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Developmental Potential in Bovine Oocytes Is Related to Cumulus-Oocyte Complex Grade, Calcium Current Activity, and Calcium Stores

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ABSTRACT

A morphological classification of the immature cumulus-oocyte complex (COC), which grossly resembled the atresia grade of its follicle source, was used in bovine oocytes to determine 1) the developmental potential by either in vitro fertilization or parthenogenetic activation, 2) the calcium current activity by whole-cell voltage clamp technique, and 3) the intracytoplasmic calcium stores by microfluorimetric evaluation. The COC classification took into account some cumulus and ooplasm features, designated as follows: A) presence of a clear and compact cumulus and translucent ooplasm, B) dark and compact cumulus and dark ooplasm, and C) dark and expanded cumulus and dark ooplasm. We found no difference between in vitro fertilization and parthenogenetically activated oocytes in terms of cleavage rate and blastocyst production. Both protocols indicated a significant variability between the three compared COC categories. The B-COCs showed the highest embryo production efficiency as well as the greatest Ca^{2+} current activity, whereas A-COCs showed an opposite pattern. The C-COCs, mostly attributed to atretic and heavily atretic follicles, showed morphological characteristics between those of A- and B-COCs. Stores of Ca^{2+} were significantly greater in A-COCs than in B- and C-COCs in the case of immature oocytes, and greater in B-COCs than in C-and A-COCs in the case of in vitro-matured oocytes. These results demonstrate that in the bovine 1) the considered morphological criteria for oocyte classification are related to developmental competence, 2) plasma membrane Ca^{2+} current in the immature oocyte is related to developmental potential, and 3) calcium stores are related to morphological quality in immature oocytes and to developmental competence in mature oocytes.

calcium, gamete biology, in vitro fertilization, ovum

INTRODUCTION

An extremely heterogeneous population of oocytes is commonly collected from ovaries to be used for in vitro technologies. Sources of variability may be the age of these cells, the growth stage, and the atresia grade of the corresponding follicle population. Inevitably, this affects the efficiency of in vitro embryo production (IVEP).

In cattle, many authors have studied the relationship between follicle population and meiotic competence of the oocytes by considering the effect of such parameters as stage of the estrous cycle [1, 2], hormonal patterns [3] and biochemical characteristics [4, 5] of the follicular fluid, diameter [1, 2, 6, 7] and atresia grade [2, 8] of the follicle, and the ovarian morphology [9]. An analysis of these parameters provided general information without solving the key questions of what the oocyte needs to acquire meiotic competence and whether follicle activity can be manipulated to improve IVEP efficiency.

Cumulus-oocyte complex (COC) morphology is related to the atresia grade of the follicle that comprises it [8]. Based on this finding, Wurth and Kruip [8] distinguished three COC morphological grades in relation to the cumulus surrounding the oocytes and the ooplasm characteristics, designated as follows: A) presence of a clear and compact cumulus and translucent ooplasm, B) dark and compact cumulus and dark ooplasm, and C) dark and expanded cumulus and dark ooplasm. This gross and simple classification avoids wasting time for follicle dissection and evaluation, and it provides consistent information regarding the in vitro developmental potential of the different COC grades. Surprisingly, the B-COC grade showed the highest IVEP potential, despite the fact that these originate mostly from atretic follicles. This was independent from cyclic activity and stage of the estrous cycle of the donor [2]. Similar findings have also been reported by other authors [10, 11] using different evaluation criteria. To our knowledge, there has been no explanation for this, though it may be due to the reduction of meiotic-arresting factor (cAMP) levels in the oocyte [12] consequent to a decrease of cumulus-oocyte communications during atresia [13].

Successful oocyte maturation is achieved by a culture system that allows the normal progression of oocyte metabolic activity, which mainly involves RNA transcription and protein synthesis [5]. These activities are necessary for oocyte meiotic completion and for early embryo development. Most of these activities occur during the first period of maturation and require intense cumulus-oocyte communication [13]. Hence, the best candidate for IVEP should be those COCs that do not belong to atretic follicles, with preserved cumulus-oocyte communication [13, 14]. This is, however, not consistent with the results described above. This contradiction might explain the low efficiency of in vitro maturation and IVEP technologies [15, 16].

Intracellular Ca^{2+} loading plays a crucial role in oocyte maturation [17, 18]. It occurs through both cumulus-oocyte communication [19, 20] and oocyte plasma membrane Ca^{2+} channels [21, 22]. This activity is largely expressed during early maturation and later decreases [22]. Intracellular Ca^{2+} release is universally recognized to be essential for oocyte activation at fertilization [23–25].

