Ca$^{2+}$ current activity decreases during meiotic progression in bovine oocytes

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Tosti, Elisabetta, Raffaele Boni, and Annunziata Cuomo. Ca$^{2+}$ current activity decreases during meiotic progression in bovine oocytes. Am J Physiol Cell Physiol 279: C1795–C1800, 2000.—By using the whole cell voltage-clamp technique, we studied changes in plasma membrane permeability at different meiotic stages of bovine oocytes. Follicular oocytes were matured in vitro and activated by Ca$^{2+}$ ionophore. Oocytes at germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), metaphase II (MII), and meiosis exit were used for electrophysiological recording. By clamping the oocytes at $-30$ mV, we found that the L-type voltage-dependent Ca$^{2+}$ channels were active at the GV stage and that their activity decreased after the GVBD stage. Furthermore, the resting potential decreased from the GV to the MI stage and increased again at MII. A significant decrease of the steady-state conductance occurred from the GV to the MI stage, followed by a sharp increase at the MII stage. With the addition of organic L-type Ca$^{2+}$ channel blockers (nifedipine and verapamil), we inhibited the Ca$^{2+}$ currents. However, only in the case of verapamil was there a decrease of in vitro maturation efficiency. Our results suggest that, in addition to the cumulus-oocyte junctions, the plasma membrane channels provide another mode of Ca$^{2+}$ entry into bovine oocytes during meiosis.

IN MAMMALS, FOLLICULAR OOCYTES ARE ARRESTED IN THE DIPLOTENE STAGE OF THE FIRST MEIOTIC PROPHASE (GERMINAL VESICLE [GV] STAGE) UNTIL THE START OF FOLICULAR MATURATION RELEASE FROM THE FOLLICULIC ENVIRONMENT (16). cAMP IS SUSPECTED TO MAINTAIN THE MEIOTIC ARREST WHEN TRANSMITTED FROM CUMULUS CELLS TO THE OOCYTE THROUGH GAP JUNCTIONS (1, 6, 15, 21). IN RESPONSE TO THE LUTEINIZING HORMONE (LH) SURGE, CUMULUS CELLS TRANSMIT A Ca$^{2+}$ SIGNAL TO THE OOCYTE (17), LEADING TO GAP JUNCTION REGRESSION (50). Simultaneously, cAMP LEVELS DECREASE, WHICH IN TURN RELEASES THE OOCYTE FROM MEIOTIC ARREST (46). THESE CONSECUTIVE EVENTS ARE PRECEDED BY A RELATIVELY LONG PHASE LASTING FROM THE GV STAGE TO GERMINAL VESICLE BREAKDOWN (GVBD), WHICH IS CHARACTERIZED BY HIGH PROTEIN SYNTHESIS AND TRANSCRIPTIONAL ACTIVITY (26). DURING THIS LAG PHASE, CUMULUS-OOCYTE COMMUNICATION IS OPEN AND THE INTRACYTOPLASMATIC cAMP LEVELS ARE HIGH (11). THE OOCYTE THEN COMPLETES MEIOSIS I BY EXTRUDING THE FIRST POLAR BODY AND BEGINS THE SECOND MEIOTIC DIVISION. THIS IS CHARACTERIZED BY A CELL CYCLE BLOCK AT METAPHASE II (MII) THAT LASTS UNTIL FERTILIZATION. SPERM-OOCYTE INTERACTION, AS WELL AS PHARMACOLOGICAL SUBSTANCES, E.G., Ca$^{2+}$ IONOPHORE OR ETHANOL, MAY INDUCE MEIOSIS COMPLETION AND TRIGGER EARLY EMBRYO DEVELOPMENT (8, 49, 57).

MEIOSIS AND MITOSIS ARE REGULATED BY TWO ENZYMES, HISTONE 1 (H1) AND MITOTEN-ACTIVATED PROTEIN (MAP) KINASE. H1, OR MATURATION-PROMOTING FACTOR (MPF), IS COMPOSED OF CYLIN B AND P34$^{edc2}$ SUBUNITS, WHICH DISPLAY A CYCLICAL ACTIVITY PEAKING AT THE METAPHASE STAGE (18, 56). MAP KINASE IS PART OF A KINASE CASCADE THAT IS LIKELY INITIATED BY c-mos (45). THIS PATHWAY SEEMS TO BE APPARENTLY INVOLVED IN MEIOTIC SPINDLE ORGANIZATION, EXTRAUSION OF THE FIRST POLAR BODY, AND MEIOTIC ARREST AT THE MII STAGE (7).

