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Inhibition of PDE3A sustains meiotic arrest and gap junction of bovine growing oocytes in *in vitro* growth culture

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Abstract

Bovine growing oocytes with a diameter of 105–115 μm from early antral follicles (1.2–1.8 mm) are able to resume meiosis, but lack the competence to mature to metaphase II. To confer full maturation competence onto the oocytes, culture systems which can support their growth and prevent their meiotic resumption during culture are needed. In this study, we cultured growing oocytes for 5 days to examine the effects of different phosphodiesterase (PDE) inhibitors on meiotic arrest and acquisition of full maturation competence of growing oocytes, and their gap junctional communication with cumulus cells. Growing oocyte-cumulus complexes (OCCs) were cultured with 3-isobutyl-1-methylxanthine (IBMX; broad-spectrum PDE inhibitor), rolipram (PDE4 inhibitor), cilostamide and milrinone (PDE3A inhibitors). The mean diameters of oocytes increased similarly in all groups. IBMX, cilostamide and milrinone induced antrum formation by OCCs and maintained meiotic arrest of oocytes during culture, whereas rolipram neither promoted antrum formation nor maintained oocyte meiotic arrest. Gap junctional communication between oocytes and cumulus cells was maintained by IBMX and cilostamide, but not by rolipram as judged by the transfer of injected lucifer yellow dye from oocytes to cumulus cells. In subsequent *in vitro* maturation, oocytes grown with IBMX, cilostamide and milrinone showed full maturation competence. These results suggest that PDE3A inhibition maintains the meiotic arrest of bovine growing oocytes and sustains their gap junctional communication with cumulus cells for 5 days, thereby contributing to their acquisition of full maturation competence.

Keywords: Antrum formation, Bovine oocyte, Gap junction, Meiotic arrest, Phosphodiesterase

1. Introduction

Mammalian ovaries contain a huge number of oocytes arrested at prophase I. During this arrested period, oocytes increase in size and acquire complete meiotic competence to resume meiosis and mature to metaphase II (MII). In the ovary, oocyte growth is coordinated with follicular development, which occurs by morphological change and active proliferation of granulosa cells and formation of an antrum

structure [1]. The size and stage of follicles at which oocytes complete their growth are dependent on species. In mice, fully grown oocytes are obtained from early antral follicles [2], whereas porcine and bovine oocytes complete their growth in follicles at the middle antral stage [3−5]. Acquisition of meiotic competence of oocytes depends on follicle size and oocyte diameter. Bovine oocytes with a diameter of <100 μm (from <1 mm follicles) are meiotically incompetent, whereas oocytes with a diameter of >120 μm (from ≥4 mm follicles) are meiotically competent to mature to MII [5].

Many approaches have been taken to grow mammalian small oocytes *in vitro* [6,7]. For example, a variety of growth culture systems have been developed to utilize incompetent small oocytes (<100 μm) collected from bovine ovaries [8−11]. Hirao and his colleagues developed a long-term growth culture system, in which bovine oocyte-granulosa cell complexes (OGCs) were cultured in medium supplemented with 4% (w/v) polyvinylpyrrolidone for 2 weeks [10]. This system supported the growth of bovine small oocytes (<100 μm), and OGCs formed an antrum structure during the culture. In addition, some of the growth culture systems for bovine incompetent oocytes led to successful production of offspring [8,10,11]. On the other hand, less emphasis has been given to the long-term growth culture of 100–120 μm oocytes. Bovine growing oocytes with diameters in this range have some unique features. They are characterized by ongoing RNA transcription [12–14], distinctive chromatin configuration [13,15], specific epigenetic marks [13,16] and organelle distribution [15], all of which are different from those of fully grown oocytes. However, oocytes of this size are able to resume meiosis, although they are incompetent to mature to MII. Thus these oocytes are considered "partially meiotic-competent", and their precocious resumption of meiosis during growth culture makes it difficult for them to grow to fully meiotic competent oocytes.

The inhibition of meiotic resumption of fully grown oocytes using phosphodiesterase (PDE) inhibitors has been well studied *in vitro* [17]. It has been reported that PDE3A inhibitors, not PDE4 inhibitors, prevent meiotic resumption of oocytes in several species [18], although the effects of inhibitors are still controversial in bovine oocytes [19–22].

Follicular oocytes are connected by gap junctions to surrounding cumulus cells which support oocyte growth and development [23,24]. In *in vitro* growth culture, the maintenance of gap junctions is essential for oocyte growth [25]. In addition to their effect on the meiotic arrest of oocytes, PDE

69 inhibitors have also recently been reported to affect the gap junctional communication between oocytes
70 and cumulus cells [12]. A broad-spectrum PDE inhibitor (IBMX) sustained gap junctional
71 communication of fully grown bovine oocytes [26], and cilostamide, a PDE3A inhibitor, prolonged the
72 gap junction functionality of bovine growing oocytes [12]. So far, however, PDE inhibitors have not
73 been applied for long-term growth culture of bovine growing oocytes.

74 In the present study, partially meiotic-competent bovine oocytes (105–115 μm) with
75 surrounding cumulus cells were cultured for growth for 5 days, and the effects of different PDE
76 inhibitors on the growth, meiotic arrest, and acquisition of full-meiotic competence of these oocytes
77 were examined. During the growth culture, we examined the effect of PDE inhibitors on the antrum
78 formation by OCCs, and at the end of the growth culture, the gap junctional communication between
79 oocytes and cumulus cells was examined.

80

81 **2. Materials and methods**

82

83 **2.1. Chemicals**

84 Unless otherwise mentioned, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

85

86 **2.2. Collection of OCCs**

87 Collection, *in vitro* growth culture, and *in vitro* maturation of OCCs were carried out as described
88 previously [10,27] with some modifications. Briefly, bovine ovaries were collected at a local slaughter
89 house and conveyed to the laboratory. The ovaries were washed once with 0.2% (w/v)
90 cetyltrimethylammonium bromide and then three times with Dulbecco's PBS containing 0.1% (w/v)
91 polyvinyl alcohol (PBS-PVA). A surgical blade (No. 10; Feather Safety Razor, Tokyo, Japan) and
92 forceps were used to make the ovarian cortical slices. Early antral follicles (1.2–1.8 mm in diameter)
93 were collected from ovarian cortical slices in 25 mM HEPES-buffered medium 199 (HEPES-199;
94 Nissui Pharmaceutical, Tokyo, Japan) containing 0.1% (w/v) PVA, 0.85 mg/mL sodium bicarbonate,
95 and 0.08 mg/mL kanamycin sulfate. The follicles were opened using a blade (No. 10) and forceps to
96 collect OCCs with growing oocytes. The diameter of oocytes (excluding the zona pellucida) was