We previously demonstrated that the presence of L-type Ca^{2+} channels in the plasma membrane decrease throughout meiosis in bovine oocytes [22]. A large variability of Ca^{2+} channel activity was, however, found between oocytes. We undertook the present study to examine if this source of variability might depend on the quality of the oocyte and its developmental potential. This potential has been evaluated by either in vitro fertilization (IVF) or parthenogenetic

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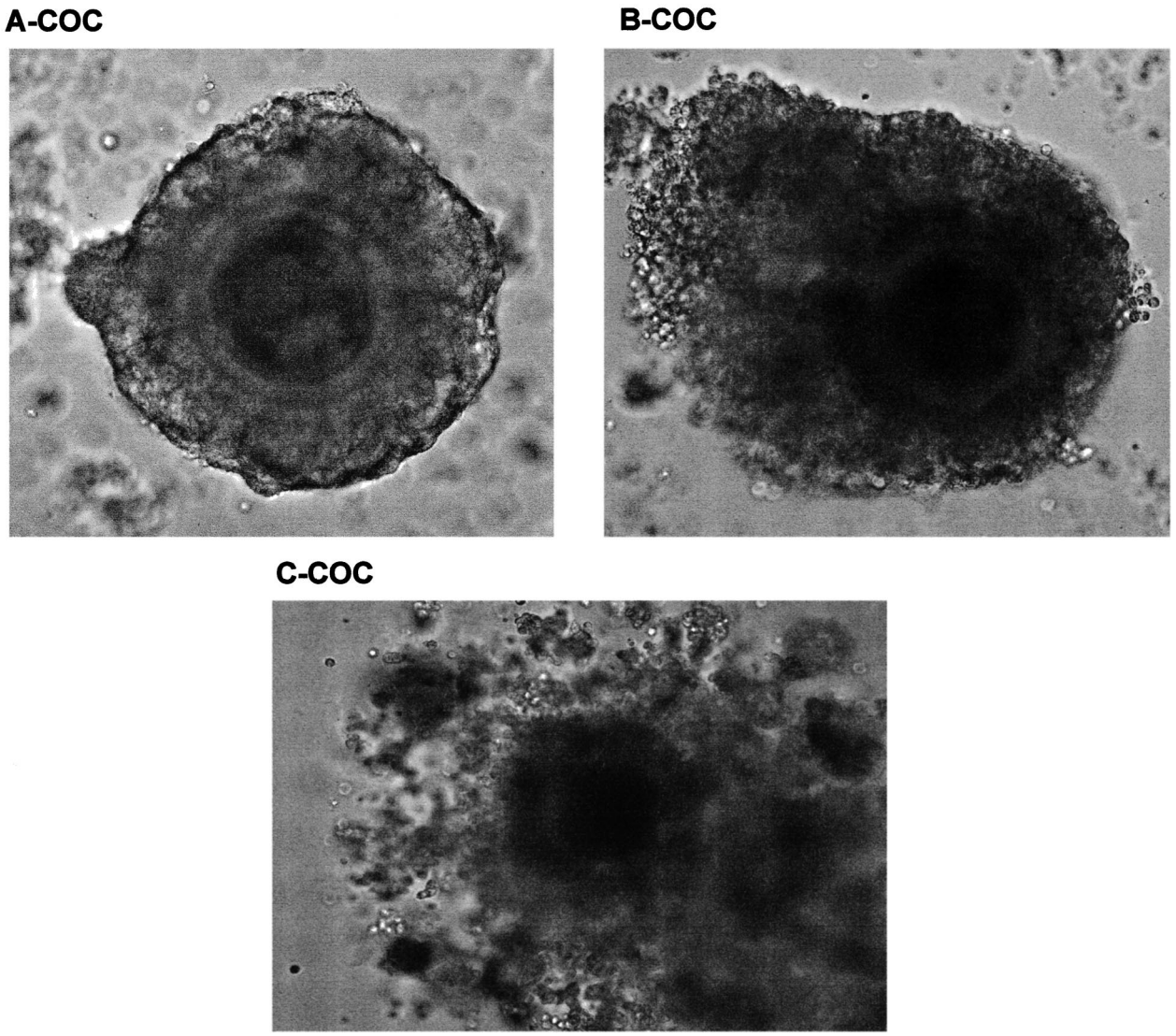

FIG. 1. Immature bovine COCs divided in three categories on the basis of morphological criteria. The A-COCs show a clear and compact cumulus surrounding completely the oocytes with at least three cell layers; the oocyte has a translucent and homogeneous ooplasm. The B-COCs show a compact and dark cumulus surrounding completely the oocytes with at least three cell layers; the oocyte has a dark and nonhomogeneous ooplasm and is surrounded by a dark corona radiata. The C-COCs show a fully, irregularly expanded cumulus with clumped, degenerated cells in a jelly matrix; the oocyte is dark with a nonhomogeneous ooplasm.

activation and related to Ca^{2+} current activity of the plasma membrane in immature bovine oocytes. In addition, we evaluated the intracytoplasmic calcium stores before and after in vitro maturation to relate this parameter to the quality and meiotic competence of the oocyte.

