Fertilization and activation currents in bovine oocytes

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One of the first events that occurs at fertilization is a transient modification of the electrical properties of the oocyte plasma membrane. The whole-cell voltage clamp technique was used to demonstrate an outward ion current and a hyperpolarization of the plasma membrane after fertilization in bovine oocytes. These electrical events, together with measurement of internal calcium concentrations, were also recorded after injection with sperm factor and exposure to parthenogenetic activators, such as Ca^{2+} ionophore, ethanol and thapsigargin. Experiments were carried out simultaneously in immature and *in vitro* matured oocytes. Significant differences were recorded in the activation current and hyperpolarization among oocyte activators and between immature and matured oocytes. However, outward ion current and Ca^{2+} release showed similar dynamics. The injection of the calcium chelator EGTA completely abolished both ion current and hyperpolarization, indicating that these electrical events

are calcium dependent. Addition of specific calcium releasers, such as 1,4,5-inositol trisphosphate (IP_3) and caffeine, triggered ion activation current and hyperpolarization indicating that IP_3 and ryanodine receptors are active in both immature and matured oocytes. Different ion channel inhibitors were used to characterize the channels underlying outward currents. Only addition of riberitoxin caused a complete inhibition of the current, indicating the involvement of high conductance Ca^{2+} -activated K^+ channels in generating activation current. In conclusion, these findings provide evidence that bovine oocyte activation is associated with Ca^{2+} -dependent electrical events. Oocytes have the potential to react to different activators even when immature; however, oocyte maturation seems to increase sensitivity to physiological activators, such as spermatozoa and sperm factor, and chemicals, such as ethanol.

Introduction

An early event that occurs at fertilization is the change in the oocyte plasma membrane potential, which transiently depolarizes in ascidians and sea urchins (Dale and De Felice, 1984; De Simone *et al.*, 1998), and hyperpolarizes in some mammalian species (Miyazaki, 1988; Gianaroli *et al.*, 1994). The whole-cell voltage clamp technique has been used to demonstrate that these electrical events are due to the gating of ion channels generating flux of ions through the plasma membrane of the fertilized oocyte (for a review, see Dale, 1994). The sperm-activated ion currents are non-specific in marine invertebrates (Dale and De Felice, 1984; De Simone *et al.*, 1998) and calcium-activated potassium currents in hamsters and humans (Miyazaki and Igusa, 1982; Dale *et al.*, 1996).

Another universal event that occurs at fertilization is the massive release of calcium that traverses the oocyte in a wave (for reviews, see Swann and Ozil, 1994; Stricker, 1999). In *Xenopus laevis* and the sea urchin, a single increase in calcium occurs at oocyte activation (Busa and Nuccitelli, 1985; Gillot and Whitaker, 1993), whereas in ascidian and mammalian oocytes, repetitive calcium

oscillations are reported up to extrusion of the second polar body (Russo *et al.*, 1996; Yoshida *et al.*, 1998) and pronuclear formation (Stricker, 1999; Swann and Parrington, 1999). The origin of these increases in calcium may be attributed to an influx through the plasma membrane or to an internal release from calcium stores. In bovine oocytes, Tosti *et al.* (2000) reported a decrease in plasma membrane Ca^{2+} currents during meiotic progression and Boni *et al.* (2002) reported a prevalence of Ca^{2+} stores in immature compared with *in vitro* matured oocytes. To date, a clear relationship at fertilization between electrical currents and Ca^{2+} release has not been demonstrated in mammals.

Oocyte activation is artificially induced by: (i) agents, such as the calcium ionophore that increase calcium content (Steinhardt and Epel, 1974; Steinhardt *et al.*, 1974) and thapsigargin (Petr *et al.*, 2000); (ii) ethanol (Nagai, 1987); (iii) inhibitors of protein synthesis and protein phosphorylation (Presicce and Yang, 1994; Liu *et al.*, 1998); and (iv) electrical pulses (Collas *et al.*, 1993). In bovine oocytes, activation by Ca^{2+} ionophore and ethanol results in a wide range of parthenogenetic development from cleavage up to blastocyst formation (Shi *et al.*, 1993; Presicce and Yang, 1994). It has been suggested that activation is mediated by an increase in Ca^{2+} that in turn leads to the exit from the second meiotic block via the inactivation of the cytostatic factor and consequently the

mitotic promoting factor (for a review, see Parrish *et al.*, 1992). Calcium mobilization seems to come from intracellular stores in most of the species studied; however, external calcium has also been demonstrated to replenish the stores for the continuation of calcium signals (Stricker, 1999). Studies on parthenogenesis represent a crucial step for understanding the basic mechanisms underlying oocyte activation and embryo development. Artificial activation and fertilization seem to share common mechanisms in many animal species, as artificial stimuli mimic both electrical responses and calcium oscillations observed at fertilization (Nuccitelli, 1980; Schlichter and Elisson, 1981; Swann, 1990; Dale *et al.*, 1996). Early studies by Eusebi and Siracusa (1983) showed that a variety of different stimuli trigger parthenogenetic development in mice and hamsters; however, the electrical events generated were not driven by common ionic mechanisms or by the classical activation potential. A clear understanding of oocyte activation mechanisms is required for assisted reproductive technologies, such as intracytoplasmic sperm injection (ICSI), nuclear transfer and cloning in cattle. Despite the large number of studies on oocyte activation, little is known about the electrical events induced by different activators or the nature of the ion channels responsible for this.