97 measured by an ocular micrometer attached to an inverted microscope. OCCs with oocyte diameters of
98 105–115 μm were selected. Some of the OCCs were used for *in vitro* growth culture, and the remainder
99 were used as an *in vivo* control (growing oocytes: GO). OCCs from middle antral follicles (4–6 mm in
100 diameter) were sucked up with follicular fluid using a syringe and needles (18 ga; Terumo, Tokyo,
101 Japan), and then the diameter of oocytes was measured. The OCCs collected from middle antral follicles
102 with oocytes of 120–130 μm in diameter served as an *in vivo* fully grown control (fully grown oocytes:
103 FO).
104
105 **2.3. *In vitro* growth culture of OCCs**
106 Collected bovine OCCs from early antral follicles were cultured for 5 days in 96-well culture plates
107 (Biocoat Collagen I Cellware; Becton Dickinson Biosciences, San José, CA) containing 200 μL culture
108 medium. Each OCC was placed in a well of a 96-well plate for individual culture. The culture conditions
109 were 5% CO_2, 5% O_2, and 90% N_2 in humidified air at 38.5°C. The basic culture medium was Minimum
110 Essential Medium Alpha (GIBCO, Invitrogen, Scotland, UK) supplemented with 4% (w/v)
111 polyvinylpyrrolidone (molecular weight 360,000), 5% (v/v) fetal bovine serum (ICN Biomedicals,
112 Aurora, OH), 4 mM hypoxanthine, 50 μg/mL ascorbic acid 2-glucoside (Hayashibara Biochemical
113 Laboratories, Okayama, Japan), 55 μg/mL cysteine, 0.05 mM dexamethasone, 100 ng/mL 17β-estradiol,
114 10 ng/mL androstenedione, 1 mM sodium pyruvate, 2.2 mg/mL sodium bicarbonate, and 0.08 mg/mL
115 kanamycin sulfate [27]. The culture medium was also separately supplemented with different PDE
116 inhibitors: 0, 2.5, 5, 50, or 100 μM of IBMX; 0, 5, 25, or 50 μM of rolipram; 0, 2.5, 5, or 10 μM of
117 cilostamide; and 0, 2.5, 5, or 25 μM of milrinone. These concentrations of PDE inhibitors were selected
118 on the basis of the IC_{50} values of PDE inhibitors and previous reports [19,28]. The day of OCC
119 collection was designated as Day 0, and half (100 μL) of the culture medium was replaced with new
120 medium on Day 3. Antrum formation by OCCs was examined on Day 3 and Day 5 by identifying visible
121 spaces surrounded by cumulus cells.
122 After 5 days of growth culture, OCCs showing complete detachment of cumulus cells from the
123 oocytes and/or oocytes with cytoplasmic degeneration were not used for further experiments. The
124 diameter of oocytes from OCCs with a healthy structure was measured using an ocular micrometer.

125 Some of the oocytes were denuded mechanically, fixed in aceto-ethanol and stained with 1% (w/v)
126 aceto-orcein. The stages of meiotic division were examined by Nomarski interference microscopy.
127 Other oocytes were used for further experiments (*in vitro* maturation and lucifer yellow dye transfer).
128 The stained oocytes were classified on the basis of morphology of the chromatin and nuclear envelope
129 [4,27,29]. Oocytes before meiotic resumption were at the filamentous chromatin (FC), stringy
130 chromatin (SC), or germinal vesicle (GV) stage (I-IV). After resumption of meiosis, the stages were
131 classified into early diakinesis (ED), late diakinesis (LD), metaphase I (MI) and metaphase II (MII).
132 Oocytes showing cytoplasmic or nuclear abnormalities were considered degenerated oocytes.
133

134 **2.4. *In vitro* maturation of oocytes**

135 The OCCs surviving after 5 days of *in vitro* growth culture were further used for *in vitro* maturation.
136 OCCs were cultured in a 50 μL droplet of maturation medium under paraffin oil in an atmosphere of
137 5% CO_2 at 38.5°C for 22 hours. About 5 OCCs were cultured in each droplet. The maturation medium
138 was bicarbonate-buffered medium 199 supplemented with 10% (v/v) fetal bovine serum, 0.1 mg/mL
139 sodium pyruvate, 0.1 IU/mL human menopausal gonadotropin (Asuka, Tokyo, Japan), 2.2 mg/mL
140 sodium bicarbonate and 0.08 mg/mL kanamycin sulfate. After 22 hours, oocytes were denuded
141 mechanically with the help of 0.1% (w/v) hyaluronidase and using a small-bore pipette. Then they were
142 fixed in aceto-ethanol and stained with 1% (w/v) aceto-orcein to examine the stage of oocyte maturation.
143 The oocytes showing female pronuclei or cytoplasmic degeneration were considered degenerated
144 oocytes. OCCs collected from early antral follicles (1.2–1.8 mm in diameter) and middle antral follicles
145 (4–6 mm in diameter) were also subjected to maturation, and served as *in vivo* control groups.
146

147 **2.5. Lucifer yellow (LY) dye transfer**

148 Healthy OCCs after 5 days of growth culture were further used for LY dye transfer. Microinjection of
149 LY to OCCs was done as described by Isobe et al. [30] with some modifications. Oocytes in OCCs
150 were microinjected with HEPES-199 containing 10% (w/v) lucifer yellow CH dilithium salt
151 (fluorescent stain). Holding and injection pipettes were prepared by drawing glass capillary tubes with
152 a pipette puller (P-97/IVF; Sutter Instrument, Novato, CA), followed by processing with a Microforge

153 (MF- 79; Narishige Scientific Instrument Lab., Tokyo, Japan). LY solution was injected into each
154 oocyte by regulating the duration and gas pressure of injection using an electric controlled pneumatic
155 microinjector (IM-30; Narishige Scientific Instrument Lab.). The volume of LY solution injected to the
156 oocytes was estimated at approximately 4 pL based on the procedure of Isobe et al. [30]. After
157 microinjection, OCCs were placed in HEPES-199 to allow the spread of dye from oocytes to cumulus
158 cells through gap junctions. The spread of LY from oocytes to the outermost layer of cumulus cells took
159 25–30 min, when the OCCs were observed under a fluorescence microscope (Olympus IX70 Multi-
160 parameter Fluorescence Microscope; Olympus, Tokyo, Japan). Therefore, all of the OCCs were
161 examined for dye transfer under a fluorescence microscope (FV1000-KDM; Olympus) after 30 min of
162 LY microinjection.

163 The OCCs were classified into two categories depending on the spread of the dye to the cumulus
164 cells. In dye-positive OCCs, approximately more than half of the cumulus cells surrounding the oocytes
165 were stained with LY dye, and in dye-negative OCCs, approximately less than half of the cumulus cells
166 surrounding the oocytes were stained with LY dye. As a negative control, freshly collected OCCs from
167 1.2–1.8 mm follicles were incubated for 15 hours with *in vitro* growth culture medium containing
168 carbenoxolone disodium salt (30 μM), a gap junction blocker [31,32]. Then the oocytes were
169 microinjected with LY. Some of the freshly collected OCCs from 1.2–1.8 mm follicles were
170 microinjected with LY just after collection as the positive control.

171

172 **2.6. Statistical analysis**

173 Mean diameters of oocytes were compared using a *t*-test: two-sample assuming unequal variances
174 (Excel software). All data from other experiments were subjected to one-way ANOVA followed by
175 Duncan's test (IBM SPSS Statistics 22). Values of $P < 0.05$ were considered significant.

176

177 **3. Results**

178

179 **3.1. Growth of oocytes**

180 The changes of oocyte diameter are shown in Figure 1. The diameter of oocytes increased significantly
181 from Day 0 to Day 5 in media with or without PDE inhibitors. But there were no significant differences
182 in the oocyte diameters after culture among the inhibitors. In all groups, the diameters of oocytes after
183 culture increased similarly to those of oocytes grown *in vivo* (Fig. 1. "FO" in each group) in the middle
184 antral follicles (4–6 mm).
185
186 **3.2. Antrum formation**
187 The typical morphologies of bovine OCCs during growth culture are shown in Figure 2. After placing
188 OCCs in the culture media, cumulus cells began to proliferate and formed a multilayered structure
189 surrounding the oocytes. After 2–3 days, some of the OCCs formed visible antra by developing spaces
190 inside the multilayers of cumulus cells (Day 3). The antra became more prominent day by day until the
191 end of culture (Day 5).
192 IBMX induced a significantly higher percentage of antrum formation by OCCs than in the
193 OCCs without IBMX (Figs. 2A and 3A). Among the IBMX groups, there was no significant difference
194 in antrum formation. Rolipram had no significant effect on antrum formation (Figs. 2B and 3B).
195 However, the cultures with cilostamide induced significantly higher percentages of antrum formation
196 compared to those without the inhibitor (Figs. 2C and 3C). Among the cilostamide groups, the
197 maximum percentage of antrum formation (88%) was observed at the concentration of 5 μM. Milrinone
198 also induced antrum formation during the growth culture of oocytes. Milrinone 5 μM ensured the
199 maximum percentage of antrum formation by OCCs (87%), which was significantly higher than that of
200 the OCCs cultured without milrinone (Figs. 2D and 3D).
201
202 **3.3. Effect of PDE inhibitors on meiotic arrest of oocytes during *in vitro* growth**
203 Bovine growing oocytes (105–115 μm) collected from early antral follicles (1.2–1.8 mm) were at the
204 FC or SC stage, and only a few were at the GV (I-IV) stage (Supplementary Table 1). On the other hand,
205 *in vivo* fully grown oocytes (120–130 μm) collected from middle antral follicles (4–6 mm) were at the
206 GV (I-IV) stage.