MATERIALS AND METHODS

Materials

If not otherwise stated, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Oocyte Source

Ovaries from slaughtered cows were collected from the abattoir and transported in a thermal bag at 27–30°C to the laboratory within 3–4 h of collection. The laboratory temperature was 30°C. Immature oocytes were collected from 2- to 8-mm follicles by an 18-gauge needle under controlled pressure (50–70 mm Hg) [26]. The COCs were isolated from the follicular fluid and washed three times with TCM199 supplemented with 0.05% (w/v) polyvinyl alcohol and 10 mM Hepes.

COC Quality Grade

At the time of COC isolation from the sediment of the collected follicular fluid, the COCs were grouped in relation to their quality grade according to the method of Wurth and Kruip [8] as follows: A-COC, presence of a clear and compact cumulus and a translucent ooplasm; B-COC, dark and compact cumulus and dark ooplasm; and C-COC, dark and expanded cumulus and dark ooplasm (Fig. 1). This grouping was performed exclusively based on the criteria shown in Figure 1. Any COCs showing characteristics intermediate to or not corresponding to these criteria were excluded.

In Vitro Maturation

The COCs were transferred into maturation medium (TCM199 supplemented with 10% fetal calf serum, 10 IU/ml of LH, 0.1 IU/ml of FSH, and 1 μg/ml of 17β-estradiol) within four-well plates (30 μl/COC; Nunclon, Roskilde, Denmark) and left in an incubator at 39.0°C in 5% CO_2 humidified air for 24 h.

Parthenogenetic Development

After maturation, COCs were freed from the cumulus cells by vortexing for 3 min and parthenogenetically activated by 5-min exposure to 7.5

μM Ca-ionophore, A23187 in Fert-TALP medium [27] as described by Liu et al. [28]. The oocytes were then transferred into four-well culture dishes containing 500 μl of Fert-TALP medium (20–25 oocytes/well) supplemented with 2.5 mM 6-dimethylaminopurine (6-DMAP) for 3.5 h of incubation at 39°C in a controlled gaseous environment (5% CO_2, 7% O_2, and 88% N_2). Finally, the oocytes were transferred into four-well dishes containing 500 μl of synthetic oviductal fluid supplemented with essential and nonessential amino acids and bovine serum albumin (SOFaaBSA medium) [29], covered with embryo-tested oil, and cultured in a controlled gaseous environment as described above for 8 days postactivation for blastocyst development. Culture plates were changed every 2 days.

IVF and In Vitro Embryo Culture

After maturation, COCs were transferred into four-well dishes containing 300 μl of Fert-TALP medium (25–30 COC/well) supplemented with 2 IU/ml of heparin. Frozen bovine sperm from an IVF-tested bull was thawed and then centrifuged at 180 × *g* for 30 min using a Percoll gradient (90:40 [v/v]). After two washes in Hepes-TALP [27], the spermatozoa were added to the COCs at a final concentration of 2×10^6 spermatozoa/ml (~10^4 spermatozoa/COC). Two days later, COCs were transferred into 1 ml of Hepes-buffered TCM199 and freed from the cumulus cells and the attached spermatozoa by vortexing for 3 min. Embryos at the 2- to 8-cell stages were then cultured in SOFaaBSA (20 μl/embryo) in a gas mixture of 5% CO_2, 7% O_2, and 88% N_2 at 39°C. The media of in vitro-cultured embryos were covered with embryo-tested oil, and the culture dishes were changed every 2 days. In vitro culture was carried out until Day 8 postinsemination for expanded blastocyst assessment.

