Effect of Different Levels of Intracellular cAMP on the In Vitro Maturation of Cattle Oocytes and Their Subsequent Development Following In Vitro Fertilization

A.M. LUCIANO, P. POCAR, E. MILANESI, S. MODINA, D. RIEGER, A. LAURIA, AND F. GANDOLFI

ABSTRACT
Serum, gonadotrophins, growth factors, and steroid hormones stimulate the in vitro maturation (IVM) of competent oocytes, acting, directly or indirectly, upon the adenylate cyclase pathway to produce the intracellular messenger, cAMP. The intracellular levels of cAMP in cattle cumulus-oocyte complexes (COC) were manipulated by adding to the collection and maturation media invasive adenylate cyclase (iAC), a toxin produced by the bacterium, Bordetella pertussis. High concentrations of iAC (1 or 5 μg/ml) in the maturation medium inhibited the resumption of meiosis, while low concentrations (0.1 or 0.01 μg/ml) resulted in high rates of maturation to the MII stage (92.6 ± 2.5 and 98.5 ± 1.4% respectively). The same low concentrations of iAC in the maturation medium resulted in rates of development to the blastocyst stage 8 days post insemination (30.1 ± 4.2 and 45.1 ± 3.9%, respectively), which were either not different, or significantly better, than those obtained after IVM in medium supplemented only with serum and gonadotrophins (36.1 ± 2.9%). Finally, the addition of 0.1 μg/ml iAC and 0.5 mM 3-isobutyl 1-methylxanthine (IBMX) in the collection medium significantly improved the blastocyst rate when IVM was performed in control medium or medium supplemented with 0.01 μg/ml iAC (31.9 ± 5.5 vs. 12.1 ± 1.6 and 45.5 ± 2.9 vs. 19.1 ± 2.3% respectively). It is concluded that the maintenance of an optimal intracellular concentration of cAMP before and during IVM ensures a high developmental competence of bovine oocytes matured in medium without serum and hormones. Mol. Reprod. Dev. 54:86–91, 1999. © 1999 Wiley-Liss, Inc.

Key Words: oocyte developmental competence; invasive adenylate cyclase; IBMX; bovine; embryo

INTRODUCTION
Oocyte quality has a profound influence on embryonic development (Sirard and Blondin, 1996; Hyttel et al., 1997; Gandolfi, 1998). However, current in vitro maturation (IVM) procedures are suboptimal, as indicated by the observations that in vivo matured oocytes, fertilized and cultured in vitro, yield almost twice as many blastocysts as in vitro matured oocytes fertilized and cultured in the same conditions (Leibfried-Rutledge et al., 1987; Marquant Le Guienne et al., 1989).

Oocyte developmental competence is gradually acquired during follicular growth, and the morphology of the follicle, or the cumulus-oocyte complex (COC) has been shown to be related to the potential of the enclosed oocytes to develop as embryos following in vitro fertilization (Gordon, 1994). More recently we also observed that ovarian morphology is related to oocyte developmental competence and can be used as a simple and noninvasive parameter for an effective selection of oocytes with better quality (Gandolfi et al., 1997).

In addition to oocyte quality, the basic maturation medium, serum, hormones, and growth factors have profound effects on oocyte maturation and on subsequent embryo development (Rose and Bavister, 1992; Brackett and Zuelke, 1993; Harper and Brackett, 1993). These substances act, directly or indirectly, upon the adenylate cyclase pathway, which produces the intracellular messenger, cAMP (Steele and Leung, 1993; Ji et al., 1996; Katzenellenbogen, 1996). However, the use of chemicals that elevate intracellular levels of cAMP can, paradoxically, inhibit the resumption of meiosis in cattle oocytes (Homa, 1988; Sirard and First, 1988; Sirard, 1990; Bilodeau et al., 1993; Aktas et al., 1995b).