207 A broad-spectrum PDE inhibitor, IBMX (50 μM), maintained the meiotic arrest of bovine
208 growing oocytes over 5 days of growth culture (Fig. 4). A PDE4 inhibitor, rolipram, did not maintain
209 the meiotic arrest of oocytes. Cilostamide, a PDE3A inhibitor, maintained the meiotic arrest of oocytes
210 over 5 days. The percentages of GV-stage oocytes were 57%, 80%, 94%, and 84% in media containing
211 0, 2.5, 5, and 10 μM cilostamide, respectively (Fig. 4). To ensure the effect of the PDE3A inhibitor,
212 another PDE3A inhibitor, milrinone, was added to the culture. Milrinone also maintained the meiotic
213 arrest of oocytes, and there was a significantly higher percentage (87%) of GV-stage oocytes when 5
214 μM milrinone was used than without milrinone (Fig. 4). At the maximum concentrations of cilostamide
215 (10 μM) and milrinone (25 μM), some oocytes were degenerated after growth culture (8% and 7% of
216 the total number of oocytes, respectively), which was similar to the level of degeneration in 100 μM
217 IBMX (Supplementary Table 1).
218
219 **3.4. *In vitro* maturation of oocytes grown with PDE inhibitors**
220 Since the broad-spectrum PDE inhibitor (IBMX) and PDE3A inhibitors (cilostamide and milrinone)
221 maintained meiotic arrest of oocytes during growth culture, but the PDE4 inhibitor did not, oocytes
222 grown with IBMX and PDE3A inhibitors were further subjected to *in vitro* maturation to assess their
223 maturation competence.
224 Growing oocytes collected from early antral follicles resumed meiosis after *in vitro* maturation,
225 but most of them remained at the early diakinesis (ED) or late diakinesis (LD) stage, and only a few
226 oocytes matured to the MII stage (GO in Fig. 5 and Supplementary Table 2). Fully grown oocytes
227 collected from middle antral follicles resumed meiosis and most of them matured to MII (FO in Fig. 5
228 and Supplementary Table 2). Oocytes grown with PDE inhibitors matured to MII at higher percentages
229 than the oocytes without PDE inhibitors. IBMX at 50 μM, cilostamide at 5 μM and milrinone at 5 μM
230 showed similar percentages of MII oocytes after *in vitro* maturation, which were comparable to the
231 percentages of fully grown oocytes *in vivo*.
232
233 **3.5. Effect of PDE inhibitors on gap junctional communication between oocytes and cumulus cells**

234 Gap junctional communication between oocytes and cumulus cells was examined in OCCs cultured
235 with each type of PDE inhibitor. For each PDE inhibitor, we selected the most effective concentration
236 for the meiotic arrest of oocytes. Typical diffusion patterns of LY from oocytes to cumulus cells are
237 shown in Figure 6. As expected, in the OCCs before *in vitro* growth culture, gap junctional
238 communication between oocytes and cumulus cells was observed as 100% dye-positive (GO in Fig. 7).
239 IBMX (50 μM) and cilostamide (5 μM) maintained the communications (dye-positive OCCs) at
240 significantly higher percentages (81% and 84%, respectively) than the culture with 25 μM rolipram and
241 without PDE inhibitor (55% and 46%, respectively). In the negative control group with carbenoxolone
242 (30 μM), only 25% of OCCs were dye-positive.
243
244 **4. Discussion**
245
246 In the ovary, oocytes and granulosa cells coordinately develop within the follicles and bidirectional
247 communication is maintained between oocytes and granulosa cells. The oocyte development includes
248 increase in diameter and acquisition of complete meiotic competence. Granulosa cell development
249 involves changes in shape, proliferation, formation of an antrum structure and differentiation to cumulus
250 and mural granulosa cells. In the present study, we used bovine OCCs containing partially meiotic-
251 competent growing oocytes collected from early antral follicles for the in *vitro* growth culture. In the
252 culture with specific PDE inhibitors, the oocytes continued their growth and acquired complete meiotic
253 competence, while at the same time the cumulus cells proliferated to form an antrum structure, and the
254 gap junctional communication was maintained between oocytes and cumulus cells.
255 The meiotic arrest of partially meiotic-competent bovine oocytes was maintained by IBMX and
256 PDE3A inhibitors (cilostamide and milrinone) during growth culture. On the other hand, PDE4 inhibitor
257 was unable to maintain the meiotic arrest of growing oocytes. The basic growth culture medium used
258 in this study contained hypoxanthine, which is a naturally occurring PDE inhibitor present in follicular
259 fluid [33]. Hypoxanthine maintained the meiotic arrest of growing oocytes collected from 11–12 day
260 old mice in the growth culture for 12 days [34]. Bovine oocyte-granulosa cell complexes containing
261 incompetent growing oocytes (90–99 μm) also maintained meiotic arrest in the growth culture for 11–

262 14 days in hypoxanthine-supplemented media [27,35]. However, partially meiotic-competent bovine
263 oocytes resumed meiosis in the basic growth culture medium in the present study. Meiotic resumption
264 of bovine fully grown oocytes has been shown to be prevented by PDE3A-specific inhibitors during *in*
265 *vitro* maturation culture [19,20]. The results of the present study using bovine growing oocytes are
266 consistent with these reports, and suggest that meiotic arrest of partially meiotic-competent bovine
267 oocytes can be attained in the same manner as that of fully grown oocytes. Since the functional PDE3A
268 is not present in bovine cumulus cells but is present in oocytes [20], it is reasonable to conclude that
269 PDE3A inhibitors directly inhibited PDE3A in partially meiotic-competent bovine oocytes to maintain
270 their meiotic arrest.

271 The LY dye transfer experiment revealed that bovine growing oocytes from 1.2–1.8 mm
272 follicles had gap junctional communication with surrounding cumulus cells. Because oocytes of this
273 size are still in the growing stage, they must take nutrients and metabolites from cumulus cells in order
274 to complete their growth. After 5 days of growth culture, IBMX and a PDE3A inhibitor (cilostamide)
275 maintained the gap junctional communication between oocytes and cumulus cells, whereas a PDE4
276 inhibitor (rolipram) did not maintain the gap junctional communication. Studies with rodent [36−39],
277 porcine [40–43] and bovine [44–48] oocytes suggest that gap junctional communication decreases with
278 the meiotic resumption of oocytes. In bovine oocytes, gap junctional communication between oocytes
279 and cumulus cells disappears after 6 hours of *in vitro* maturation culture [44]. In the present study, it is
280 thought that the PDE3A inhibition prevented meiotic resumption of bovine growing oocytes during
281 growth culture, which in turn prevented the disconnection of gap junctional communication between
282 oocytes and cumulus cells.