Electrophysiology

Electrical recording was performed at 38.5°C on oocytes (n = 10 per each COC category, n = 4 replicates) at the germinal vesicle stage. Before micromanipulation, the oocytes were freed from the cumulus as described above, and the zona pellucida was removed by incubating the oocytes in 0.5% Pronase for 1.5–2 min at 37°C. The oocytes were then washed and incubated in TCM199 supplemented with Hepes and 10% fetal calf serum. The zona-free oocytes were subsequently placed in a recording chamber containing 2 ml of Ham F10 (Mascia Brunelli, Milan, Italy) at 38.5°C. Micromanipulations were performed by a Wild manipulator mounted on a Nikon Diaphot epifluorescence microscope (Nikon, Badhoevedorp, The Netherlands). Oocytes were voltage clamped by standard techniques [22]. Patch pipettes of 10 MΩ resistance and 1–2-μm tip diameter were filled with an intracellular-like solution (ICS) [22] containing 70 mM KCl, 7 mM NaCl, 10 mM EGTA, 10 mM Hepes, pH 7.4, and 280 mOsm. After obtaining a gigaΩ-seal, we set the pipette voltage to the desired negative potential (−30 mV) and ruptured the patch. Observation of a stable negative resting potential signaled access to the cytosol. Depolarizing and hyperpolarizing voltage steps of 10 mV and 500 msec were applied to generate the voltage-dependent currents. Electrical modifications consequent to the addition of 7.5 μM Ca^{2+}-ionophore A23187 to the bath solution were also recorded. Currents were recorded on a List EPC7 amplifier (List Medical, Darmstadt, Germany), and data were stored on a VCR tape for subsequent analysis.

$[Ca^{2+}]_i$ Determinations

Immature (n = 30) and in vitro-matured (n = 30), zona-free oocytes (n = 5 replicates) equally distributed for each COC category were placed in a recording chamber containing 2 ml of Ca^{2+}-free TALP medium [27] supplemented with 1 mM EGTA at 38.5°C and injected by standard techniques (see *Electrophysiology*) with the fluorescence dye Calcium Green dextran 10 000 (Molecular Probes, Leiden, The Netherlands). This dye was diluted in dimethyl sulfoxide and adjusted to 0.5 mM with the ICS solution. The total volume injected in all the experiments corresponded to 1%–2% of the total cell volume. The Ca^{2+} stores were evoked by 7.5 μM Ca-ionophore addition [30]. The Ca^{2+} values were monitored using a computer-controlled photomultiplier system. Briefly, a digital video microscopy system based on a Zeiss Axiovert 135 microscope (Zeiss, Göttingen, Germany) and an ORCA-100 Hamamatsu 12-bit digital camera (Hamamatsu Photonics Italia, Arese, Milan, Italy) was controlled by a Macintosh G3 workstation (Apple Italia, Cologno Monzese, Italy). This computer was used to control the microscopy system and to perform all the image acquisitions/elaborations by the Openlab software (Improvision, Coventry, U.K.). To exclude the variations of fluorescent intensity by different volumes of injected dye, the fluorescent signals were corrected for variation in dye concentration by normalizing fluorescence (*F*) against baseline fluorescence (F_0) to obtain reliable information regarding transient Ca^{2+} changes from baseline values (relative fluorescence variations [RFV] = $F - F_0/F_0$).

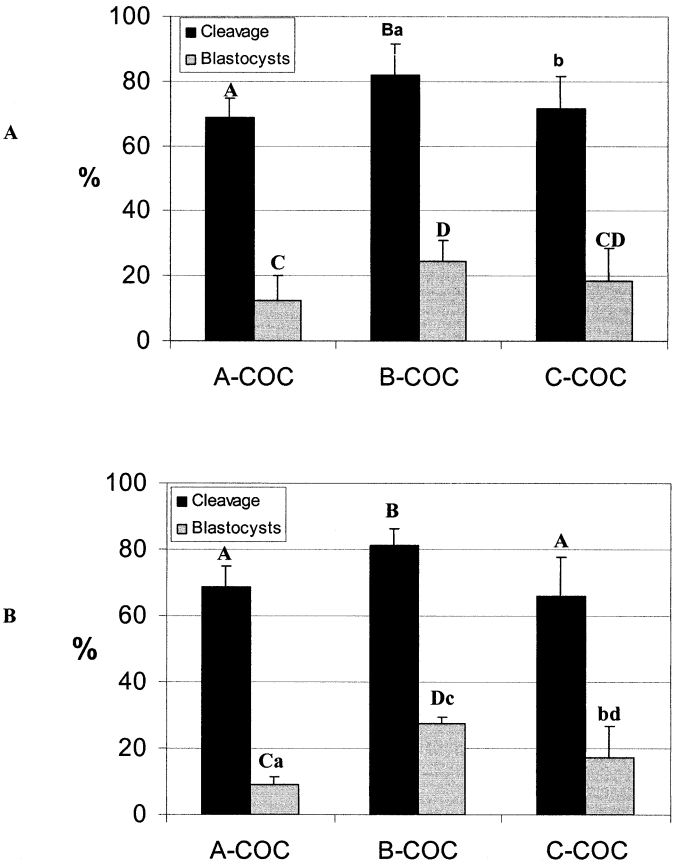

FIG. 2. Mean cleavage and expanded blastocyst rates, with relative standard deviations, yielded in the three considered COC categories by in vitro fertilization (**A**) and parthenogenetic activation (**B**) protocols. Bars with capital letters (A vs. B, C vs. D) are significantly different ($P < 0.01$). Bars with lowercase letters (a vs. b, c vs. d) are significantly different ($P < 0.05$).