The aim of the present study was to investigate the first electrical modifications that occur in bovine oocytes after activation and to relate these modifications to Ca^{2+} dynamics. This analysis was carried out in both immature and *in vitro* matured oocytes to define the origin of such mechanisms.

Materials and Methods

Materials

If not otherwise stated all chemicals were purchased from Sigma Chemical Co. (St Louis, MO) and were of tissue culture grade where appropriate.

Oocyte source and *in vitro* maturation

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 follicles of 2–8 mm in diameter by an 18 G needle under controlled pressure (50–70 mmHg). Cumulus–oocyte complexes (COC) were isolated from the follicular fluid and washed three times with washing medium (TCM-199 supplemented with 0.1% (w/v) polyvinyl alcohol and 10 mol Hepes l^{-1}). The COC were then transferred into maturation medium (TCM-199 supplemented with 10% (v/v) fetal calf serum, 10.0 iu LH ml^{-1} , 0.1 iu FSH ml^{-1} and 1 μg oestradiol ml^{-1}) (40 μl per COC) and kept in an incubator at 39°C in 5% (v/v) CO_2 humidified air for 24 h. At the time of each experimental session, immature and mature oocytes were freed from the cumulus cells by vortexing for 3 min in washing medium. The zona pellucida

was removed after incubation for 1.0–1.5 min in 0.5% (w/v) protease dissolved in washing medium. The zona pellucida-free oocytes were then incubated for 10 min at 39°C in TCM-199 supplemented with 10% (v/v) fetal calf serum and 10 mol Hepes l^{-1} and, finally, transferred in Fert–Tyrode's albumin lactate pyruvate (TALP) (Parrish *et al.*, 1986) medium supplemented with Hepes (wash–TALP) and kept in an incubator until the electrical recording or calcium measurement was performed.

Immature COC were selected on the basis of morphology (De Loos *et al.*, 1989). In brief, at least three layers of cumulus cells entirely surrounded the oocytes, which were assumed to be at the germinal vesicle stage up to 4 h after their removal from the enclosed follicle.

Oocytes matured *in vitro* were obtained at the end of the maturation period (24 h) and selected on the basis of the presence of a clear polar body and a homogeneous cytoplasm. For each experiment, a batch of ten oocytes was fixed in acetic–ethanol (1:3) solution and after 24 h stained with 1% (w/v) lacmoid solution to determine the number that had reached metaphase II (MII). The proportion of oocytes at MII ranged between 80% and 100% throughout the entire experimental period.

Electrophysiology

Electrical recording was performed at 39°C on oocytes in either the immature or *in vitro* matured stages. Zona pellucida-free oocytes were placed in a recording chamber containing 200 μl wash–TALP. Oocytes were voltage-clamped using standard techniques. Patch pipettes of 10 M Ω resistance and 1–2 μm in tip diameter were filled with an intracellular-like solution (ICS) containing 70 mmol KCl l^{-1} , 7 mmol NaCl l^{-1} , 10 mmol EGTA l^{-1} , 10 mmol Hepes l^{-1} , pH 7.4, and 280 mOsm. After obtaining a giga Ω -seal, the pipette voltage was set to the desired negative potential and the patch ruptured. Observation of a stable negative resting potential signalled access to the cytosol. The permeabilization of the plasma membrane after the oocyte activation was verified by applying depolarizing and hyperpolarizing voltage steps of 10 mV and 500 ms before and at the peak current to generate the voltage-dependent currents. All microinjections were performed in the patch clamp configuration with an Eppendorf transjector (Hamburg) with an estimated injection volume corresponding to 1–2% (v/v) of the oocyte. All electrical traces were recorded on a List EP7 amplifier and data were stored on a VCR tape for subsequent analysis.