IT IS WELL KNOWN THAT Ca$^{2+}$ IS INVOLVED IN OOCYTE MATURATION (REF. 25 FOR REVIEW). IN THE HAMSTER (19) AND MOUSE (5), A SERIES OF SPONTANEOUS Ca$^{2+}$ OSCILLATIONS OCCUR IN THE OOCYTE AFTER ISOLATION FROM THE FOLLICLE UPTO THE GVBD STAGE. AFTER THESE OSCILLATIONS HAVE SUBSIDED, Ca$^{2+}$ DOES NOT AFFECT FURTHER MEIOTIC PROGRESSION. IN BOVINE AND PIG, NO Ca$^{2+}$ OSCILLATIONS OCCUR DURING MEIOSIS PROGRESSION; HOWEVER, Ca$^{2+}$ IS NECESSARY FOR MEIOTIC PROGRESSION SINCE 1,2-BIS(2-AMINO-PHENOXY)ETHANE-NNNN'-TETRAACETIC ACID (BAPTA), A Ca$^{2+}$ CHALCER, CAUSES A DELAY OR BLOCK OF MEIOSIS (22). AT FERTILIZATION, A NEW SERIES OF OSCILLATIONS RELATED TO MEIOTIC COMPLETION OCCURS (36). THE Ca$^{2+}$ IONOPHORE, A-23187, INDUCES MEIOSIS RESUMPTION IN OOCYTES BLOCKED EITHER AT THE GV STAGE (55) OR MII (30, 48, 53) STAGE. BECAUSE EXTRACELLULAR Ca$^{2+}$ IS REQUIRED FOR IN VITRO GVBD (14) AND FOR FIRST MEIOTIC DIVISION (41), IT APPEARS THAT Ca$^{2+}$ ION TRANSPORT THROUGHOUT THE PLASMA MEMBRANE PLAYS A FUNCTIONAL ROLE IN MATURATION.

L-TYPE Ca$^{2+}$ CHANNELS ARE INVOLVED IN NUMEROUS PHYSIOLOGICAL PROCESSES (2, 24). THESE VOLTAGE-GATED CHANNELS HAVE BEEN FOUND IN OOCYTES OF THE MARINE INVERTEBRATES (TUCINATES) (10) AS WELL AS IN MAMMALIAN OOCYTES (39). IN THE MOUSE, MURNAUNE AND DE FELICE (38) SHOWED A SELECTIVE INCREASE OF THESE CHANNELS ON THE

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oocyte plasma membrane after puberty, corresponding to meiotic competence occurrence.

In this study, we have analyzed the electrical properties of the plasma membrane in bovine oocytes at different meiotic stages, focusing primarily on the activity of L-type voltage-dependent Ca\(^{2+}\) channels.

**MATERIALS AND METHODS**

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

**Oocyte source.** Ovaries from slaughtered cows were collected from the abattoir and transported in a thermal bag at 30–35°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 mmHg). Cumulus-oocyte complexes (COC) were isolated from the follicular fluid and washed three times with TCM199 supplemented with 5% FCS and 10 mM HEPES. The COC were then transferred into maturation medium (TCM199 supplemented with 10% FCS, 10 IU/ml LH, 0.1 IU/ml follicle-stimulating hormone, and 1 μg/ml 17β-estradiol) and left in an incubator at 39°C in 5% CO\(_2\), 5% O\(_2\), and 88% N\(_2\) for 1 day. Zygotes and embryos were cultured in SOF medium containing amino acids and BSA (51) in the previous gas mixture for 8 days postactivation for blastocyst development.

**Electrophysiology.** Electrical recording was performed at 37°C on oocytes at the following stages: GV, promptly isolated from follicles; GVBD, after 8 h of maturation; metaphase I (MI), after 12 h of maturation; and MII, after 24 h of maturation (47). Meiosis exit occurred 15–16 h after Ca\(^{2+}\) ionophore treatment, corresponding to the time span related to the decrease of MPF and MAP kinases (29) and the increase in a-gustagonist (30). For each experiment, batches of oocytes (control groups) were submitted to the same A-23187 treatment, followed by 3.5 h of incubation in culture dishes (Nunclon, Nunc, Denmark) and cultured in a gas mixture of 5% CO\(_2\), 7% O\(_2\), and 88% N\(_2\). At the time of the electrophysiological studies, batches of oocytes (control groups) were treated with a 20-min incubation with either FITC-conjugated wheat germ lectin at the GV and GVBD stages to visualize the nuclear membrane or with FITC-conjugated anti-a-tubulin at the MI and MII stages to visualize the meiotic spindle. Finally, after being washed twice with PBS, the oocytes were scanned with an Olympus Fluoview confocal microscope.