The objective of the experiments described in this report was to determine the effect of manipulating the intracellular levels of cAMP ([cAMP]) in cattle cumulus oocyte complexes (COCs) during collection and IVM on nuclear maturation and on subsequent embryonic development following in vitro fertilization.

The COCs were exposed to invasive adenylate cyclase (IAC), a toxin produced by the bacterium, Bordetella pertussis, which can be internalized by mammalian cells (Confer et al., 1984). This results in the increased...
production of cAMP within the host cell from its own pool of adenosine 5'-triphosphate (ATP) (Hanski and Farfel, 1985). The resulting [cAMP]i is a function of the concentration of iAC present in the medium (Friedman et al., 1987). Previous work has shown that iAC is effective in rat (Aberdam et al., 1987) and cattle (Aktas et al., 1995b) COCs.

We tested the effect of different concentrations of iAC in maturation medium (free of serum, serum proteins, gonadotrophins, and growth factors) on nuclear maturation of cattle oocytes, and on cleavage and development to the blastocyst stage, following in vitro fertilization. In addition, we determined the effect of controlling the [cAMP]i of COCs during the collection and selection procedures that precede IVM, on maturation, cleavage, and development to the blastocyst stage. Groups of COCs matured in medium containing fetal calf serum (FCS) and human menopausal gonadotrophin (hMG) were included as positive controls in all experiments.

**MATERIALS AND METHODS**

A purified lot of iAC (kindly provided by Dr. E.L. Hewlett, University of Virginia Medical School) was used for all the experiments. It had an enzyme specific activity of 4066 µmol/min/mg with a toxin specific activity in Jurkat cells of 2.8 µmol cAMP/mg toxin/mg cell protein (E.L. Hewlett, personal communication). All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO), unless otherwise stated.

**Oocyte Collection, Selection and In Vitro Maturation**

Bovine ovaries were obtained from a local abattoir, and transported to the laboratory, within 2 hr, in Dulbecco's phosphate balanced saline (PBS), maintained at 32–34°C. All subsequent procedures were conducted at a constant temperature of 36°C.

Oocyte aspiration and selection procedures were performed either in basic Collection Medium (bCM) made of TCM-199, supplemented with 1 mg/ml polyvinyl alcohol (PVA), 25 mM Hepes, 10 µg/ml heparin, or in complete Collection Medium (cCM) made of bCM supplemented with 0.5 mM 3-isobutyl 1-methylxanthine (IBMX, Sigma no. i-7018), and 0.1 µg/ml iAC. The IBMX and iAC were included in order to preserve the cAMP concentration in the COCs as close as possible to that present when they were within the follicle, as suggested by Aktas et al. (1995b).

The COCs were retrieved from the ovaries by aspiration of 2–5 mm follicles with an 18-gauge needle on a 10 ml syringe containing a small volume of collection medium. The final proportion of follicular fluid to collection medium was maintained as closely as possible to 3:1.

The COCs were examined under a stereo microscope, and only those that were medium-brown in colour, with three or more complete layers of cumulus cells and a finely granulated homogenous ooplasm were used. Selected COCs were then washed two times in the same medium used for collection and two times in the maturation medium used according to the experimental designs described below. The whole procedure was performed in approximately 30 min.

The maturation medium was TCM-199 supplemented with 0.68 mM L-glutamine, 25 mM NaHCO3, and 1 mg/ml PVA. To this was added 0 to 5 µg/ml iAC, depending on the experiment, or 10% (v/v) FCS (Sigma no. F-7524) plus 0.1 i.u./ml hMG (Pergovet, Serono, Rome, Italy), as a positive control. The COCs were cultured in groups of 20–30 COCs in 500 µl of maturation medium for 24 hr, in four-well dishes (Nunc, Roskilde, Denmark) at 38.5°C under 5% CO2 in humidified air.