$[\text{Ca}^{2+}]_i$ determinations

Immature and *in vitro* matured zona pellucida-free oocytes were placed in a recording chamber containing 2 ml of either wash–TALP medium or Ca^{2+} -free TALP medium supplemented with 1 mmol EGTA l^{-1} at 39°C and injected using the standard techniques described above with the fluorescence dye calcium green dextran (M_r 10 000) (Molecular Probes, Leiden). This dye was diluted in ICS with

a concentration in the pipette of 0.5 mmol l⁻¹. Ca²⁺ stores were evoked by the addition of spermatozoa, 5 µmol Ca²⁺ ionophore l⁻¹, 7% (v/v) ethanol, 10 µmol thapsigargin l⁻¹, 20 mmol caffeine l⁻¹ and by injecting 5 µmol 1,4,5-inositoltrisphosphate (IP₃) l⁻¹ and sperm factor with a subsequent injection. Ca²⁺ values were monitored using a computer-controlled photo-multiplier system. In brief, a digital video microscopy system on a Zeiss (Göttingen) Axiovert 135 microscope and an ORCA-100 Hamamatsu (Hamamatsu Photonics Italia, Arese) 12-bit digital camera were controlled by a Macintosh G3 workstation. This computer was used to control the microscopy system and to perform all the image acquisitions and elaborations using the Openlab program (Improvision, Coventry). The fluorescent signals were corrected for variation in dye concentration by normalizing fluorescence (*F*) against baseline fluorescence (*F*₀), to obtain reliable information about transient Ca²⁺ changes from baseline values ($(F-F_0)/F_0$) and to exclude variations in fluorescence intensity by different volumes of injected dye.

In vitro fertilization

Frozen straws of semen from bulls, previously tested by IVF procedures, were thawed in water at 30°C for 30 s. The spermatozoa were layered on to a discontinuous (90/40: v/v) Percoll gradient and centrifuged at 180 g for 30 min. The resultant pellet of spermatozoa was washed twice in sperm-TALP medium by centrifugation at 180 g for 5 min. Finally, the preparation was adjusted to approximately 10 × 10⁶ spermatozoa ml⁻¹ and an equal volume of 200 µg heparin ml⁻¹ in sperm-TALP medium was added. After 3 h of incubation at 39°C, the sperm preparation was added to zona pellucida-free *in vitro* matured patch-clamped oocytes within a 2 ml wash-TALP bath. During the time course, > 50 control oocytes were fertilized and the resulting embryos were cultured according to Boni *et al.* (2002). The cleavage and blastocyst rates were always > 75% and > 20%, respectively.

Sperm factor

Bovine sperm factor was prepared according to Swann (1990) with some modifications. In brief, 18 frozen semen straws from eight different bulls, previously tested by IVF procedures, were thawed in water at 30°C. The spermatozoa were layered on to a discontinuous (90/40: v/v) Percoll gradient and centrifuged at 180 g for 30 min. The resultant pellet of spermatozoa was washed twice in sperm-TALP medium by centrifugation at 180 g for 5 min. Finally, the pellet was resuspended in 2 ml of intracellular-like medium (120 mmol KCl l⁻¹, 20 mmol Hepes l⁻¹, 500 µmol EGTA l⁻¹, 10 mmol glycerophosphate l⁻¹, 1 mmol dithiothreitol l⁻¹, 200 µmol phenylmethanesulfonyl fluoride l⁻¹, pH 7.5 and 300 mOsm) and mechanically homogenized for 20 min on ice. The cell rupture was monitored under a microscope. The resulting homogenate was ultracentrifuged at 180 000 g for 120 min. The supernatant was filtered through a 0.2 µm

pore-size filter, separated into aliquots and stored at -80°C until use. The sperm factor was injected into the oocytes as mentioned above. The sperm equivalent injected was estimated to be two to four.

Oocyte activation

Immature and *in vitro* matured bovine oocytes were exposed to Ca²⁺ ionophore A23187 (from 100 nmol l⁻¹ to 5 µmol l⁻¹) or 7% (v/v) ethanol. Ca²⁺ dependence of the ion currents triggered by these substances was tested by injecting the oocytes with the Ca²⁺ chelator EGTA at final concentration of 1 µmol l⁻¹ and loading for 10 min before exposure to activators. IP₃ and caffeine, which act through the IP₃ and ryanodine calcium release receptors were used to test further the calcium dependence of the ion activation current. IP₃ was dissolved in ICS and microinjected at a concentration ranging from 5 to 500 µmol l⁻¹. Caffeine was dissolved in Fert-TALP medium at 200 mmol l⁻¹ by heating at 70°C (Patel *et al.*, 1997) and added to the recording bath at a final concentration of 20 mmol l⁻¹. The effect of IP₃ and caffeine was evaluated by pre-loading the oocytes with specific inhibitors of the receptors, such as heparin (1 mg ml⁻¹) and procaine (50 µmol l⁻¹).

Finally, thapsigargin, a potent inhibitor of sarco/endoplasmic reticulum Ca²⁺-ATPase, was added to the bath at a final concentration of 10 µmol l⁻¹.