Statistical Analysis

The developmental potential, measured as cleavage and blastocyst rates among the COC classes and the IVF and parthenogenetic procedures, was compared by ANOVA [31] after arcsine transformation. General linear model procedure of ANOVA [31] was used to analyze variation sources in resting potentials as well as plasma membrane Ca^{2+} channel activity and Ca^{2+} stores among the considered COC classes.

RESULTS

A total of 808 collected COCs belonging to the three morphological categories were randomly submitted to parthenogenetic or IVF procedures (n = 6 replicates). The resulting mean cleavage and expanded blastocyst rates are shown in Figure 2. The analysis between COC categories showed a significantly higher efficiency ($P < 0.01$) of cleavage rate and blastocyst production in B-COCs. A lower efficiency of cleavage rate was found both in A- and C-COCs, but the lowest blastocyst efficiency belonged to A-COCs. Within each COC category, no difference was found between IVF and parthenogenetic activation procedures.

In whole-cell voltage clamp configuration, we recorded a resting potential of the oocytes that was significantly higher in B- and C-COCs than in A-COCs (−21.8 ± 5.3

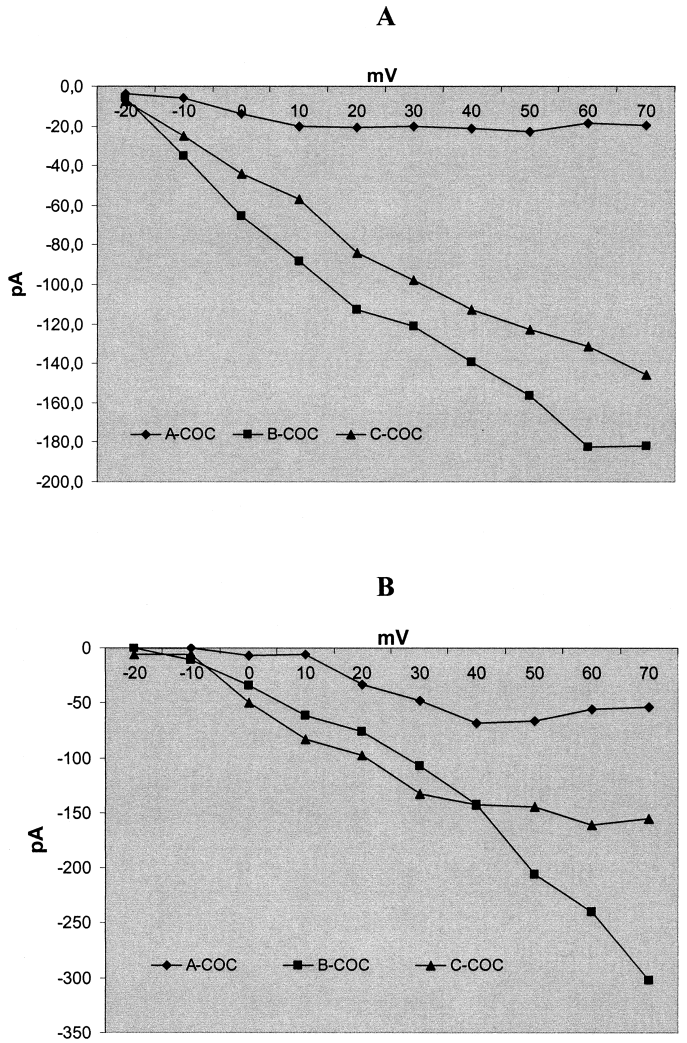

FIG. 3. Current-voltage (I/V) curves of the L-type Ca^{2+} currents on plasma membrane of germinal vesicle oocytes. Clamp was at −30 mV voltage. **A**) Values of the three considered COC categories under standard culture conditions. **B**) Values recorded in the same oocytes soon after the addition of 7.5 μM Ca^{2+} ionophore.

mV and −21.3 ± 7.9 mV vs. −11.8 ± 4.0 mV; $P < 0.01$; data not shown).

