endogenous oocyte adenylate cyclase activity. Forskolin treatment of COCs and DOs prevented GVBD in a greater proportion of oocytes compared to those that were cultured in control medium (Figs. 2A and 2B). Only 25% of COCs reached the MII stage following forskolin treatment compared to 46% of forskolin-treated DOs ($P = 0.017$). Combined treatment with forskolin and milrinone maintained a significantly greater proportion of COCs (43%GV) and DOs (35% GV) at the GV stage when compared to treatment with milrinone alone. Combined forskolin-milrinone treatment delayed meiotic progression through to metaphase II in COCs much more effectively than in DOs (MI: COC 48%; DO 27%; $P = 0.0132$) (MII: COC 9%; DO 38%; $P = 0.0001$). While combined forskolin and rolipram treatment prevented GVBD at a greater frequency (38% GV) than either agent alone in COCs (Fig. 2A), this was not the case in DO (9% GV; Fig. 2B).

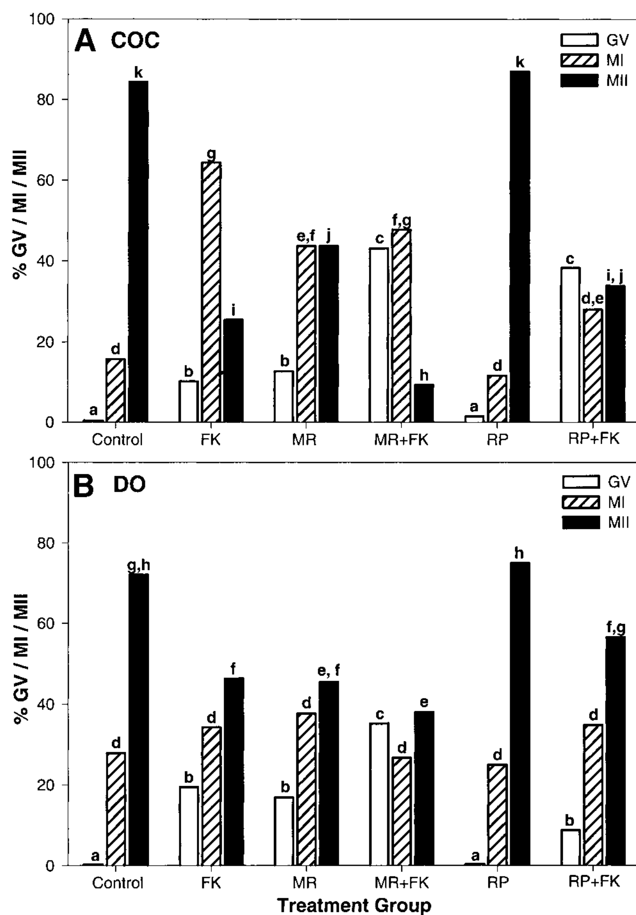

FIG. 2. Effect of the type 3 (milrinone; 50 μm) and type 4 (rolipram; 50 μm) PDE inhibitors on the spontaneous meiotic maturation of cumulus- oocyte complexes (A) and denuded oocytes (B) following a 16 h culture period. Oocytes were cultured in milrinone- or rolipram-supplemented culture media with or without forskolin and were then assessed for meiotic progression and classified as GV, MI, or MII stage. A mean number of 80 oocytes were used in each treatment group from three replicate experiments. Means within the same meiotic stage and with no common superscripts are significantly different (Chi squared analysis, $P < 0.05$).

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TABLE 1
The Effect of Two PDE3 Inhibitors, Milrinone and Cilostamide, on COC and DO Spontaneous Meiotic Maturation Following culture for 16 h

Oocyte type	Treatment**	*n* oocytes/ treatment***	% GV*	% MI*	% MII*
	Control	108	0^{a}	10.2^{d}	90.0^{h}
COC	Milrinone	90	6.7^{b}	42.2^{e}	51.1^{g}
	Cilostamide	103	20.4^{c}	44.7^{e}	35.0^{f}
	Control	80	0^{a}	15.2^{d}	84.8^{h}
DO	Milrinone	83	8.4^{b}	38.6^{e}	53.0^{g}
	Cilostamide	77	29.9^{c}	33.8^{e}	$36.4^{f,g}$

* Different letter superscripts indicate significant treatment differences ($P < 0.05$) within the same meiotic stage ($^{a\text{-}c}$GV, $^{d\text{-}e}$MI, $^{f\text{-}h}$MII; Chi-square).