Pharmacological studies

The nature of the channels generating the ionophore-induced current was determined by incubating oocytes with some ion channel inhibitors: (i) inhibitors of voltage-gated K⁺ channels, such as tetraethylammonium chloride (TEA) (5–10 mmol l⁻¹ for 10–30 min) and DendrotoxinK (from 25 nmol l⁻¹ to 500 µmol l⁻¹ for 5 min); (ii) inhibitors of high, low and medium conductance Ca²⁺-activated K⁺ channels, such as riberiotoxin (from 100 nmol l⁻¹ to 2.5 µmol l⁻¹ for 2–5 min), Apamin (1 µmol l⁻¹ for 2–5 min) and rCharibdotxin (10–100 nmol l⁻¹ for 2–5 min), respectively; (iii) inhibitors of Cl⁻ channels, such as Clorotoxin (1 µmol l⁻¹ for 5–10 min) and 4,4'-diisocyanatostilbene-2,2'-disulphonic acid (DIDS) (from 100 µmol l⁻¹ to 1 mmol l⁻¹ for 2–5 min). All the above toxins were purchased from Alomone Labs (Jerusalem).

Statistical analysis

If not otherwise stated, a total of ten oocytes at each stage, distributed within at least three replications, was submitted for the following electrophysiological, calcium and pharmacological determinations. Differences between peak values of electrical currents and calcium dynamics generated by spermatozoa, sperm factor and oocyte activators were tested by ANOVA of the General Linear Model (GLM) procedure (SAS, 1988). Hyperpolarization was evaluated by normalizing the more negative resting potential observed at the peak current against the baseline resting potential and analysed by ANOVA.

Results

Electrical events

Oocytes matured *in vitro* were whole-cell voltage clamped at -30 mV. After the addition of heparin-capacitated bovine spermatozoa to the oocytes matured *in vitro*, a bell-shaped outward ion activation current was recorded 313 ± 80 s after the addition of spermatozoa. The time interval between the beginning of the current and its peak (PR) was 337 ± 35 s (Figs 1a and 2a). This fertilization current was accompanied by a progressive hyperpolarization of the plasma membrane resting potential that reached a maximum at the peak current (Fig. 2b) and progressively repolarized up to the resting values. This pattern was then used as a reference for the study of the various activation mechanisms examined. Microinjection of the bovine sperm factor soluble fraction generated an immediate ion activation current and hyperpolarization (Figs 1b and 2a,b). However, the dynamics of these electrical events were different from those evoked by the spermatozoon; sperm factor induced an ion activation current characterized by a faster slope than the spermatozoon (Fig. 1a,b), but with lower (2.3 versus 4.4 ; $P < 0.01$) hyperpolarization and higher ion activation current peak (504 versus 334 pA; $P < 0.05$) values (Fig. 2b).

All oocyte activators evoked electrical responses with specific dynamics. However, on the basis of their short PR none of these substances mimicked the sperm-induced ion activation current. Differences in peak values of these ion activation current and hyperpolarization were found among these activators (Fig. 2a,b). Ca^{2+} ionophore elicited the highest response at $5 \mu\text{mol l}^{-1}$ and a high response was maintained up to 100 nmol l^{-1} (data not shown).

Activation currents and hyperpolarization were also generated in the immature oocytes but with different dynamics from those of matured oocytes. The sperm penetration triggered a lower electrical current ($P < 0.01$) but of a similar duration to that of matured oocytes. This current was characterized by repetitive oscillations. Activation peak current induced by the sperm factor was significantly ($P < 0.01$) lower in immature oocytes than in oocytes matured *in vitro* (Fig. 2a); however, this difference was not significant in relation to hyperpolarization values (Fig. 2b). In immature oocytes, ethanol generated a lower ($P < 0.01$) ion activation current and hyperpolarization and a longer PR (148 ± 92 s versus 40 ± 22 s, $P < 0.01$) than in matured oocytes (Fig. 1c, 2a,b). In the case of Ca^{2+} ionophore, the amplitude of the ion activation current was significantly ($P < 0.01$) higher in the immature than in matured oocytes and showed an increased initial slope rate of the current (Figs 1d, 2a). In addition, in immature oocytes, thapsigargin showed a higher ion activation current ($P < 0.05$) and hyperpolarization ($P < 0.01$) than in matured oocytes (Figs 1e and 2a,b).

Exclusively for the Ca^{2+} ionophore treatment, ramps of 10 mV depolarizing and hyperpolarizing steps were applied from the voltage clamp of -30 mV, which generated a series

of whole cell currents. $I(V)$ curves were linear (data not shown). At the peak current, an increase of 17 nS (2.3 ± 0.6 -fold) and 9.1 nS (1.6 ± 0.3 -fold) steady state conductance was recorded in immature and in matured oocytes, respectively.