By clamping the oocytes at −30 mV and applying ramps of −10 mV depolarizing and hyperpolarizing steps, a series of whole-cell currents were generated. Typical leak-subtracted currents from the clamp value of −30 mV are due to L-type Ca^{2+} channels [22] and are shown in Figure 3A for each COC category. Their amplitude, calculated as the difference between the peak and the steady state at a test potential of +30 mV, was significantly higher in B- and C-COCs than in A-COCs ($P < 0.01$). Soon after the current recording, each oocyte was exposed to the Ca^{2+}-ionophore A23187 according to the parthenogenetic activation protocol described in *Materials and Methods*. The resting potentials significantly hyperpolarized in the oocytes of all COC categories, reaching the highest values in B-COCs; these values (mean ± SD) were significantly different from those in C-COCs (−58.9 ± 6.4 mV vs. −44.3 ± 0.8 mV; $P < 0.01$; data not shown). The A-COCs rose to a value between those of the B- and C-COCs, which was significantly different from the latter values (−52.3 ± 5.1 mV vs. −44.3 ± 0.8 mV; $P < 0.05$). The obtained leak-subtracted currents

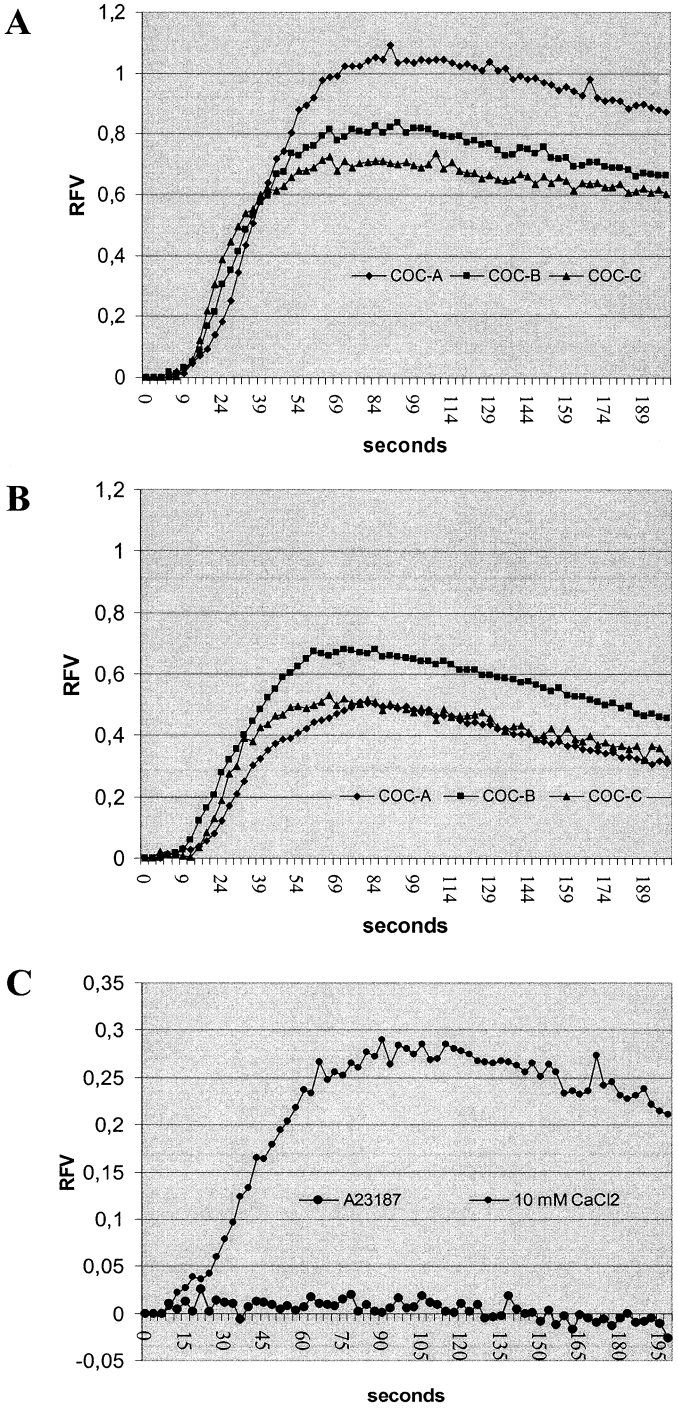

FIG. 4. Increase in $[Ca^{2+}]_i$ following ionophore (A23187) challenge measured as RFV in the three considered COC categories. **A**) Mean values of immature oocytes. **B**) Mean values of in vitro-matured oocytes. **C**) Mean values of immature oocytes loaded with 150 nM EGTA and exposed to addition of 7.5 μM Ca^{2+} ionophore in standard culture conditions or after addition to the holding medium of 10 mM $CaCl_2$.