**RESULTS**

A total of 335 oocytes (14 replications) were parthenogenetically activated and produced a cleavage rate of 81.5 ± 6.2% and an expanded blastocyst production of 22.7 ± 4.7%. Examination of the fixed oocytes showed that the majority reached the stages studied, i.e., GV, 92.6% (25/27); GVBD, 85.7% (30/35); MI, 71.4% (20/28); MII, 84.2% (32/38); and meiosis exit, 73.3% (22/30).

The resting potentials of the oocytes at particular meiotic stages are shown in Fig. 1A. These potentials did not differ from the GV stage to the GVBD stage, decreased significantly (P < 0.05) again at the MII stage, and, finally, decreased at the meiosis exit stage. By clamping the cells at −30 mV and applying ramps of 10-mV depolarizing and hyperpolarizing steps, a series of whole cell currents were generated. The outward currents suggested a rectifier channel similar to that described in the human oocyte by De Felice et al. (13). To obtain steady-state conductance, we plotted the peak current amplitude against the tip potential. This resulted in a linear relationship (Fig. 1B). The steady-state conductance current-voltage (I-V) significantly decreased (P < 0.01) from the GV to the GVBD and GV stages; it increased (P < 0.01) at the MII stage and, finally, decreased again at the meiosis exit stage (Fig. 1C).

From the voltage clamp of −30 mV to test potentials, we observed an inward component of current activating in 30 ms and slowly inactivating in 250 ms, reaching a plateau in 500 ms. Typical leak-subtracted currents from −30 mV and I-V curves for the leak-subtracted currents at −30-mV voltage clamp are shown in Fig. 2 for each stage. Their amplitude, calculated as the difference between the peak and the steady
state, significantly ($P < 0.01$) decreased from GVBD to the subsequent stages.

At −30-mV voltage clamp, the addition of 10 mM external Ca$^{2+}$ to the bath increased the inward component at all stages. The I-V relationship of the peak amplitude at high Ca$^{2+}$ concentration is shown in Fig. 2. The inward component increased at all the examined stages, with a maximum difference at the GV stage. Moreover, the pattern in the high Ca$^{2+}$ regime was shifted toward more positive voltage values (Fig. 2).

High Ca$^{2+}$ also caused a transient hyperpolarization of the plasma membrane at both the GV and MII stages. Ca$^{2+}$ currents were completely inhibited in the GV and GVBD stages by adding to the bath either nifedipine or verapamil at concentrations >5 μM. Moreover, maturing GV oocytes in the presence of either 100 μM verapamil or 10 μM nifedipine caused decreased ($P < 0.01$) cleavage efficiency (45 or 62% vs. 89%) and blastocyst development (10 or 31% vs. 32%) in the case of verapamil.

DISCUSSION

In this study, we have shown that the plasma membrane of bovine oocytes undergoes profound electrical modification throughout meiosis. In particular, differences among meiotic stages were found in the resting potential as well as in steady-state conductance and voltage-gated Ca$^{2+}$ channel activity. Little is known about the relationship between the resting potential of plasma membrane and the cell cycle. A change in plasma membrane polarization and ion permeability has already been described during maturation in invertebrate (37), amphibian (54), and mammalian (38, 43, 44) oocytes. We measured a stable negative resting potential and changes related to oocyte activation (12).

It is likely that in the bovine oocyte, a low resting potential in both the GV and MII stages is associated with a “standby” status. In the case of GV, we suggest that the plasma membrane awaits for a signal to resume meiosis, in which there is a large exchange of Ca$^{2+}$ ions. As soon as cell cycle progression is resumed, the plasma membrane depolarizes. In such a case, however, it is difficult to explain the resting potential of GVBD, which represents the first stage of meiosis resumption. This may reflect meiosis vs. mitosis, bearing in mind that GV to GVBD is a period in which the metabolic oocyte activity is high (20, 26), cumulus-oocyte communication is intact (50), and cytoplasmic cAMP is elevated (11, 58).

The plasma membrane permeability, measured as steady-state conductance, is high at the GV stage and decreases during meiosis with little restoration at the MII stage. A similarity between these values at the GV and MII stages was reported in the mouse (38). Hence, the highest permeability corresponds to the two meiotic arrest phases. It is feasible that the high-ion exchange is related to the large metabolic activity of the GV stage or to the preparation of the plasma membrane for fertilization at the MII stage.