Controls performed at the beginning of each experiment using sham injections or the addition of the activator solvents at the concentrations used did not elicit any electrical event. In most of the electrical recordings, the agonist-induced activation ion currents were preceded by a transient inward current, accompanied by a depolarization of the resting potential. In immature oocytes the amplitude of this current was significantly higher compared with that of matured oocytes (146 ± 112 pA versus 55 ± 38 pA, $P < 0.05$). At times, this event did not occur, probably masked by the negative shift. The use of a Ca^{2+} free medium supplemented with 1 mmol EGTA l^{-1} inhibited these electrical events after Ca^{2+} ionophore treatment.

Effect of calcium on electrical events

Oocytes were injected with EGTA at a final concentration of 1 mmol l^{-1} , to investigate whether intracellular calcium is involved in the fertilization and activation currents in bovine oocytes. The subsequent addition of $5 \mu\text{mol Ca}^{2+}$ ionophore l^{-1} did not elicit an activation current or hyperpolarization in either immature or matured oocytes. Occasionally, after EGTA injection, a small inward current and a depolarization indicating a capacitive mechanism (Clapham, 1995) induced by depletion of the intracellular stores were observed.

Specific calcium releasers, such as IP_3 and caffeine, also triggered activation current and hyperpolarization. Microinjection of IP_3 (5 and $500 \mu\text{mol l}^{-1}$) induced a marked electrical response (Figs 1f and 2a,b). This current was recorded even when a lower IP_3 dose ($5 \mu\text{mol l}^{-1}$) was used and was characterized by high slope current. A significant ion activation current difference ($P < 0.01$) between immature and matured oocytes was found at both $5 \mu\text{mol l}^{-1}$ and $500 \mu\text{mol l}^{-1}$ (797 ± 292 pA versus 342 ± 69 pA and 1230 ± 507 pA versus 847 ± 419 pA, respectively). Caffeine (20 mmol l^{-1}) also generated ion activation current and hyperpolarization (Figs 1g and 2a,b) with higher values ($P < 0.01$) in immature oocytes.

The mechanisms underlying the Ca^{2+} release mobilized by IP_3 and caffeine were investigated by using antagonists of calcium release. The IP_3 generated current was abolished when oocytes were pre-injected with heparin. A previous injection of procaine resulted in the inhibition of caffeine-induced ion activation current and hyperpolarization. The addition of Ca^{2+} ionophore to heparin and procaine pre-loaded oocytes did not inhibit these electrical events.

Immediately after the closure of activation currents, the addition of Ca^{2+} ionophore to the same oocytes ($n = 5$ for each agonist used) did not cause any or caused minimal additional electrical events. Oocyte activation in Ca^{2+} -free medium did not affect either activation current or hyperpolarization.

Fig. 1. Typical traces of the activation current intensity in immature (black trace) and *in vitro* matured (grey trace) bovine oocytes that were whole-cell voltage clamped at -30 mV. The bovine oocytes were (a) exposed to bovine spermatozoa, (b) injected with bovine sperm factor, (c) exposed to 7% (v/v) ethanol, (d) exposed to $5 \mu\text{mol Ca}^{2+}$ ionophore, A23187 l^{-1} , (e) injected with $5 \mu\text{mol}$ 1,4,5-inositoltrisphosphate (IP_3) l^{-1} , (f) exposed to 20 mmol caffeine l^{-1} and (g) exposed to $10 \mu\text{mol}$ thapsigargin l^{-1} . Top scale bar refers to (a) and the lower scale bar refers to (b–g).

Calcium release

The patterns of the first Ca^{2+} transient release after exposure of the oocytes to the ion activation current inducers (Fig. 3) reflect the electrical activation curves and confirm higher values in immature oocytes after exposure to Ca^{2+} ionophore, thapsigargin, IP_3 and caffeine (Fig. 3d–g).

However, spermatozoa, sperm factor and ethanol showed higher values in oocytes matured *in vitro* (Fig. 3a,b,c). Calcium curves were longer and more persistent than the electrical curves (Figs 1 and 3). IP_3 injection resulted in the highest peak in both immature and matured oocytes; in immature oocytes the peak occurred within 3 s after the

Fig. 2. (a) Activation current intensities (mean $\pm$ SEM) and (b) hyperpolarizations (mean $\pm$ SEM) expressed as the ratio of the peak (mV) and the resting value (mV) recorded in immature (■) and *in vitro* matured (□) bovine oocytes that were voltage clamped at -30 mV and exposed to bovine spermatozoa (sperm), 7% (v/v) ethanol (ET-OH), 5 μ M Ca^{2+} ionophore (A23187) I^{-1} , 20 mmol caffeine I^{-1} , 10 μ M thapsigargin (Thap) I^{-1} or injected with bovine sperm factor (SF) and 5 μ M 1,4,5-inositoltrisphosphate (IP_3) I^{-1} . (c) Intracellular calcium variations (mean $\pm$ SEM) in immature (■) and *in vitro* matured (□) bovine oocytes injected with calcium green dextran (10 000 M_r) after exposure to bovine spermatozoa (sperm), 7% (v/v) ethanol (ET-OH), 5 μ M Ca^{2+} ionophore (A23187) I^{-1} , 20 mmol caffeine I^{-1} and 10 μ M thapsigargin (Thap) I^{-1} or injection with bovine sperm factor (SF) and 5 μ M 1,4,5-inositoltrisphosphate (IP_3) I^{-1} . Data were expressed as variations of the relative fluorescence intensity (RFI). Significant difference between immature and *in vitro* matured bovine oocytes: $^{A,B}P < 0.01$; $^{a,b}P < 0.05$.