**Oocyte In Vitro Fertilization**

A straw containing cryopreserved bull spermatozoa (CIZ, S. Miniato, Italy) was thawed in water, at 32°C, and the cells were layered on top of a 45–90% Percoll gradient made with modified Tyrode's medium (TALP, Bavister et al., 1983) and centrifuged for 30 min at 600g. The sperm pellet was washed once in the same medium, counted and diluted to a final concentration of $1 \times 10^5$ spermatozoa/ml fertilization medium. The fertilization medium was TALP supplemented with 0.6% (w/v) BSA fatty acid free (Sigma no. A-8806), 10 µg/ml heparin, 20 µM penicillamine, 1 µM epinephrine, and 100 µM hypotaurine. After the maturation period, COCs were washed three times in TALP medium supplemented with 20 mM Hepes (TALP wash) and once in fertilization medium. Groups of 20–30 COCs in a volume of 10 µl were added to 300 µl of fertilization medium and incubated for 18–20 hours, in four-well dishes, at 38.5°C under 5% CO2 in humidified air.

**Embryo Culture**

Bovine oviduct epithelial cells were isolated by scraping the lumen of the ampullary region with a Pasteur pipette and then washed twice in 5 ml of Heparin-buffered (25 mM) medium TCM-199, supplemented with 0.4% (w/v) BSA (Sigma no. A-3311). Clusters of cells were cultured for 24–48 hr in TCM-199 supplemented with 0.68 mM L-glutamine, 25 mM NaHCO3 and 10% (v/v) newborn calf serum (Sigma N-4762), before being aliquotted in 20 µl drops, containing approximately 5,000 cells.

At the end of the fertilization period, the COCs were removed from the fertilization drops and denuded by vortexing, at half speed, for 3 min in 500 µl of medium TALP wash. The putative zygotes were washed twice in TALP wash and once in TCM-199 supplemented as described above. Finally they were transferred, in groups of 20–30, to the culture drops. Incubation was performed under mineral oil at 38.5°C under 5% CO2 in humidified air. Cleaved eggs were transferred into a fresh drop 2 days after insemination. Every 2 days, thereafter, 20 µl of fresh medium were added to each drop and then removed to maintain a constant volume.
The aim of this experiment was to determine the effect of different concentrations of iAC on cumulus expansion and nuclear maturation. Aspiration and selection in bCM was followed by IVM in control medium (TCM 199 supplemented with PVA) or in medium supplemented with FCS + hMG, as described above. COC aspiration and selection in cCM, was followed by culture in maturation medium containing 0.01, 0.1, 1, or 5 µg/ml iAC. The COCs were then examined for cumulus expansion under a stereo microscope. For evaluation of meiotic stage, the oocytes were denuded, fixed by immersion in a solution of acetic acid and ethanol (1:3) for 24 hr, stained with 0.1% (w/v) Lacmoid solution in a solution of acetic acid and ethanol (1:3) and then fertilized, cultured, and evaluated for cleavage and development to the blastocyst stage, exactly as described for experiment 2.

**Experimental Design**

**Experiment 1.** The aim of this experiment was to determine the effect of different concentrations of iAC on maturation rates and subsequent embryonic development. The COCs were aspirated and selected either in bCM or in cCM as described above. The COCs were then cultured in maturation medium containing 0 (control) or 0.01 µg/ml iAC, or FCS + hMG for 24 hr, and then fertilized, cultured, and evaluated for cleavage and development to the blastocyst stage, exactly as described for experiment 2.

**Statistical Analysis**

Each treatment of each experiment was replicated at least three times. The data are expressed as mean ± SEM. In experiments 1 and 2, results were analyzed using one-way ANOVA followed by Duncan’s New Multiple Range Test. In experiment 3, two-way ANOVA was used. For the proportional data, the arcsines of the proportions were calculated and the transformed values used for statistical analysis.