The I-V relationship of the leak-subtracted peak currents at different holding voltages, as well as the results obtained at high Ca$^{2+}$ regime and the sensitivity at pharmacological agents, strongly suggest that these currents represent L-type Ca$^{2+}$ channels. This is in agreement with previous findings in mouse (38) and invertebrate (10) oocytes. L-type Ca$^{2+}$ channels have been demonstrated to underlay meiosis resumption in mussel (52), *Pleurodeles* (40), and in mammalian (39) oocytes. In bovine oocytes, the predominance of these channels at the GV and GVBD stages suggests a role for Ca$^{2+}$ during the first meiotic resumption. Indeed, during maturation, the activity of plasma membrane Ca$^{2+}$ channels decreases. This pattern may support the cytosolic Ca$^{2+}$ rise at GV in addition to the LH and/or the growth factor-mediated Ca$^{2+}$ surge via cumulus-oocyte communication (23, 31). In contrast, the low plasma membrane Ca$^{2+}$ channel activity at the
MII stage argues for a minor role of external Ca$^{2+}$, whereas intracellular Ca$^{2+}$ mobilization mechanisms appear to be more important for oocyte activation and fertilization.

The mechanism of how Ca$^{2+}$ affects meiosis progression is unclear. However, we know that 1) BAPTA delays kinase activity and inhibits maturation (22); 2) Ca$^{2+}$ may influence protein synthesis that is essential in maturational processes (28); and 3) Ca$^{2+}$ modulates gap junction functionality, allowing cumulus-mediated intracytoplasmatic cAMP levels (58). In bovine oocytes, it has been shown that Ca$^{2+}$ participates in the progression of meiosis, although spontaneous Ca$^{2+}$ oscillations do not occur as in hamster and mouse oocytes (19, 22). D-Myo-inositol 1,4,5-trisphosphate (IP$_3$)-induced Ca$^{2+}$ release has been suggested as a primary mechanism for maturation of bovine oocytes because the cytoplasmic IP$_3$ receptors increase in number dur-
The new information and insight into the mechanisms at fertilization, it mainly depends on intracellular Ca$^{2+}$

because we parthenogenetically activate tors, which are poorly expressed in bovine oocytes (22).

LH-mediated Ca$^{2+}$ rise, Ca$^{2+}$ entry arises through Ca$^{2+}$ channels on the oocyte plasma membrane other than via gap junction cumulus-oocyte communication. Because the oocyte plasma membrane does not contain LH receptors, the initial Ca$^{2+}$ influx comes from cumulus cells. This may cause a change in membrane potential and gating of voltage-dependent Ca$^{2+}$ channels. The intracytoplasmatic Ca$^{2+}$ rise may undergo a self-amplifying mechanism (Ca$^{2+}$-induced Ca$^{2+}$ release, IP$_3$-induced Ca$^{2+}$ release, or Ca$^{2+}$-induced IP$_3$ release) (3). If such a mechanism exists, it could potentiate the cumulus-oocyte communication necessary for metabolic exchange and the high cAMP levels during early maturation. High Ca$^{2+}$ would then close the gap junctions (27), causing a drop in cAMP.

We have also shown that the MI stage is characterized by an increase of steady-state conductance due to K$^+$ channels that is not accompanied by Ca$^{2+}$ channel activity. In mammals, sperm-mediated oocyte activation is accompanied by a hyperpolarization of the plasma membrane due to Ca$^{2+}$-activated K$^+$ channels (9, 34, 35). Because we parthenogenetically activate oocytes by using Ca$^{2+}$ ionophore, thus simulating the sperm-mediated Ca$^{2+}$ surge, our data support the idea that in bovine oocytes external Ca$^{2+}$ is not involved in meiosis exit. Indeed, the intracytoplasmatic Ca$^{2+}$ surge may activate K$^+$ channels. The decrease of Ca$^{2+}$ channels during maturation may be correlated with the maturation of Ca$^{2+}$ release mechanisms occurring at MI (32). These findings suggest that whereas external Ca$^{2+}$ influences sperm-mediated Ca$^{2+}$ elevation at fertilization, it mainly depends on intracellular Ca$^{2+}$ stores.

In conclusion, during meiosis the plasma membrane of bovine oocytes undergoes a progressive depolarization and Ca$^{2+}$ channel depletion. These findings provide new information and insight into the mechanisms and dynamics of meiosis.

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