Fig. 3. Typical traces of the intracellular calcium release response in immature (black trace) and *in vitro* matured (grey trace) bovine oocytes that were pre-injected with the fluorescence dye, calcium green dextran 10 000. An increase in calcium was elicited by (a) exposure to bovine spermatozoa, (b) injection with bovine sperm factor, (c) exposure to 7% (v/v) ethanol, (d) exposure to 5 μmol Ca^{2+} ionophore, A23187 l^{-1} , (e) injection with 5 μmol 1,4,5-inositoltrisphosphate (IP_3) l^{-1} , (f) exposure to 20 mmol caffeine l^{-1} and (g) exposure to 10 μmol thapsigargin l^{-1} . Data were expressed as variations of the relative fluorescence intensity. Top scale bar refers to (a) and the lower scale bar refers to (b–g).

injection and quickly decreased up to the baseline value (Fig. 3f).

Ion channel characterization

Voltage-gated K^+ channel inhibitors, such as TEA and dendrotoxin, were ineffective in blocking the ion activation current and the hyperpolarization induced by the Ca^{2+} ionophore. Among the inhibitors of Ca^{2+} -activated K^+ channels, only incubation with rIberiotoxin at 100 nmol l^{-1} for 2 min caused a complete inhibition in 27% of the immature oocytes and 34% in the matured oocytes, whereas a reduction of the peak amplitude was observed in 47% of the immature oocytes and 24% of the matured oocytes ($n = 12$ for both the stages). Pre-incubation of the oocytes with Cl^- channels blockers, such as clorotoxin and DIDS, before the Ca^{2+} ionophore treatment, did not inhibit the electrical events.

Discussion

The interaction of the spermatozoon with the oocyte causes a series of physiological changes in the oocyte known as activation. The present study demonstrated that bovine oocytes matured *in vitro* that were exposed to spermatozoa undergo a bell-shaped outward ion current and hyperpolarization of the plasma membrane potential. Different substances acting as oocyte activators evoke similar electrical events, and in both immature and matured oocytes, these events are calcium dependent.

Modifications of the resting potential of the oocyte plasma membrane are described at fertilization in several mammalian species. In hamster and mouse oocytes, repetitive hyperpolarizing responses (Igusa *et al.*, 1983) are described, whereas in rabbits, the spermatozoon causes a small depolarization of resting potential (McCulloh *et al.*, 1983). In the human oocyte, hyperpolarization occurs as a unique, long lasting event (Gianaroli *et al.*, 1994). Ion currents associated with the hyperpolarization have been recorded so far only in human oocytes (Gianaroli *et al.*, 1994). This current showed similar peak amplitude with respect to the activation currents observed in bovine oocytes in the present study, but with a different time course (120 and 20 min in humans and cows, respectively). In the immature oocyte, the activation current showed a lower response compared with that of matured oocytes. This finding indicates that although sensitive to sperm action, the immature oocyte has not yet developed the necessary processes for fertilization. Moreover, the long duration of the ion activation current in the immature oocyte may be attributed to polyspermy due to the lack of cortical granule migration under the plasma membrane, a typical event of maturation (Hosoe and Shioya, 1997). This pattern has not been recorded in other species and highlights the difference between immature and matured oocytes.

The injection of a soluble sperm fraction into the immature and *in vitro* matured bovine oocytes triggered an

outward current and hyperpolarization similar to that generated by the spermatozoon although with a shorter time course. Several authors (Dale *et al.*, 1985; Stice and Robl, 1990; Homa and Swann, 1994; Wu *et al.*, 1997; Lee *et al.*, 2001; Okitsu *et al.*, 2001) described activation events after the injection of either homologous or heterologous sperm extracts into oocytes of different species. Electrical currents elicited by sperm factor were only described in human oocytes showing differences in time course and peak amplitude compared with the sperm-induced ion current (Dale *et al.*, 1996). Data from the present study are in agreement with this finding and show that sperm factor induces outward current for a shorter duration than at fertilization and with a higher peak amplitude. In addition, this finding further supports the hypothesis that a cytosolic sperm component is involved in triggering the activation events associated with fertilization in bovine oocytes, as described by Damiani *et al.* (1996). The higher electrical events induced by spermatozoa and sperm factor in matured oocytes indicates activation competence at maturation.