** Milrinone (MR; 50μM), cilostamide (CM; 50 μm).

*** A mean number of 90 oocytes were used in each treatment group from three replicate experiments.

Effect of Two Different PDE3 Subtype Inhibitors on the Spontaneous Maturation of COCs and DOs

Bovine COCs and DOs were treated with two different type 3 PDE inhibitors, milrinone and cilostamide. Treatment of COCs with 50 μm cilostamide resulted in a significantly ($P < 0.05$) higher frequency of oocytes maintained at the GV stage (20% GV) compared to those treated with 50 μm milrinone (7% GV; Table 1). A similar effect was seen in DOs, although the inhibitory effects of both type 3 PDE inhibitors—especially cilostamide—were more pronounced in DOs than in COCs. Cilostamide also prevented significantly ($P < 0.05$) more COCs from progressing through to the MII stage compared to milrinone treated COCs. A similar, although not statistically significant trend was also noted in DOs (Table 1).

Effect of Type 3 PDE Isoenzyme Inhibitor on the Spontaneous Maturation of COCs Following Extended Culture

The effect of increasing time of culture in milrinone-supplemented medium on spontaneous maturation of bovine COCs was examined (Fig. 3). Continued exposure of COCs to milrinone for up to 36 h maintained approximately 15% of oocytes at the GV stage, but only transiently inhibited progression to MII. At 16 h, ~35% of oocytes were at the MI stage and ~55% at the MII stage. With increasing time in milrinone, the proportion at MI significantly decreased ($P < 0.05$) while those in MII increased until at t = 36 h the proportions were 1% MI and 81% MII. There is a significant linear relationship (Chi-Squared test for trend) among t = 16, 20, 24, 28, and 36 h MR treated oocytes and the proportion of oocytes at both the MI and MII stages ($P < 0.0001$). Control-treated oocytes were cultured for each of the time points (line graphs; 16, 20, 24, 28, and 36 h), but were omitted from the figure for graph simplicity (~0% GV, ~7% MI, and ~93% MII).

Reversibility of Milrinone-Inhibition of Spontaneous Maturation

The reversibility of milrinone inhibition of GVBD was also examined by culture of COCs for an initial 16 h period in milrinone-supplemented medium, followed by two washes and culture for a subsequent 20 h in control medium. In contrast to oocytes cultured in milrinone supplemented medium for the full 36 h (~17% GV), the percentage of oocytes at the GV, MI, and MII stages were the same for both milrinone (16 h)-control(20 h) and control (36 h)-treated oocytes (Fig. 3), indicating that the inhibitory effect of milrinone on spontaneous meiotic progression was completely reversible.

Effect of Type 3 and Type 4 PDE Inhibitors on the cAMP Content of COC, CDO, and DO

In bovine oocytes, GVBD occurs after 6–9 h of culture (Sirard *et al.,* 1989). To test whether treatment of oocytes

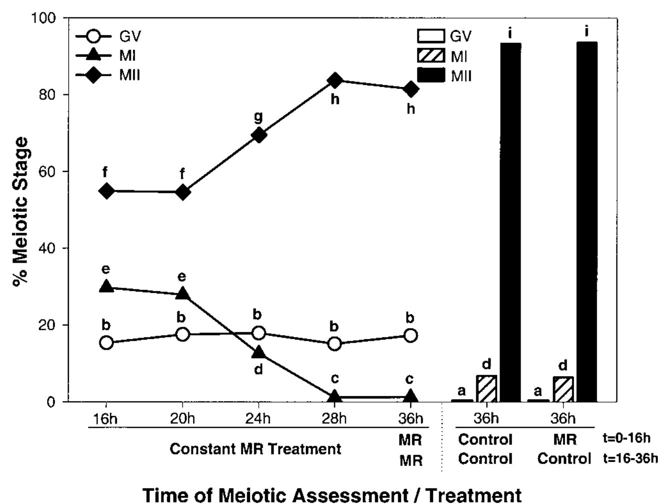

FIG. 3. Effect of the type 3 (milrinone; 100 μm) PDE inhibitor on the spontaneous meiotic maturation of cumulus-oocyte complexes following extended culture. COCs were cultured in control or milrinone-supplemented media and cohorts of oocytes then fixed and assessed for meiotic progression at 16, 20, 24, 28, and 36 h (line graphs; data from 16 to 28 h control-treated oocytes have been omitted for simplicity). In addition, oocytes were cultured in milrinone-supplemented media for an initial 16 h before being washed and transferred to control-media for a subsequent 20 h to examine milrinone's reversibility/toxic effects on meiosis. A mean number of 80 oocytes were used in each treatment group from three replicate experiments. Means in both the bar and line graphs within the same meiotic stage with no common superscripts are significantly different (Chi squared analysis, $P < 0.05$).