283 The increase in the diameters of oocytes after growth culture was similar between oocytes
284 cultured with or without PDE inhibitors. When bovine oocyte-granulosa cell complexes collected from
285 early antral follicles (0.4−0.7 mm) were cultured, steroid hormones (17β-estradiol and androstenedione)
286 promoted oocyte growth and connection through transzonal projections with granulosa cells [27].
287 Although oocyte-granulosa cell complexes cultured without steroid hormones did not maintain the
288 transzonal projections, the oocytes increased in diameter after 14 days of growth culture. In our study,

289 the oocytes appeared to continue growing after being placed in the growth culture, although the gap
290 junctional communication with the cumulus cells was reduced at the end of growth culture.

291 When oocyte-cumulus complexes were cultured with IBMX or PDE3A inhibitors, oocytes
292 acquired higher maturation competence than those cultured without any inhibitor. This indicates that
293 the oocytes whose meiotic resumption was arrested by the inhibitors gained maturational competence.
294 Bovine growing oocytes <110 μm in diameter have been shown to engage in RNA synthesis at a 3-fold
295 greater rate than fully grown oocytes of ≥120 μm in diameter [5]. RNA synthesis in the oocytes
296 continues after antrum formation in cows [49] and pigs [3] until the oocytes reach full size. A recent
297 study indicated that RNA synthesis of bovine growing oocytes is regulated by gap junctional
298 communication between oocytes and cumulus cells through a cAMP-mediated mechanism [12]. In that
299 study, when gap junctional communication was experimentally interrupted by 1-heptanol, oocyte RNA
300 synthesis suddenly ceased, and cilostamide, a PDE3A inhibitor, prevented the effect of 1-heptanol.
301 Moreover, the authors reported that 24 hour-culture of bovine growing oocytes with cilostamide before
302 *in vitro* maturation enhanced the ability of oocytes to mature and undergo early embryonic development
303 [12]. It has also been shown that short-term pre-maturation culture of fully grown oocytes with IBMX
304 and dbcAMP increased the ability of oocytes to mature and contribute to embryo production in cows
305 [50] and pigs [51]. Considering that gap junctional communication regulates oocyte RNA synthesis
306 through a cAMP-mediated mechanism [12], and that IBMX and PDE3A inhibitors maintained the gap
307 junctional communication between oocytes and cumulus cells for the 5 day culture period in the present
308 study, it appears that the inhibitors supported the RNA synthesis of bovine growing oocytes for their
309 growth and acquisition of full meiotic competence to MII.

310 After 5 days of growth culture, there was a significant increase in antrum formation by the
311 OCCs cultured with IBMX and PDE3A inhibitors, whereas the antrum formation was not promoted by
312 the PDE4 inhibitor. This result indicates that inhibition of PDE3A, which is functional specifically in
313 oocytes [20], promotes antrum formation by surrounding cumulus cells. Several factors have been
314 identified that regulate follicular antrum formation [52]. Shen et al. [53] reported that porcine granulosa
315 cells cultured with oocytes formed an antrum structure, although they did not form an antrum structure
316 in the absence of oocytes. Among the oocyte-secreted factors, growth-differentiation factor 9 (GDF 9)

317 [54,55] and bone morphogenetic protein 15 (BMP15) [56] have been shown to be essential for granulosa
318 cell proliferation in mice. Although it is not known whether these oocyte-derived factors act on antrum
319 formation, it is interesting to speculate that inhibition of oocyte PDE3A promotes the production of
320 some oocyte-derived antrum formation factors. Further study will be needed to reveal the relationship
321 between oocyte PDE3A and follicular antrum formation.

322 In summary, inhibition of PDE3A was shown to maintain the meiotic arrest of bovine growing
323 oocytes and to sustain their gap junctional communication with cumulus cells for 5 days of growth
324 culture, thereby contributing to the acquisition of full maturation competence of the oocytes.

325

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332

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334

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477

Figure legends

Fig. 1.

Effect of PDE inhibitors (A: IBMX; B: rolipram; C: cilostamide; and D: milrinone) on the growth of bovine oocytes. In each graph (A–D), the white box labeled by GO (left) represents the diameter of oocytes isolated from early antral follicles (1.2–1.8 mm) and the white box labeled FO (right) represents the diameter of oocytes isolated from middle antral follicles (4–6 mm). The numbers of oocytes (n) used for each experiment are shown at the bottom of each box. The mean diameters of oocytes are shown at the top of each box. In each graph, boxes with the same pattern (except the white boxes) indicate the whisker plot of oocyte diameters before (Day 0) and after (Day 5) growth culture. The numbers below each graph represent the concentration (μM) of PDE inhibitors used for growth culture. Boxes with different letters (a, b) are significantly different (*t*-test; $P < 0.05$).

Fig. 2.

Typical morphologies of bovine oocyte-cumulus complexes (OCCs) during *in vitro* growth culture. OCCs were cultured for 5 days with IBMX (A), rolipram (B), cilostamide (C), and milrinone (D) at different concentrations. OCCs growth-cultured without PDE inhibitor are labeled "None". The top, middle and bottom panels represent Day 1, 3 and 5 of culture, respectively, and the different concentrations (μM) of PDE inhibitors are shown in increasing order from left to right. The scale bar represents 200 μm.

Fig. 3.

Antrum formation of bovine OCCs during *in vitro* growth culture with PDE inhibitors (A: IBMX; B: rolipram; C: cilostamide; and D: milrinone). Antrum formation was confirmed by examining spaces formed inside the cumulus cell layers on Day 3 and Day 5 of the culture period. The numbers of OCCs (n) used for each experiment are shown in each graph. Different types of lines indicate different concentrations (μM) of the respective PDE inhibitor. Data are shown as average percentages from at least three replicated cultures. The letters "a–c" denote significantly different values ($P < 0.05$).

Fig. 4.

Effect of PDE inhibitors on the meiotic arrest of bovine growing oocytes during growth culture. The percentages of oocytes at the germinal vesicle (GV I-IV) stage after 5 days of *in vitro* growth culture are shown in the bar graphs. White bar graphs represent oocytes without PDE inhibitors and bars with the same pattern represent the same PDE inhibitor at different concentrations. The concentrations (μM) of PDE inhibitors are given below the bar graphs. Data are shown as the means ± SEM from at least three replicated cultures. Bars with different letters (a–g) are significantly different ($P < 0.05$).

Fig. 5.

Maturation ability of bovine oocytes grown with PDE inhibitors. The average percentages of oocytes that matured to metaphase II (MII) are shown in the bar graphs. In each panel, the white bar labeled GO (left) represents the mean percentage of oocytes collected form early antral follicles (1.2–1.8 mm) that matured to MII. The white bar labeled FO (right) represents the mean percentage of oocytes collected from middle antral follicles (4–6 mm) that matured to MII. Oocytes grown without PDE inhibitor are shown with gray bars. Bars with the same pattern represent the same PDE inhibitor at different concentrations. Concentrations (μM) of PDE inhibitors are given below the bar graphs. Data are shown as the means ± SEM from at least three replicated cultures. Bars with different letters (a–e) are significantly different ($P < 0.05$).

Fig. 6.

Representative images of lucifer yellow (LY) dye transfer from bovine oocytes to cumulus cells. LY solution was microinjected into each oocyte and allowed to spread from oocytes to cumulus cells for 30 min. The OCCs showing spread of LY from the oocyte to approximately more than half of the cumulus cells were designated as dye-positive (A). The OCCs showing spread of LY approximately less than half of the cumulus cells are designated as dye-negative (B); their appearance was similar to the negative control OCCs (C) treated with carbenoxolone (30 μM) for 15 hours before LY microinjection. The scale bar represents 40 μm.