It is well known that oocyte activation may be induced by chemical substances (Tarkowski, 1975; Whittingham, 1980). Artificial stimuli cause a variety of electrical responses, indicating the presence of different mechanisms mobilized at activation (Eusebi and Siracusa, 1983). Bovine oocytes undergo embryo development up to the blastocyst stage after Ca^{2+} ionophore or ethanol treatment (Wang *et al.*, 1999) with the same efficiency yielded by IVF (Boni *et al.*, 2002). However, parthenogenetic embryos show different characteristics from IVF embryos (Winger *et al.*, 1997) and fail to generate viable offspring (Fukui *et al.*, 1992). In the present study, outward currents and hyperpolarization were also generated by treating oocytes with Ca^{2+} ionophore and ethanol. Although these substances activate the oocyte with a similar efficiency (Liu *et al.*, 1998), they induce different electrical responses in immature and matured oocytes. In fact, matured oocytes were very sensitive to ethanol and generated an activation current and hyperpolarization significantly higher and with a PR shorter than in immature oocytes. The opposite pattern is found when Ca^{2+} ionophore as well as the other Ca^{2+} releasers were used. After exposure to spermatozoa, sperm factor and ethanol, the matured oocytes showed a higher reaction than immature oocytes, whereas the opposite has been recorded in the case of calcium releasers.

The increase in intracellular free calcium is universally recognized to be necessary for oocyte activation (Homa *et al.*, 1993; Whitaker and Swann, 1993; Carroll *et al.*, 1996). Single or repetitive calcium transients occur at fertilization in all species studied (Stricker, 1999). The fertilization current is calcium independent in tunicates (Dale, 1987) and calcium dependent in sea urchins (De Simone *et al.*, 1998). In mammals, electrical activation events are due to K^+ conductance, which is dependent on calcium release in hamsters (Igusa and Miyazaki, 1983) and humans (Gianaroli *et al.*, 1994), and calcium independent in mice (Igusa *et al.*,

1983). In the present study, all activators increased internal Ca^{2+} . In addition, many similarities were found between the amplitudes of electrical currents and the dynamics of the increase in calcium either among the activators used or between mature and immature oocytes. However, comparison between these two dynamics is due to physiological (intracellular free-calcium mobilization versus membrane channel gating) and technical reasons such as buffering of calcium by the dye. In mammalian models, the ability of the oocyte to release Ca^{2+} after fertilization increases during maturation, which also occurs when calcium releasers are used (for a review, see Carroll *et al.*, 1996). The first transient is of higher amplitude and is longer lasting in mature oocytes. In addition, the number of transients generated after oocyte activation markedly increases in mature oocytes. This increase is related to the increase of the intracellular calcium stores during maturation (Jones *et al.*, 1995). In bovine oocytes, Boni *et al.* (2002) demonstrated that calcium stores decrease during maturation and are related to oocyte quality. In the present study, Ca^{2+} release after oocyte activation was demonstrated to be related to various activating stimuli rather than the store *per se*. This finding is consistent with the oocyte electrical response under the same stimulation. Different responses to activating stimuli may be attributed to the high selectivity of calcium releasers with respect to spermatozoa, sperm factor and ethanol, as these factors trigger higher but shorter responses. However, consistent with the results of the present study, in other species it has been shown that there is: (i) a higher electrical response to spermatozoa and sperm factor with respect to calcium releasers (Lee *et al.*, 2001) and (ii) calcium releasers cannot induce repetitive oscillations, such as those triggered by spermatozoa and sperm factor (Carroll *et al.*, 1994; Parrington *et al.*, 1999).

In bovine oocytes, multiple calcium transients follow fertilization (Sun *et al.*, 1994). The first transient was observed 4 h after insemination, a period necessary for penetration of the zona pellucida. The present study used zona pellucida-free matured oocytes and a marked shortening of gamete interaction time was observed, as an increase in calcium occurred 1 min after addition of spermatozoa and reached its maximum peak after 12 min. In immature oocytes, an increase in calcium did not differ with respect to matured oocytes. The discrepancy between this finding and the results after the electrical stimulation may be attributed to: (i) the subsequent fusion of surplus spermatozoa due to removal of the zona pellucida causing repetitive calcium release and (ii) the dissociation constant (K_d) of our fluorochrome which may have transformed repetitive sperm entry in a cumulative calcium response. Injection of sperm factor into oocytes has been shown to elicit repetitive Ca^{2+} increase in many mammalian species (Swann, 1990; Homa and Swann, 1994; Wu *et al.*, 1997). In the present study, it was shown that injection of bovine sperm factor into bovine oocytes induces a Ca^{2+} increase in immature and matured oocytes with a pattern consistent with electrical currents. In addition, ethanol induced an increase in calcium, as

already observed (Cuthbertson, 1983) in both mature and immature mouse oocytes with a pattern resembling electrical currents. Taken together, these data clearly indicate a relationship between calcium increase and electrical currents.