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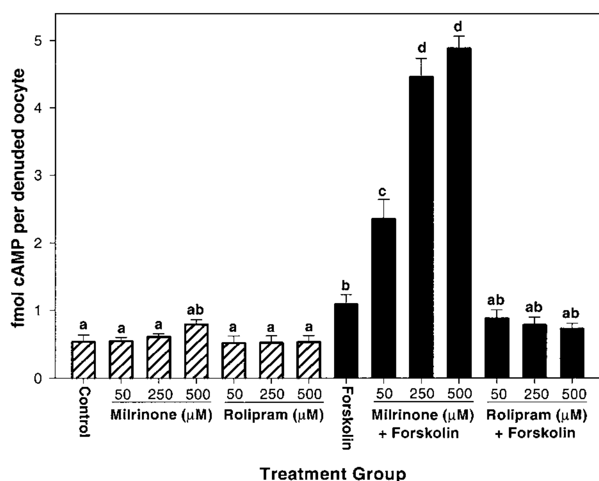

FIG. 4. Effect of increasing concentrations of the type 3 (milrinone) and type 4 (rolipram) inhibitors ± forskolin (0.1 mM) on the cAMP content of denuded oocytes following a 5 h culture period. Oocytes were cultured in milrinone- or rolipram-supplemented culture media with or without forskolin and were then assessed for cAMP content. Values are expressed as the mean concentration of cAMP per oocyte ± SEM of three replicates using 19–24 DOs per treatment replicate. Means with different superscripts indicate significantly different amounts of cAMP between individual treatments (One-way ANOVA, $P < 0.05$).

with the type 3 PDE inhibitor milrinone prevents the spontaneous GVBD and meiotic progression of isolated oocytes by increasing intraoocyte cAMP, cAMP concentrations were measured in cumulus-enclosed, complex-derived, and denuded oocytes after 5 h of culture (Figs. 4 and 5). Oocytes were also treated with the type 4 PDE inhibitor rolipram to determine whether its dissimilar effect on nuclear maturation was due to a separate potency in inhibiting cAMP degradation.

Neither the type 3, nor the type 4 PDE inhibitor alone elevated DO cAMP levels, even at the very high concentration of 500 μm (Fig. 4). In combination with forskolin, the PDE3 inhibitor elevated oocyte cAMP levels in a dose dependant manner above that of forskolin alone (1.09 fmol per oocyte), with a maximal concentration achieved at 250 μm (4.46 fmol per oocyte). Forskolin treatment combined with 500 μm milrinone, the highest dose tested, did not elevate cAMP above that of 250 μm. The PDE4 inhibitor failed to increase oocyte cAMP in combination with forskolin, even after treatment with 500 μm (Fig. 4).

Cumulus-oocyte complexes (COCs), oocytes cultured and assayed with their cumulus mass intact, were used to evaluate the effect of the PDE inhibitors on intracomplex cAMP concentrations. Treatment of COCs with the type 3 PDE inhibitor milrinone and the type 4 PDE inhibitor rolipram alone failed to elevate cAMP levels above that of control-treated COCs (Fig. 5A). In contrast, treatment of COCs with forskolin alone induced a 50-fold increase in cAMP over that of control-treated COCs. Combined treatment of COCs with forskolin and either milrinone or rolipram failed to induce COC cAMP levels significantly different to those complexes treated with forskolin alone, although the numerical value of rolipram and forskolin treatment combined was approximately double that for forskolin alone or in combination with milrinone.