(Fig. 3B) followed the same patterns observed in the preactivated oocytes (Fig. 3A) with regard to the differences between the three COC categories; an increase ($P < 0.05$), calculated at a test potential of +50 mV, occurred only in A- and B-COCs. These currents, however, increased their amplitude and shifted toward more positive voltage values.

Figure 4 shows the patterns of $[Ca^{2+}]_i$ expressed as RFV ($F - F_0/F_0$) of the three COC categories after exposure to the Ca^{2+}-ionophore treatment in immature (Fig. 4A) and in

vitro-matured (Fig. 4B) oocytes. Data were pooled for the three COC categories, and the peak values of Ca^{2+} release were compared for immature and mature oocytes. The Ca^{2+} stores (mean ± SD) significantly decreased from immature to in vitro-matured oocytes (0.864 ± 0.217 RFV vs. 0.534 ± 0.200 RFV; $P < 0.01$; data not shown). This 38.2% total decrease of calcium content involved all three COC categories as follows; A-COC, 51.6%, B-COC, 15.5%, and C-COC, 26.7%. In immature oocytes, calcium stores were higher in A-COCs and progressively decreased in B-COCs (1.055 ± 0.184 RFV vs. 0.824 ± 0.171 RFV; $P < 0.05$) and then in C-COCs (1.055 ± 0.184 RFV vs. 0.712 ± 0.128 RFV; $P < 0.01$). After in vitro maturation, the calcium-store patterns changed with a higher content in B-COCs than in C- and A-COCs (0.696 ± 0.135 RFV vs. 0.522 ± 0.190 RFV and 0.511 ± 0.177 RFV; $P < 0.05$). These variations in relative fluorescence intensity are related to the increase of free calcium from the intracytoplasmic calcium stores, both because the oocytes were loaded in calcium-free medium supplemented with EGTA and because the injection of 150 mM EGTA in ICS into the oocytes (n = 5) inhibited any change in fluorescence following A23187 treatment (Fig. 4C). A small fluorescence increase was, however, observed when 10 mM $CaCl_2$ were added to the holding medium, but this effect was partially attributed to the reduction in the volume of the oocyte due to an increase in external osmotic pressure (Fig. 4C).

DISCUSSION

The present study demonstrated that, in bovine oocytes, developmental competence is related to plasma membrane Ca^{2+} current activity. The Ca^{2+} stores reflect the quality of immature oocytes and the developmental competence of in vitro-matured oocytes.

The higher IVEP efficiency found in B-COCs, either by IVF or parthenogenetic activation, agrees with the results of Wurth and Kruip [8] and of Wurth et al. [2], as well as with those of Blondin and Sirard [10] and Hazeleger et al. [11], even if the latter authors used different evaluation criteria that do not allow for direct comparison. Considered together, these findings support the idea that follicular atresia causes physiological modifications inside the COC, which positively affect the developmental competence of bovine oocytes. This competence is preserved throughout advanced stages of follicular degeneration and is expressed as IVEP efficiency. Surprisingly, this competence is low in A-COCs, a class mostly attributed to nonatretic follicles [32]. Meiotic arrest in follicular oocytes is maintained by high cAMP concentrations in the follicular fluid, which are transmitted to the oocyte via cumulus-oocyte intercellular processes [12], and cumulus-oocyte intercellular communication decreases throughout follicular atresia [13]. The decrease of intercellular communication likely affects the stability of meiotic arrest and facilitates oocyte-maturation mechanisms. Results of studies on ovum pick-up (OPU) in cattle also support these findings [33]. The new follicular wave following each OPU session becomes atretic due to follicle dominance, a mechanism leading to a single ovulatory follicle in uniparous species [34]. The increase of the interval between two consecutive OPU sessions from 3 to 4 days causes the progression of follicle selection with a consequent increase of atresia as well as of IVEP efficiency ([33], unpublished data).

The comparison between IVF and parthenogenetic activation did not show significant differences when considering the total data of the three COC categories or within each COC category (data not shown). This finding agrees with that of Liu et al. [28], who, comparing different activation protocols, found the combination of Ca^{2+} ionophore and 6-DMAP to be the best procedure, with results that did not differ from those obtained with IVF. A perfect overlapping of IVF and activation procedures occurred, however, only when IVF was performed using semen from a previously tested bull, when the highest IVF efficiency was expressed. Parthenogenetic activation overcomes problems associated with individual sperm variability by defining a chemical protocol that allows full expression of the developmental potential of the oocyte. This procedure is highly related to IVF and shows a high sensibility, as demonstrated by the discriminate evaluation of COC categories. Based on these characteristics, this procedure is proposed as a reliable tool for evaluation of meiotic competence in bovine oocytes and, generally, as a check-tool for overall IVEP procedures.