**RESULTS**

**Effect of iAC Concentration in the Maturation Medium on Nuclear Maturation and Cumulus Expansion (Experiment 1)**

The effects of supplementation of the maturation medium with iAC depended on the enzyme concentration used. As summarized in Table 1, none of the oocytes cultured in the highest concentration of iAC (5 µg/ml) matured to the MII stage, while the maturation rate in the presence of lower concentrations (0.1 and 0.01 µg/ml) was significantly greater than that in control medium, and not different from the rate obtained in maturation medium supplemented with FCS + hMG. A concentration of 0.01 µg/ml iAC yielded a high maturation rate but no cumulus expansion, while high concentrations of iAC stimulated cumulus expansion but maturation was totally or partially inhibited.

**Effect of iAC Concentration in the Maturation Medium on Cleavage and Development to the Blastocyst Stage (Experiment 2)**

As shown in Table 2, the cleavage rates of oocytes matured in the presence of 0.01 or 0.1 µg/ml iAC were significantly greater than that of oocytes matured in control medium, and not different from that of oocytes matured in the presence of FCS + hMG. Development to the blastocyst stage was similarly greater for oocytes matured in the presence of 0.01 or 0.1 µg/ml iAC than for those matured in control medium. Moreover, the rate of development to the blastocyst stage was not
different between the 0.1 µg/ml iAC and FCS+hMG group, and significantly greater in the 0.01 µg/ml iAC group than in the FCS+hMG group. There was no significant effect of treatment on blastocyst cell numbers.

**Effect of iAC in the Collection and Maturation Media on Cleavage and Development to the Blastocyst Stage (Experiment 3)**

As shown in Table 3, the rates of cleavage and development to the blastocyst stage were significantly greater when the COC collection medium contained 0.5 mM IBMX and 0.1 µg/ml iAC (cCM), than when the collection medium contained no IBMX or iAC (bCM). The use of cCM had no effect only when IVF was performed in medium supplemented with serum and gonadotrophins. However, the presence of IBMX and iAC in the collection medium followed by IVF in control medium, yielded a rate of development to the blastocyst stage that was not different from the rate for oocytes matured in the presence of FCS+hMG. As in experiment 2, collection of the COCs in cCM followed by maturation in medium containing 0.01 µg/ml iAC yielded the highest rate of development to the blastocyst stage, significantly greater than that for oocytes matured in the presence of FCS+hMG.

**DISCUSSION**

The results of these studies demonstrate that low concentrations of iAC stimulate the nuclear maturation of cattle oocytes while high concentrations inhibit nuclear maturation. Concentrations of 1 and 5 µg/ml of iAC markedly inhibited oocyte maturation, whereas 0.01 and 0.1 µg/ml iAC resulted in high rates of maturation to MII, which were not different from that for COCs matured in medium supplemented with FCS+hMG.

These observations are partially consistent with those of Aktas et al. (1995b) and Bilodeau et al. (1993), who observed an inhibitory effect of high [cAMP] on the nuclear maturation of cattle oocytes. However, to our knowledge, there are no previous reports of a stimulatory effect on nuclear maturation of low concentrations of iAC or other agents that modulate intracellular cAMP. The high maturation rates seen with low concentrations of iAC were not simply the result of a lack of an inhibitory effect, because the maturation rates with 0.1 and 0.01 µg/ml iAC were significantly better than those obtained with control medium.

The concentrations of iAC used in these experiments cannot be directly compared to those used by Aktas et al. (1995a,b) because the concentration of iAC was expressed in arbitrary units in those reports. However, it can be noted that an iAC concentration equivalent to 0.25 µg of the preparation used in our experiments is 0.1 µg/ml or 5 µg/ml iAC. Therefore we can infer that 1 unit is approximately equivalent to 0.25 µg of the preparation used in our experiments.