Complex-derived oocytes (cultured with their cumulus mass intact and then denuded before assay) were used to evaluate the effect of the surrounding cumulus cells on intraoocyte cAMP levels (Fig. 5B). On average, control-treated COCs (Fig. 5A) contained approximately 3.5 times the amount of cAMP measured in similarly treated CDOs (Fig. 5B). CDOs treated with either of the PDE inhibitors alone did not induce an increase in oocyte cAMP above that of the control (Fig. 5B). Forskolin treatment of CDOs alone induced a 47-fold increase in the amount of oocyte cAMP compared to that of control-treated CDOs. Treatment of CDOs with the type 3 and 4 PDE inhibitors in combination with forskolin failed to induce significantly different levels of cAMP compared to forskolin alone (Fig. 5B). cAMP levels in DOs treated with forskolin alone were not significantly different from controls (Fig. 5C). However, milrinone treatment of DOs in combination with forskolin produced intraoocyte cAMP levels 3.9 and 2.8 times those levels measured after treatment with milrinone alone and forskolin alone, respectively (Fig. 5C). In contrast, combined rolipram and forskolin treatment of DOs failed to induce cAMP levels significantly different from that of controls. These results suggest the type 3, but not the type 4 PDE inhibitor, is able to elevate intraoocyte cAMP.

Effect of Type 3, Type 4, and Nonspecific PDE Inhibitors on Mural Granulosa Cell cAMP Synthesis

MGCs treated with either the type 4 PDE inhibitor or the nonspecific PDE inhibitor combined with rhFSH caused a significant ($P < 0.05$) increase in the production of cAMP above that of control-treated and type 3 PDE inhibitor-treated granulosa cells (Fig. 6). No PDE inhibitor effects were observed in the absence of rhFSH. Compared to controls, combined treatment of 120 mIU rhFSH and rolipram induced a 2.5-fold increase in cAMP, and 120 mIU rhFSH combined with IBMX induced an almost fourfold increase in cAMP concentration in media from treated granulosa cells. In contrast, the type 3 PDE inhibitor milrinone in combination with high rhFSH concentrations failed to significantly elevate cAMP production above that of control-treated cells (Fig. 6).

A dose response of the type 4 PDE inhibitor in combination with three different concentrations of rhFSH (Fig. 7A) and forskolin (Fig. 7B) increased production of cAMP by MGCs with increasing concentrations of rolipram. Rolipram treatment alone, even at the highest concentration of 100 μm, did not induce a significant increase in cAMP production. MGCs treated with FSH or forskolin alone

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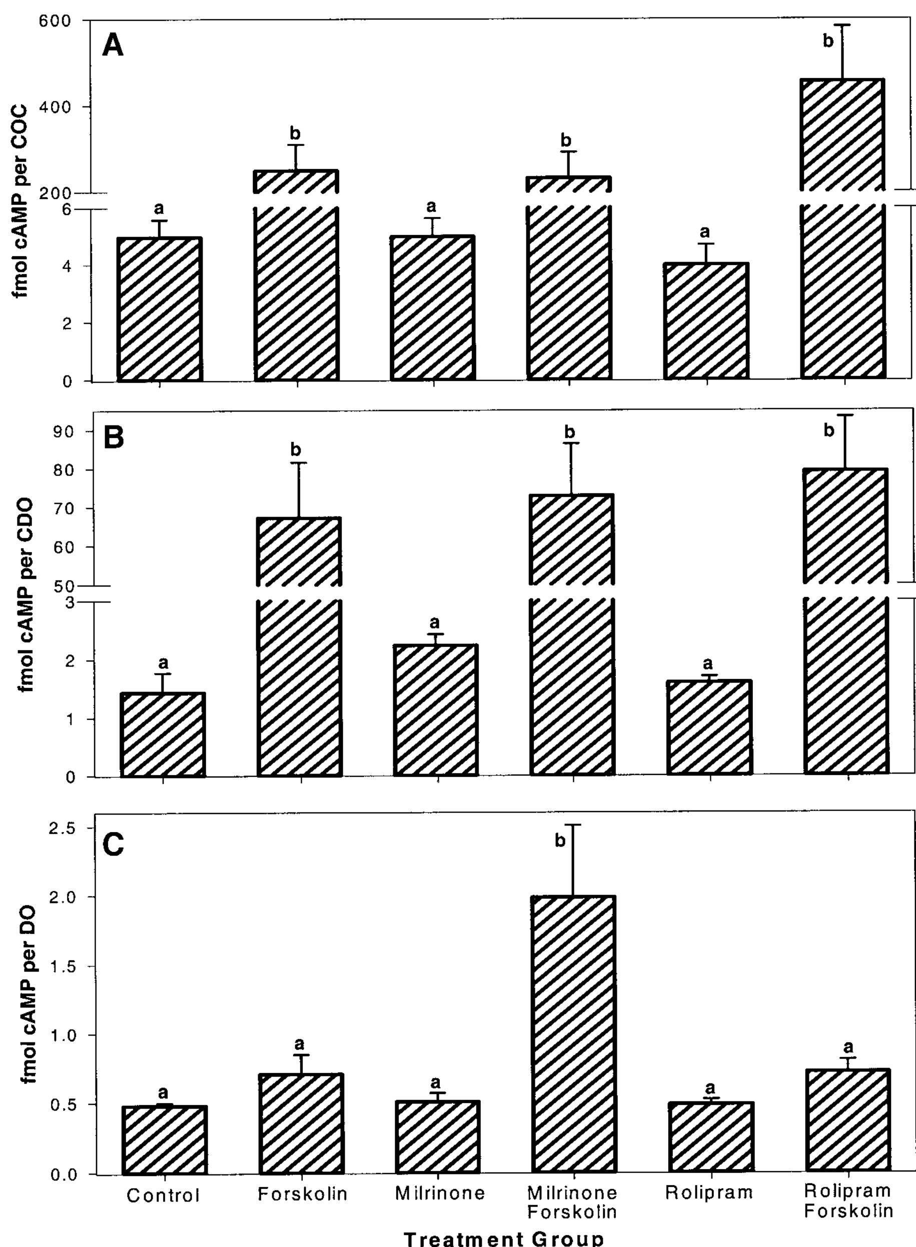