The resting potential is related to several developmental events. During maturation, oscillations in resting potential of the oocyte are correlated to meiotic progression both in marine invertebrates [35] and mammals [21]. In bovine oocytes, we previously demonstrated [22] that membrane depolarization is related to a decrease in K^+ permeability. In this paper, the resting potential varied significantly in the three COC categories and showed a negative relationship with developmental competence. This pattern was preserved following addition of Ca^{2+} ionophore, which caused a transient hyperpolarization, possibly consequent to Ca^{2+}-activated K^+ channels, as described in matured mammalian oocytes [36].

The role of calcium during oocyte maturation (for review, see [17]) is relevant. In bovine oocytes, nuclear maturation is inhibited by Ca^{2+}-chelating agents [37]. Recent research also demonstrated a Ca^{2+} need for cytoskeleton assembly in normal cytoplasmic maturation [38]. Plasma membrane Ca^{2+}-channel activity and cumulus-oocyte intercellular communicative devices ensure calcium influx into the oocyte [39]. Both these sources of calcium influx decrease throughout maturation [22]. Within the three immature COC classes, we found a relationship between plasma membrane Ca^{2+} current activity and developmental competence of the oocyte. These differences could also explain the high variability that we found in a previous study [22], in which this classification was not considered. The increase of Ca^{2+} current activity throughout the process of atresia could represent a compensation process to Ca^{2+}-loading mechanisms consequent to the progressive decrease of cumulus-oocyte intercellular communication. The increase of the Ca^{2+} peak amplitude observed after Ca^{2+}-ionophore addition is consistent with sensitization of Ca^{2+} channels [40]. We have no explanation for the shift of the peak amplitude toward more positive voltage values with caused byrespect to the control. A similar pattern was, however, described previously [22] after external Ca^{2+} increase.

The role of Ca^{2+} in fertilization mechanisms is well known. As a consequence of sperm-egg interaction, an increase in egg intracellular Ca^{2+} has been found in all animal species studied so far (for review, see [25]). Although such a rise in Ca^{2+} plays a key role in egg activation, its origin is still controversial, and three hypotheses have been proposed regarding how this occurs; by Ca^{2+} conduit, by membrane receptor, or by soluble sperm factor. Moreover, recent research by Sato et al. [41] demonstrated that intracellular sperm injection is more effective than sperm factor in sensitizing Ca^{2+} channels of the endoplasmic reticulum. What-

ever the case, internal Ca^{2+} stores always seems to be involved in fertilization. The dynamics of intracytoplasmic calcium loading in the oocyte significantly changed from the immature to mature stages and within each stage between COC categories. The decrease in Ca^{2+} stores throughout maturation reflects the pattern of Ca^{2+} current activity on the plasma membrane [22] and the decrease in the intercellular communicative devices observed throughout meiosis [14]. This finding suggests that less need of Ca^{2+} stores exists in bovine oocytes that are ready for fertilization, and it highlights a difference between bovine and mouse [40] oocytes, as previously described by Homa [17].

In the case of immature oocytes, we found a relationship between Ca^{2+} stores and oocyte quality. Although the A-COC class showed the lowest plasma membrane Ca^{2+} current activity, it turned out to have the largest Ca^{2+} store. This finding suggests that, at this stage, the role of plasma membrane channels on Ca^{2+} entry is minimal in comparison to cumulus-oocyte communicative devices. The progressive diminution of the intercellular route of Ca^{2+} entry throughout maturation gives rise to a new pattern of Ca^{2+} stores within the three COC classes. At the end of maturation, Ca^{2+} stores appear to be related more to developmental competence than to oocyte quality. A relation between Ca^{2+} stores and developmental competence was also described for in vitro-matured bovine oocytes by Damiani et al. [42] using this approach to evaluate differences between calf and cow oocytes.

In conclusion, in the present study, we found a definite relationship between the morphological features of the bovine oocyte population and their developmental potential. The plasma membrane Ca^{2+} currents of immature oocytes and the intracellular Ca^{2+} stores in mature oocytes are good markers for the developmental potential of the oocytes. On the other hand, Ca^{2+} stores are related to morphological quality in the case of immature oocytes. The design of new protocols for IVEP may better match the needs of the best-quality oocyte class with a positive cascade effect on in vitro embryo technologies.

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