Fig. 7.

Effect of PDE inhibitors on the maintenance of gap junctional communication between bovine oocytes and cumulus cells. Lucifer yellow (LY) was microinjected into the oocytes (GO) before *in vitro* growth culture as the positive control, and *in vitro* grown oocytes (none, IBMX 50 μM, rolipram 25 μM, and cilostamide 5 μM) at the end of the culture (Day 5). The transfer of LY from oocytes to surrounding cumulus cells was examined and the OCCs were classified as dye-positive or dye-negative. Carbenoxolone was used as a gap junction blocker. Oocytes before growth culture were incubated with carbenoxolone (30 μM) for 15 hours then microinjected with LY and served as the negative control. The numbers of oocytes (n) used for each experiment are shown inside the respective bars. Data are shown as the means ± SEM from at least three replicated cultures. Bars with different letters (a–d) are significantly different ($P < 0.05$).

Fig. 1.

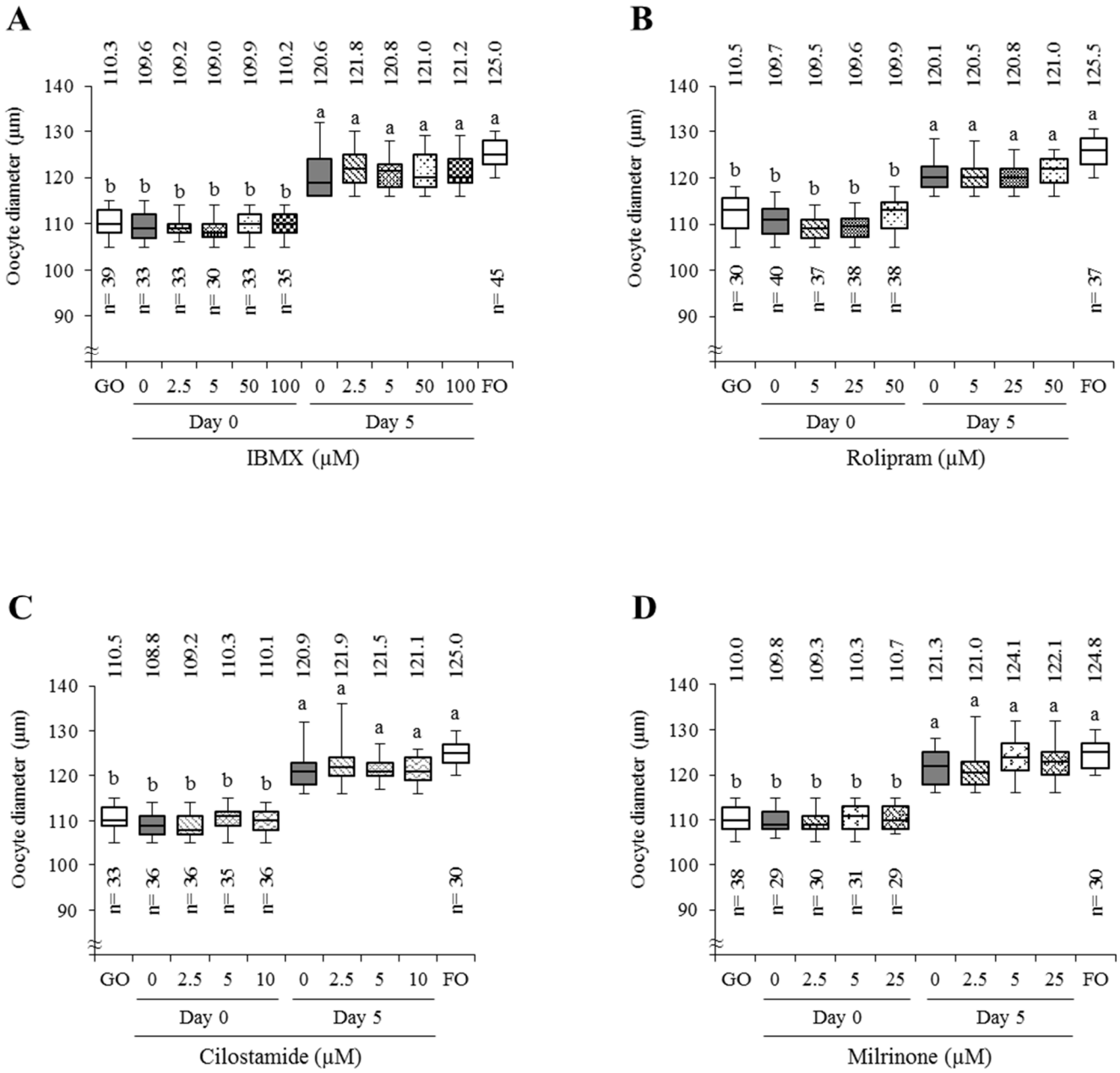

A
110.3 109.6 109.2 109.0 109.9 110.2 120.6 121.8 120.8 121.0 121.2 125.0
Oocyte diameter (µm)
140
130
120
110
100
90
n= 39 n= 33 n= 33 n= 30 n= 33 n= 35 n= 45
GO 0 2.5 5 50 100 0 2.5 5 50 100 FO
Day 0
Day 5
IBMX (µM)
B
110.5 109.7 109.5 109.6 109.9 120.1 120.5 120.8 121.0 125.5
n= 30 n= 40 n= 37 n= 38 n= 38 n= 37
GO 0 5 25 50 0 5 25 50 FO
Rolipram (µM)
C
110.5 108.8 109.2 110.3 110.1 120.9 121.9 121.5 121.1 125.0
n= 33 n= 36 n= 36 n= 35 n= 36 n= 30
GO 0 2.5 5 10 0 2.5 5 10 FO
Cilostamide (µM)
D
110.0 109.8 109.3 110.3 110.7 121.3 121.0 124.1 122.1 124.8
n= 38 n= 29 n= 30 n= 31 n= 29 n= 30
GO 0 2.5 5 25 0 2.5 5 25 FO
Milrinone (µM)

Fig. 2.