FIG. 5. Effect of the type 3 (milrinone; 10 μm) and type 4 (rolipram; 10 μm) PDE inhibitors ± forskolin (0.1 mM) on the cAMP content of three different types of oocytes following a 5 h culture period: those cultured and assayed with their cumulus investment intact (COCs) (A); those cultured as COCs but denuded of their cumulus investment before assay (CDOs) (B); and those denuded of all cumulus cells before culture and assay (DOs) (C). Oocytes of each of the three types were cultured in milrinone- or rolipram-supplemented culture media with or without forskolin and were then assessed for cAMP content. Values are expressed as the mean concentration of cAMP per oocyte or complex ± SEM of three replicates using 4–7 COCs, 12–15 CDOs, and 21–24 DOs per treatment replicate. Means within the same graph/oocyte type with different superscripts indicate significantly different amounts of cAMP between individual treatments (One-way ANOVA, $P < 0.05$).

produced increased levels of cAMP, with the highest doses producing levels 3.5- and 5.2-fold above that of control treated cells, respectively. Rolipram combined with FSH or forskolin dramatically elevated MGC cAMP production.

DISCUSSION

This study demonstrates that specific PDE subtypes have differing activities within the two compartments of the

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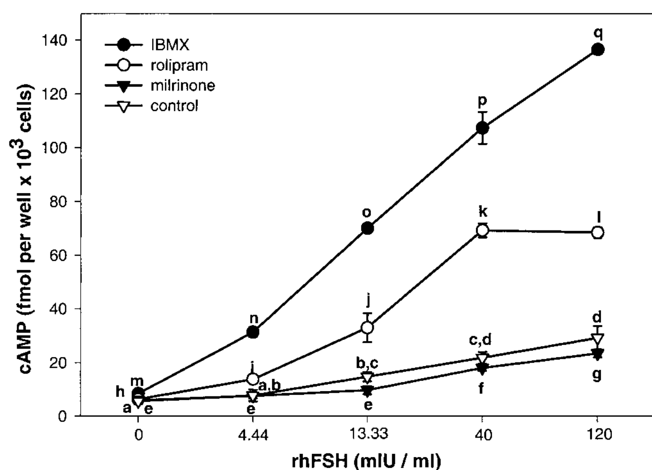

FIG. 6. Effect of the type 3 (milrinone; 10 μm), type 4 (rolipram; 10 μm), and the nonspecific (IBMX; 200 μm) PDE inhibitors on the production of cAMP by mural granulosa cells. Granulosa cells were cultured with each of the PDE inhibitors and increasing concentrations of rhFSH for a 24 h-incubation period. Values are expressed as mean ± SEM concentration of cAMP in the culture media after incubation. Each point represents a mean of three replicates. Means with no common superscripts indicate significant differences between amount of cAMP within an individual treatment (control[a–d], milrinone[e–g]; rolipram[h–l] and IBMX[m–q]; One-way ANOVA, SPSS package, $P < 0.05$).