No cumulus expansion was observed at the lowest iAC concentration used (0.01 µg/ml) despite the fact that most of the oocytes reached MII. Cumulus expansion is induced in vitro by FSH (Eppig, 1979a; Ball et al., 1983; Salustri et al., 1990) and epidermal growth factor (EGF, Downs, 1989; Lorenzo et al., 1994) via a mechanism that appears to be mediated by cAMP (Dekel and Kraicer, 1978; Eppig, 1979b; Ball et al., 1983). In mouse and rat this process requires a factor that is secreted by the oocyte and acts downstream from

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**TABLE 2. Effect of iAC Concentration in the Maturation Medium on Bovine Oocytes Developmental Competence**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>COCs (n)</th>
<th>% Cleaved 2 d.p.i.</th>
<th>% Blastocyst 8 d.p.i.</th>
<th>Blastocyst cell number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>93</td>
<td>69.4 ± 5.2a</td>
<td>12.1 ± 1.6b</td>
<td>17.8 ± 3.1a</td>
</tr>
<tr>
<td>iAC 0.01 µg/ml</td>
<td>101</td>
<td>85.4 ± 2.9a</td>
<td>45.1 ± 3.9b</td>
<td>52.5 ± 4.5b</td>
</tr>
<tr>
<td>iAC 0.1 µg/ml</td>
<td>137</td>
<td>83.9 ± 7.2b</td>
<td>30.1 ± 4.2a</td>
<td>37.1 ± 5.1b</td>
</tr>
<tr>
<td>FCS + hMG</td>
<td>92</td>
<td>86.6 ± 1.9b</td>
<td>31.3 ± 2.4a</td>
<td>36.1 ± 2.9b</td>
</tr>
</tbody>
</table>

*Different superscripts within the same column indicate significant differences (P ≤ 0.05).

**TABLE 3. Effect of Collection and Maturation Media Composition on Developmental Competence of Bovine Oocytes**

<table>
<thead>
<tr>
<th>Media supplementation during</th>
<th>Collection</th>
<th>Maturation</th>
<th>COCs (n)</th>
<th>% Cleaved 2 d.p.i.</th>
<th>% Blastocyst 8 d.p.i.</th>
<th>Blastocyst cell number</th>
</tr>
</thead>
<tbody>
<tr>
<td>bCM</td>
<td>Control</td>
<td>73</td>
<td>69.5 ± 5.3a</td>
<td>12.1 ± 1.6a</td>
<td>17.9 ± 3.1a</td>
<td>87.7 ± 24.9</td>
</tr>
<tr>
<td>cCM</td>
<td>Control</td>
<td>72</td>
<td>86.8 ± 1.8b</td>
<td>31.9 ± 5.5b</td>
<td>36.1 ± 3.5b</td>
<td>78.6 ± 25.4</td>
</tr>
<tr>
<td>bCM</td>
<td>iAC 0.01 µg/ml</td>
<td>82</td>
<td>71.2 ± 0.3a</td>
<td>19.1 ± 2.3ab</td>
<td>26.7 ± 3.2ab</td>
<td>79.7 ± 26.1</td>
</tr>
<tr>
<td>cCM</td>
<td>iAC 0.01 µg/ml</td>
<td>89</td>
<td>87.9 ± 2.6b</td>
<td>45.5 ± 2.9b</td>
<td>52.4 ± 4.5c</td>
<td>79.7 ± 26.1</td>
</tr>
<tr>
<td>bCM</td>
<td>FCS + hMG</td>
<td>72</td>
<td>86.6 ± 1.9b</td>
<td>31.3 ± 2.4b</td>
<td>36.1 ± 2.9b</td>
<td>79.7 ± 26.1</td>
</tr>
<tr>
<td>cCM</td>
<td>FCS + hMG</td>
<td>84</td>
<td>91.5 ± 2.8b</td>
<td>25.6 ± 5.3ab</td>
<td>23.3 ± 5.1ab</td>
<td>79.7 ± 26.1</td>
</tr>
</tbody>
</table>