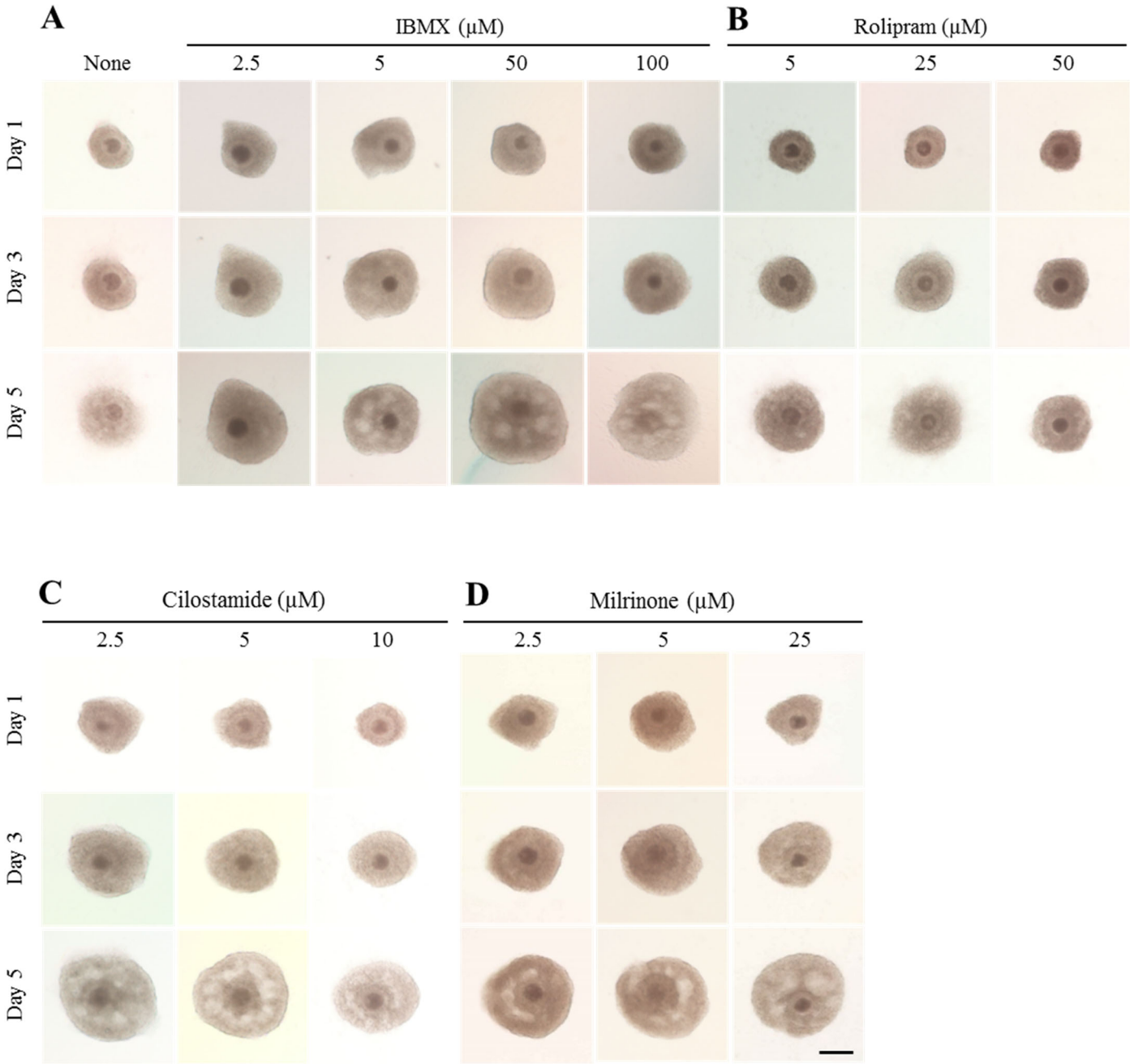

A
IBMX (μM)
None
2.5
5
50
100
B
Rolipram (μM)
5
25
50
Day 1
Day 3
Day 5
C
Cilostamide (μM)
2.5
5
10
D
Milrinone (μM)
2.5
5
25
Day 1
Day 3
Day 5

Fig. 3.

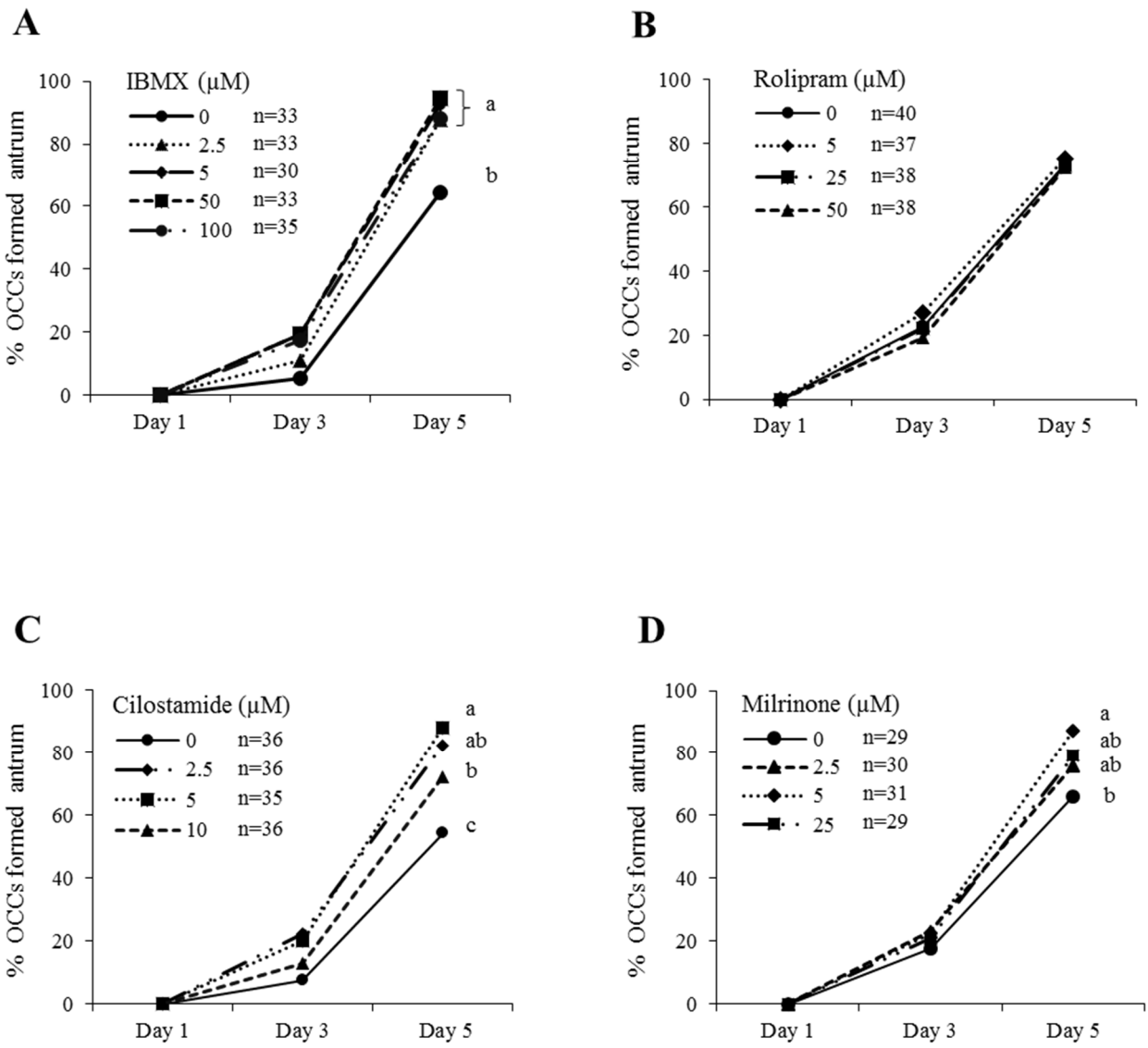

A
IBMX (μM)
0 n=33
2.5 n=33
5 n=30
50 n=33
100 n=35
a
b
% OCCs formed antrum
Day 1
Day 3
Day 5
B
Rolipram (μM)
0 n=40
5 n=37
25 n=38
50 n=38
C
Cilostamide (μM)
0 n=36
2.5 n=36
5 n=35
10 n=36
a
ab
b
c
D
Milrinone (μM)
0 n=29
2.5 n=30
5 n=31
25 n=29
a
ab
ab
b

Fig. 4.