bovine follicle—the type 3 PDE in the oocyte and the type 4 PDE in the granulosa cells. The type 3 PDE inhibitor, but not the type 4, prevented the resumption of spontaneous meiotic maturation and elevated intraoocyte cAMP in cultured denuded oocytes. In contrast, the type 4 PDE inhibitor had no effect on the oocyte, but elevated mural granulosa and cumulus cell cAMP production.

The PDE3, but not the PDE4 inhibitor, significantly delayed the meiotic progression of both cumulus-oocyte complexes and denuded oocytes, implying the bovine oocyte contains an active, milrinone-sensitive PDE3. The inhibitory effect of milrinone was not transient—those oocytes arrested at the GV stage remained so for at least 36h (the longest incubation time measured)—and was not due to toxic effects on meiosis as indicated by its demonstrated reversibility. The effectiveness of cilostamide (an alternate PDE3 inhibitor) in preventing GVBD and delaying progression of meiosis to MII provides further evidence that the inhibitory effect of milrinone is indeed due to inhibition of PDE3 and not a nonspecific or artifactual effect. The absence of a PDE4 inhibitor effect on meiosis in denuded oocytes is consistent with previously published results that type 4 PDEs are found primarily in the somatic compartment of the rodent follicle and not in the oocyte (Reinhardt *et al.*, 1995; Tsafriri *et al.*, 1996).

Although evidence for the participation of cAMP-dependant pathways in the maintenance of meiotic arrest is compelling in rodents, the situation is more complex in higher order mammals. Due to the similarity between PDE subtype sequences across species (Conti and Jin, 2000), it is likely that the presence of subtypes similar in regulation and function should also act similarly in other mammalian species. Despite this, rodent oocytes are experimentally more responsive than those of ungulates to cAMP and its analogues, as well as to inhibitors of PDEs (for a review see (Schultz, 1991); rodent (Tsafriri *et al.*, 1996; Wiersma *et al.*, 1998); bovine (Aktas *et al.*, 1995a; Sirard and First, 1988).