*Different superscripts within the same column indicate significant differences (P ≤ 0.05).
the FSH-induced increase in intracellular cAMP (Buccione et al., 1990; Vanderhyden, 1993). Conversely, the presence of an oocyte is not required for cumulus expansion in the pig (Prochazka et al., 1991; Vanderhyden, 1993). Our data indicate that, in cattle, cumulus expansion in vitro is not directly linked to nuclear maturation because, in two of the treatments (5 and 0.01 iAC µg/ml), we observed cumulus expansion without oocyte maturation, and oocyte maturation without cumulus expansion, respectively. The inhibitory effect of an elevated level of intracellular cAMP on meiotic resumption has been well characterized (Bilodeau et al., 1993; Aktas et al., 1995a,b; Rosehellekant and Bavister, 1996; Richard et al., 1997), but the degree of cumulus expansion was not described in those studies. Our observations may suggest that, in cattle, nuclear maturation and cumulus expansion are triggered by two different levels of [cAMP], such that the cAMP level required to stimulate meiotic resumption is lower than that required to induce cumulus expansion.

In addition to stimulating nuclear maturation, low concentrations of iAC in the IVM medium, also resulted in increased rates of cleavage following in vitro fertilization, and increased rates of development to the blastocyst stage. Most interestingly, the maturation of oocytes in the presence of the lowest concentration of iAC (0.01 µg/ml) resulted in a blastocyst rate that was significantly greater than that for oocytes matured in the presence of FCS + hMG. This is particularly noteworthy since the IAC-supplemented medium is completely defined, without any serum, serum derivatives, or hormones. The use of iAC in maturation medium may, therefore, offer a significant advantage in the development of efficient methods for the in vitro production of embryos, because it may be possible to eliminate blood derivatives that represent a health hazard.

The mechanism by which iAC enters the cell is unclear (Otero et al., 1995), but it has been suggested that the COOH-terminal portion of the toxin creates a channel in the membrane through which the NH2-terminal fragment is translocated (Rogel and Hanski, 1992). This allows iAC to modify [cAMP] without the need of specific receptors. The physiological modulation of [cAMP] in COCs is exerted by gonadotrophins and growth factors which act through specific receptors. It seems likely that iAC exerts its beneficial effect on oocyte maturation by directly inducing the optimal [cAMP] by bypassing the specific receptors for physiological effectors such as FSH and EGF.

It has been previously demonstrated that there is a significant decrease in [cAMP] in COCs during collection from the follicle, and that this decrease can be prevented by inducing IBMX or iAC in the collection medium (Bilodeau et al., 1993; Aktas et al., 1995b). The results of experiment 3 indicate that the presence of IBMX and iAC in the COC collection medium has significant effects on subsequent development. Both the cleavage rate and the rate of development to the blastocyst stage were significantly greater when the collection medium contained IBMX and iAC, regardless of whether the maturation medium contained iAC. The presence of IBMX and iAC in the collection medium was sufficient to yield a rate of development to the blastocyst stage that was equal to that following maturation in the presence of FCS + hMG. These results suggest that the [cAMP] of COCs at the time of collection from the follicle is critical for subsequent development, and that attention must be paid to the collection and selection procedures in order to optimize in vitro embryo production.

In conclusion, culture of cattle COCs in the presence of low concentrations of IAC, without serum, serum proteins, gonadotrophins, or growth factors, results in rates of nuclear maturation, cleavage, and development to the blastocyst stage that are not different from, or significantly greater than, those obtained by maturation in the presence of FCS + hMG. Moreover, the presence of IBMX and iAC in the COC collection medium, followed by maturation in control medium, resulted in rates of cleavage and development to the blastocyst stage not different from that for COCs matured in the presence of FCS + hMG. The use of IAC may provide an approach to the development of completely chemically defined media for oocyte collection and maturation.

ACKNOWLEDGMENT

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REFERENCES


EFFECT OF [CAMP] ON OOCYTE COMPETENCE

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