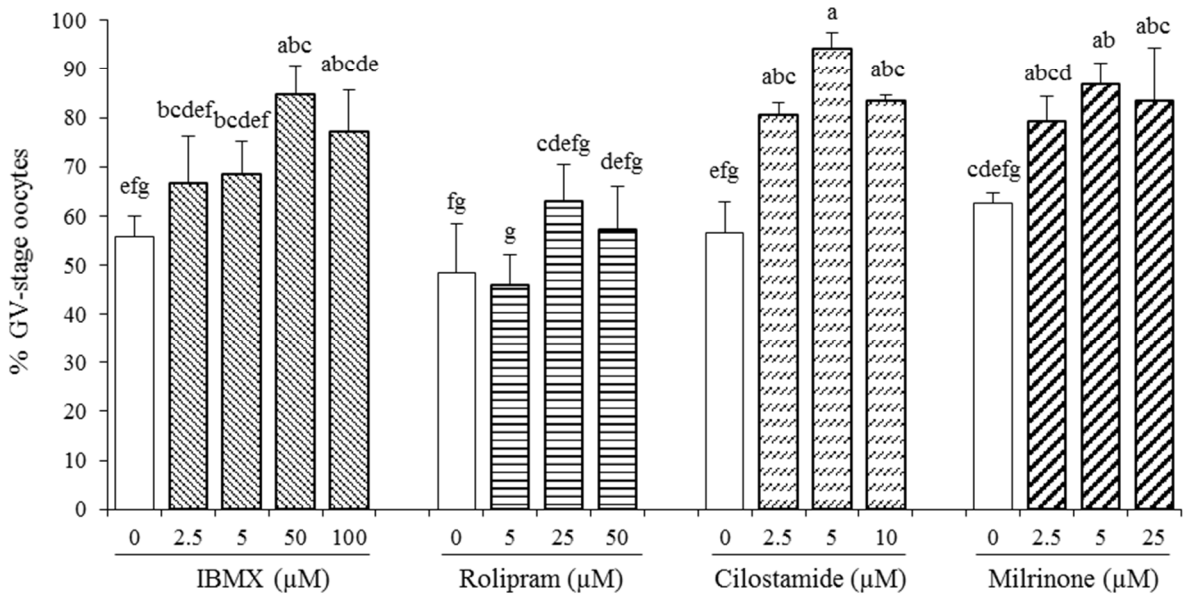

% GV-stage oocytes
100
90
80
70
60
50
40
30
20
10
0
efg
bcdef
bcdef
abc
abcde
fg
g
cdefg
defg
efg
abc
a
abc
cdefg
abcd
ab
abc
0
2.5
5
50
100
0
5
25
50
0
2.5
5
10
0
2.5
5
25
IBMX (μM)
Rolipram (μM)
Cilostamide (μM)
Milrinone (μM)

Fig. 5.

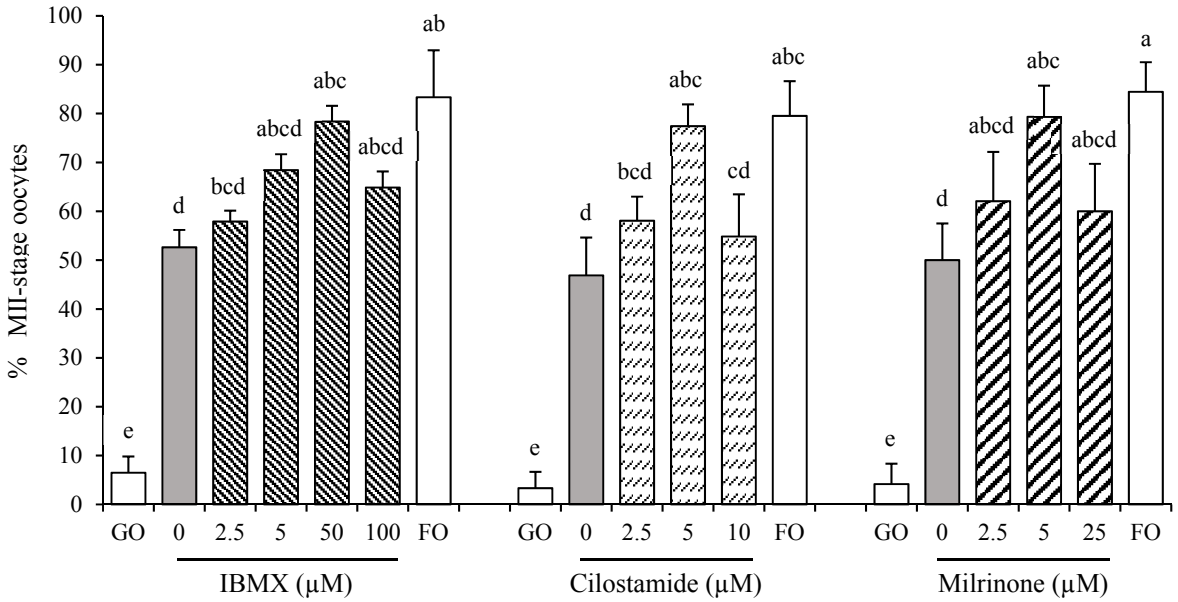

% MII-stage oocytes
100
90
80
70
60
50
40
30
20
10
0
e
d
bcd
abcd
abc
abcd
ab
e
d
bcd
abc
cd
abc
e
d
abcd
abc
abcd
a
GO
0
2.5
5
50
100
FO
GO
0
2.5
5
10
FO
GO
0
2.5
5
25
FO
IBMX (μM)
Cilostamide (μM)
Milrinone (μM)

Fig. 6.

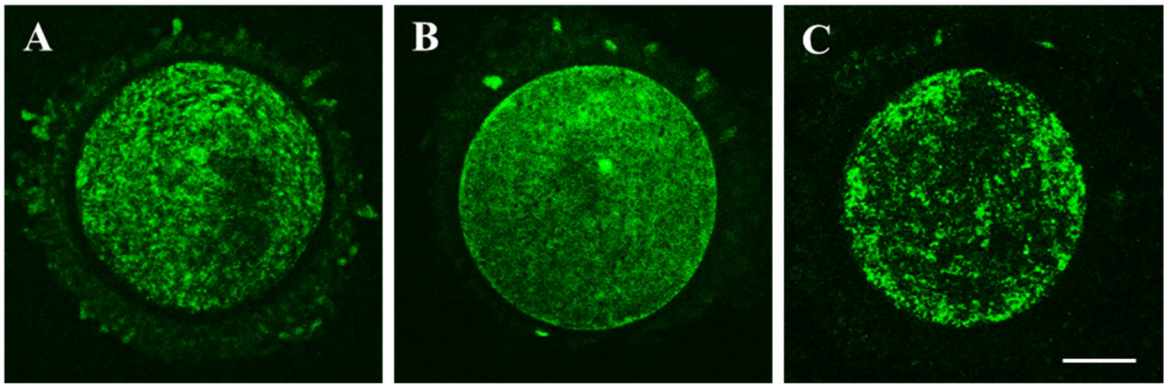
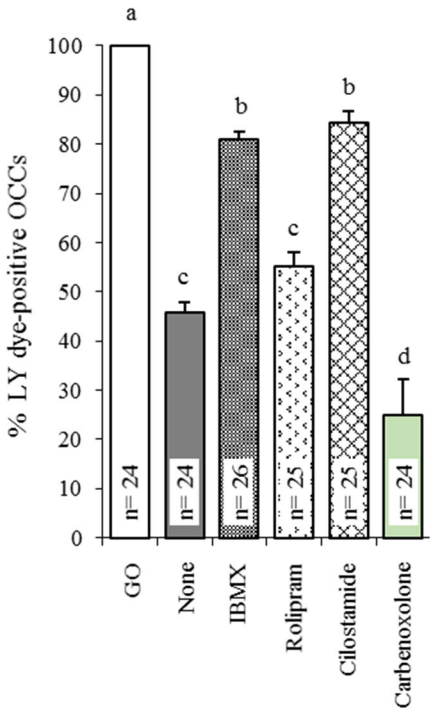


Fig. 7.