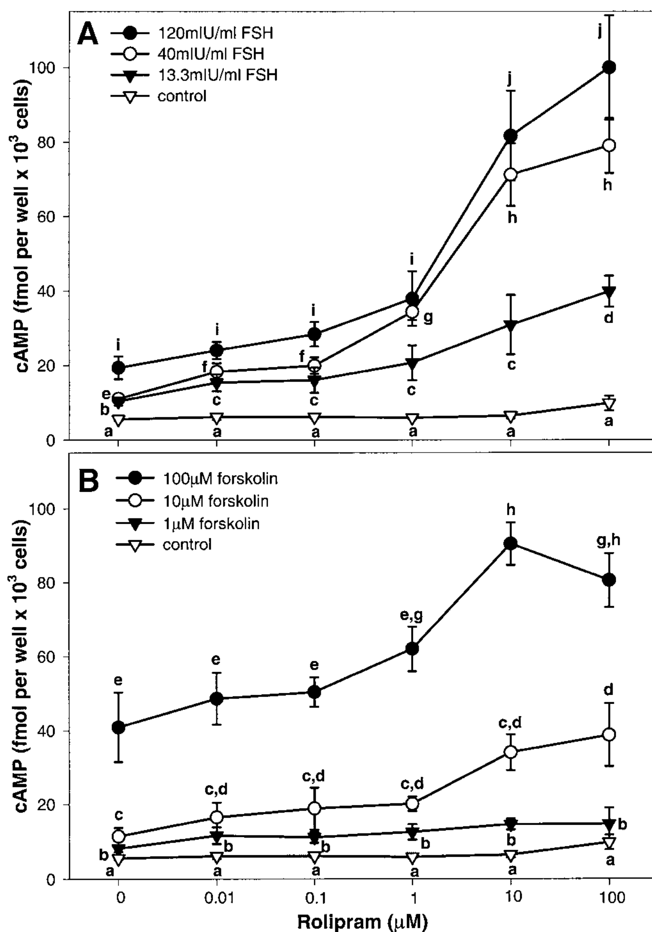

FIG. 7. Effect of increasing doses of the type 4 PDE inhibitor rolipram on the production of cAMP by mural granulosa cells in response to stimulators of cAMP - (A) rhFSH and (B) forskolin. Granulosa cells were cultured with increasing concentrations of rolipram and three different concentrations of rhFSH or forskolin for a 24 h incubation period. Values are expressed as mean ± SEM concentration of cAMP in the culture media after incubation. Each point represents a mean of three replicates. Means with no common superscripts indicate significant differences between amount of cAMP within an individual treatment (Fig. 5a: control[a], 13.3 mIU FSH[b–d]; 40 mIU FSH[e–h] and 120 mIU FSH[i–j]; Fig. 5b: control[a], 1 μm FK[b]; 10 μm FK [c–d] and 100 μm FK [e–h]; One-way ANOVA, SPSS package, $P < 0.05$).

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Bovine oocytes recovered from slaughterhouse ovaries are extremely heterogeneous in terms of quality and developmental competence, probably due to the mixed age, breed, and reproductive status (i.e. follicle size, day of estrous cycle, level of atresia and influence of dominant follicles) of cows passing through the abattoir on a given day (Fry *et al.,* 1997; Machatkova *et al.,* 2000). In our laboratory for example, milrinone maintains ~15% of bovine oocytes (present study), but 80–90% of mouse oocytes at the GV stage (Gilchrist *et al.,* 2001) *in vitro*. Accordingly, the reduced efficacy of PDE3 inhibitors in bovine compared to rodent oocytes may be related to greater follicular heterogeneity typical for bovine studies using ovaries collected at abattoirs.

Although adenylate cyclase has been localised to the membrane of the bovine oocyte (Kuyt *et al.,* 1988), in the absence of stimulation its basal activity may be too low to allow milrinone alone to elevate oocyte cAMP levels sufficiently to maintain arrest of oocytes in GV stage. Combined treatment of DO with forskolin and milrinone (but not rolipram) increased oocyte cAMP. This is the first report that distinct PDEs can selectively control cAMP levels in the granulosa and germ cell compartments of the bovine follicle, with PDE3 being active in the oocyte, and PDE4 in the cumulus cells. This supports the finding in rodent (Reinhardt *et al.,* 1995; Tsafriri *et al.,* 1996; Wiersma *et al.,* 1998) and preliminary observations in bovine (Mayes, 2000) studies, that PDE subtypes are compartmentalised in the bovine follicle and provides a mechanism for the differential regulation of cAMP through the compartmental expression of different phosphodiesterase subtypes.

Measurements of cAMP levels in oocytes exposed to the various agents as DO and COC provide compelling evidence for two sources of cAMP in the oocyte: one, internally generated (within the oocyte), with levels determined by a balance between synthesis in the oocyte by adenylate cyclase (weak stimulation by forskolin) and degradation by PDE3 (inhibited by milrinone, but not rolipram); and two, externally generated (from cumulus cells), where levels are strongly stimulated by forskolin alone but are not greatly influenced by either PDE inhibitor. Regulation of intraoocyte cAMP apparently occurs primarily by control of its degradation (by very active PDE3), rather than by endogenous synthesis. While exposure of COC to forskolin increased cAMP levels in both cumulus cells and CDO, neither the PDE3 inhibitor milrinone, nor the PDE4 inhibitor rolipram altered basal or forskolin-stimulated cAMP levels. Therefore, in contrast to intraoocyte cAMP level regulation, cAMP levels in COC seem to be regulated primarily by control of synthesis in the cumulus cells (by very active adenylate cyclase)—with its degradation by PDE4 being of lesser importance. The considerably higher intraoocyte levels of cAMP achieved in CDO than in DO indicate that cumulus cells are the major source, with internally generated cAMP being quantitatively less important. The transfer of cAMP from cumulus cells to oocyte appears an efficient process, with approximately 25% of that produced in COC in response to stimulation with FK appearing in the CDO derived therefrom. As a consequence of this efficient transfer, much higher levels of cAMP are attained in oocytes derived from COC stimulated maximally with forskolin and rolipram combined (80 fmol/oocyte) compared to DO maximally stimulated with forskolin and milrinone combined (2 fmol/oocyte).

In attempting to relate observed changes in cAMP levels to changes in meiotic stage attained under the various treatments, account must be taken of the heterogeneity of oocytes under study, as discussed above. It is evident that only a minority of oocytes was capable of inhibition of GVBD under the conditions of the study, irrespective of treatment. Forskolin by itself reduced GVBD in a small proportion of oocytes (10% of COC; 20% of DO), and only approximately 40% of oocytes remained in GV stage when exposed to FK + MR either as COC or as DO. Of the remaining oocytes (those that underwent GVBD irrespective of treatment), the stage reached after 16 h in culture differed depending on whether exposed to treatment as DO or as COC. Just under half of those treated with FK and/or MR as DO completed meiosis (reached MII stage), whereas the majority of those exposed to FK + MR as COC proceeded only to MI stage during the 16 h culture. It is of interest to relate these maturation results to maximal intraoocyte cAMP levels attained during 5 h culture under the same treatments; the cAMP level attained in DO when maximally stimulated with FK + MR was 2 fmol/oocyte, compared to approximately 70 fmol/oocyte when stimulated as COC with FK + MR. These results suggest that a (minor) subpopulation of the oocytes is capable of complete inhibition of meiosis by treatments that result in a modest increase in intraoocyte cAMP level of ≤2 fmol/oocyte. In contrast, the majority of oocytes, which undergo GVBD spontaneously irrespective of treatment, respond to considerably higher cAMP levels (up to 80 fmol/oocyte) with inhibition at a later meiotic stage.

An additional level of meiotic control has been proposed in rodent oocytes, whereby the inhibitory effect of elevated intraoocyte cAMP can be over-ridden by an uncharacterised factor(s) of somatic cell origin, for example follicular fluid meiosis-activating sterol (FF-MAS) secreted by follicular somatic cells in response to gonadotrophic stimulation (Byskov *et al.,* 1997). Secretion of FF-MAS by rodent granulosa cells is itself a cAMP-dependent process. Although direct evidence for FF-MAS in regulation of oocyte maturation in nonrodent species is lacking, the results of our studies may offer tenuous support for a similar mechanism of meiotic control in bovine oocytes. Thus, the addition of the PDE4 inhibitor rolipram to COC cultured with FK was able to partially reverse the inhibitory effect of FK alone on progression of maturation from MI to MII stage (Fig. 2A), while eliciting an approximate doubling (albeit not statistically significant) of mean cAMP levels attained in the COC culture (Fig. 5). This finding, together with the lack of a similar effect of combined FK + RP on either progression of meiosis or cAMP levels in DO and CDO, is consistent

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with a role of factor(s) secreted by cumulus cells in response to high levels of cAMP that override the meiosis-inhibiting action of cAMP on completion of meiosis.

Measurements of cAMP production by mural granulosa cells were consistent with those of cumulus cells, indicating that the follicular somatic cells contain the rolipram-sensitive type 4 PDEs, but not the milrinone-sensitive type 3 PDEs. In conjunction with FSH or forskolin treatment, cAMP production by mural granulosa cells was increased by treatment with the PDE4 inhibitor rolipram and the nonspecific PDE inhibitor IBMX, but not by those treated with PDE3 inhibitor milrinone. Although the subtype-specific localisation of PDEs has been confirmed in rodent follicles using *in situ* hybridisation (Tsafriri *et al.,* 1996), the results of the present study are the first to demonstrate this in a nonrodent species. In the absence of stimulation by FSH or forskolin, granulosa cells did not produce increased cAMP concentrations following treatment by any of the PDE inhibitors.

In conclusion, this study has utilised the compartmentalisation of PDE4 and PDE3 within the bovine follicle to alter cAMP levels in the somatic and germ cell compartments, respectively. The oocyte is responsive to the PDE3 inhibitor but not the PDE4 inhibitor in terms of both cAMP and the maintenance of meiotic arrest. On the other hand, the granulosa cells respond to the PDE4, but not to the PDE3 inhibitor, and cumulus cell production of cAMP is a significant contributor to the total cAMP content of the oocyte. The selective PDE inhibitors are powerful experimental tools to study the oocyte and surrounding cumulus cells in separation, and to demonstrate the functions of specific PDEs in regulation of cAMP levels in the two follicular compartments. Such studies should provide new insights into the fundamental mechanisms regulating mammalian oocyte maturation, with potential for development of improved methods of *in vitro* embryo production for assisted reproduction applications in livestock and humans.

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