% LY dye-positive OCCs
100
90
80
70
60
50
40
30
20
10
0
a
c
b
c
b
d
n= 24
n= 24
n= 26
n= 25
n= 25
n= 24
GO
None
IBMX
Rolipram
Cilostamide
Carbenoxolone

Supplementary Table 1 Effect of PDE inhibitors on meiotic arrest of bovine growing oocytes during *in vitro* growth culture.

PDE inhibitor	Conc. (μM)	No. of oocytes examined	No. (%) of oocytes at the stage						
			FC	SC	GV	ED	LD	MI	DG
in vivo (105–115 μm)		39	21 (54)	17 (44)	1 (2) ^{h}	0	0	0	0
IBMX	0	33	0	0	19 (55) ^{fg}	7 (24) ^{ab}	4 (12)	2 (6) ^{ab}	1 (3)
	2.5	33	0	0	22 (67) ^{cdefg}	6 (18) ^{abcd}	3 (9)	1 (3) ^{ab}	1 (3)
	5	30	0	0	21 (68) ^{cdefg}	4 (14) ^{bcd}	4 (14)	1 (4) ^{ab}	0
	50	33	0	0	28 (85) ^{abcd}	2 (6) ^{bcd}	3 (9)	0	0
	100	35	0	0	27 (77) ^{bcdef}	3 (9) ^{bcd}	2 (5)	0	3 (9)
in vivo (120–130 μm)		45	0	0	45 (100) ^{a}	0	0	0	0
in vivo (105–115 μm)		30	20 (66)	8 (27)	2 (7) ^{h}	0	0	0	0
Rolipram	0	40	0	0	19 (48) ^{fg}	11 (27) ^{ab}	6 (14)	4 (11) ^{a}	0
	5	37	0	0	18 (46) ^{g}	12 (33) ^{a}	6 (19)	1 (2) ^{ab}	0
	25	38	0	0	25 (63) ^{defg}	9 (24) ^{ab}	3 (8)	1 (5) ^{ab}	0
	50	38	0	0	23 (57) ^{efg}	8 (23) ^{abc}	4 (13)	3 (7) ^{ab}	0
in vivo (120–130 μm)		37	0	0	37 (100) ^{a}	0	0	0	0
in vivo (105–115 μm)		33	19 (58)	13 (39)	1 (3) ^{h}	0	0	0	0
Cilostamide	0	36	0	0	21 (57) ^{efg}	7 (21) ^{abcd}	5 (15)	0	3 (7)
	2.5	36	0	0	29 (80) ^{abcd}	4 (11) ^{bcd}	2 (6)	1 (3) ^{ab}	0
	5	35	0	0	33 (94) ^{ab}	1 (3) ^{d}	1 (3)	0	0
	10	36	0	0	30 (84) ^{abcd}	3 (8) ^{bcd}	0	0	3 (8)
in vivo (120–130 μm)		30	0	0	30 (100) ^{a}	0	0	0	0
in vivo (105–115 μm)		38	26 (68)	10 (27)	2 (5) ^{h}	0	0	0	0
Milrinone	0	29	0	0	18 (62) ^{defg}	5 (17) ^{abcd}	3 (11)	0	3 (10)
	2.5	30	0	0	24 (79) ^{abcde}	0	3 (11)	1 (3) ^{ab}	2 (7)
	5	31	0	0	27 (87) ^{abc}	1 (3) ^{cd}	3 (10)	0	0
	25	29	0	0	24 (83) ^{abcd}	1 (3) ^{cd}	2 (7)	0	2 (7)
in vivo (120–130 μm)		30	0	0	30 (100) ^{a}	0	0	0	0

FC: Filamentous chromatin stage, SC: Stringy chromatin stage, GV: Germinal vesicle stage (I-IV), ED: Early diakinesis, LD: Late diakinesis, MI: Metaphase I, and DG: Degenerated. In each PDE inhibitor section, the meiotic stages of oocytes before growth culture are shown as "*in vivo* (105–115 μm)" and *in vivo* fully grown oocytes are shown as "*in vivo* (120–130 μm)". Data are shown in total number (%) of oocytes from at least three replicated cultures. Values with different superscripts (a–h) are significantly different within the same meiotic stage ($P < 0.05$). Conc.: concentration.

Supplementary Table 2 Meiotic competence of bovine oocytes grown with PDE inhibitors.

PDE inhibitor	Conc. (μM)	No. of oocytes examined	No. (%) of oocytes at the stage					
			GV	ED	LD	MI	MII	DG
in vivo (105–115 μm)		46	1 (2)	27 (60) [a]	12 (24) [ab]	0	3 (7) [e]	3 (7) [bcde]
IBMX	0	38	0	4 (10) [b]	3 (8) [bc]	3 (8) [ab]	20 (53) [d]	8 (21) [abc]
	2.5	38	0	2 (5) [b]	3 (8) [bc]	1 (3) [b]	22 (58) [bcd]	10 (26) [a]
	5	38	0	3 (8) [b]	3 (8) [bc]	1 (3) [b]	26 (68) [abcd]	5 (13) [abcde]
	50	37	0	3 (8) [b]	2 (5) [bc]	1 (3) [b]	29 (79) [abc]	2 (5) [bcde]
	100	37	0	2 (5) [b]	3 (8) [bc]	1 (3) [b]	24 (65) [abcd]	7 (19) [abcd]
in vivo (120–130 μm)		18	0	1 (6) [b]	1 (6) [bc]	1 (6) [ab]	15 (82) [ab]	0
in vivo (105–115 μm)		33	0	19 (58) [a]	12 (36) [a]	1 (3) [b]	1 (3) [e]	0
Cilostamide	0	32	0	4 (13) [b]	3 (9) [bc]	6 (19) [a]	15 (47) [d]	4 (12) [abcde]
	2.5	31	0	2 (6) [b]	3 (10) [bc]	3 (10) [ab]	18 (58) [bcd]	5 (16) [abcde]
	5	31	0	1 (3) [b]	0	3 (10) [ab]	24 (77) [abc]	3 (10) [abcde]
	10	31	0	3 (10) [b]	3 (10) [bc]	2 (6) [ab]	17 (55) [cd]	6 (19) [abc]
in vivo (120–130 μm)		36	0	2 (6) [b]	2 (6) [bc]	2 (6) [ab]	29 (80) [abc]	1 (2) [de]
in vivo (105–115 μm)		27	0	19 (70) [a]	5 (18) [bc]	1 (4) [ab]	1 (4) [e]	1 (4) [cde]
Milrinone	0	28	0	4 (14) [b]	2 (7) [bc]	2 (7) [ab]	14 (50) [d]	6 (22) [ab]
	2.5	29	0	2 (7) [b]	3 (10) [bc]	2 (7) [ab]	18 (62) [abcd]	4 (14) [abcde]
	5	29	0	2 (7) [b]	1 (4) [bc]	0	23 (79) [abc]	3 (10) [abcde]
	25	30	0	3 (10) [b]	2 (7) [bc]	0	18 (60) [abcd]	7 (23) [ab]
in vivo (120–130 μm)		33	0	3 (9) [b]	1 (3) [c]	1 (3) [b]	28 (85) [a]	0

GV: Germinal vesicle stage (I-IV), ED: Early diakinesis, LD: Late diakinesis, MI: Metaphase I, MII: Metaphase II, and DG: Degenerated. In each PDE inhibitor section, the maturation competence of growing oocytes before growth culture are shown as "*in vivo* (105–115 μm)" and *in vivo* fully grown oocytes are shown as "*in vivo* (120–130 μm)". Data are shown in total number (%) of oocytes from at least three replicated cultures. Values with different superscripts (a–e) are significantly different within the same meiotic stage ($P < 0.05$). Conc.: concentration.