Preloading oocytes with EGTA inhibited the electrical events indicating calcium dependence of activation currents. Ca^{2+} releasers were tested to evaluate this dependence further. The use of Ca^{2+} ionophore and thapsigargin elicited either activation current and hyperpolarization or Ca^{2+} release; the dynamics of these events differed among activators and between immature and matured oocytes. The type of Ca^{2+} stores involved in oocyte activation mechanisms was characterized using IP_3 and caffeine (Berridge, 1993). The use of these two Ca^{2+} releasers evoked a high response in terms of activation current and hyperpolarization, as well as Ca^{2+} release. It is noticeable that so far IP_3 -induced fertilization-like potential has been described in non-mammalian oocytes (Kline and Nuccitelli, 1985; Slack *et al.*, 1986; Dale, 1988) and in human oocytes (Gianaroli *et al.*, 1994). The present study describes for the first time that caffeine induces an ion activation current in mammalian oocytes.

Preloading oocytes with inhibitors of both receptors (heparin and procaine, respectively) abolished electrical responses, demonstrating the activity of both receptors in immature and *in vitro* matured oocytes. This finding is in agreement with results of Yue *et al.* (1995) who showed the independent action of IP_3 and ryanodine receptors in matured bovine oocytes. In the present study, IP_3 and caffeine evoked a higher response in terms of activation current and Ca^{2+} release in immature oocytes. This finding is in contrast to a study by Damiani *et al.* (1996) who showed the inability of bovine immature oocytes to respond to low IP_3 dosages. It is difficult to explain the differences in the results, but as the present study used the same IP_3 dosages (5 and 500 $\mu\text{mol l}^{-1}$) as those used by Damiani *et al.* (1996), it is likely that the oocyte quality chosen in the present study may have affected the results (Boni *et al.*, 2002). In bovine oocytes, it has been demonstrated that Ca^{2+} increase at fertilization is an IP_3 independent mechanism (Sun *et al.*, 1994); however, results from the present study indicate that oocyte activation mechanisms cannot be attributed entirely to a single and known receptor pathway. In fact, heparin and procaine, injected separately into the oocyte, did not inhibit the Ca^{2+} ionophore-induced activation events. This finding indicates that Ca^{2+} ionophore activation is mediated by unspecific calcium release or by a pathway involving calcium release through ryanodine and IP_3 receptor synergetic co-operation.

The origin of calcium mobilization in the oocyte differs among species (Igusa and Miyazaki, 1983; Kline and Kline, 1992; Wang *et al.*, 1999). In bovine oocytes, a calcium influx through oocyte plasma membrane channels seems to be unnecessary for Ca^{2+} increase, as it does not differ in calcium-free medium. In addition, activation current and hyperpolarization do not differ under these conditions and

this confirms their dependence by intracellular calcium mobilization. However, the inability to regenerate a second set of ionophore-induced ionic events after the activation currents supports the hypothesis that a desensitization or transient depletion of calcium stores affects their capacity to be activated by Ca^{2+} ionophore.

A common characteristic of the activation current in most of the experiments performed was an initial inward current which corresponded to a small depolarization. The use of Ca^{2+} -free medium abolished the initial inward current without affecting the subsequent activation current dynamics. This finding indicates that the electrical events at oocyte activation begin with an initial external calcium entry. The higher values found in immature oocytes further sustain this hypothesis on the basis of our previous study demonstrating a prevalence of plasma membrane calcium currents in immature oocytes (Tosti *et al.*, 2000). Finally, the role of this first calcium entry does not seem crucial for oocyte activation as the oocyte is also activated in Ca^{2+} -free medium.

The activation current and hyperpolarization observed in the present study indicate the involvement of K^+ channels in oocyte activation. In a previous study, it was found that K^+ permeability in the bovine oocyte was high at the meiotic block stages, that is, in immature and matured oocytes (Tosti *et al.*, 2000). Both the fertilization potential and fertilization current have been related to Ca^{2+} -activated K^+ currents in hamsters and humans (Miyazaki and Igusa, 1982; Dale *et al.*, 1996). Ca^{2+} -activated K^+ channels are ubiquitous of different conductance channels (Latorre *et al.*, 1989). They are known to link membrane excitability and cell metabolism (Behrens *et al.*, 1988; Latorre *et al.*, 1989). On the basis of the calcium dependence of activation currents in the present study, several inhibitors of both K^+ - and Ca^{2+} -activated K^+ channels were screened and activation current and hyperpolarization were inhibited only by iberiotoxin, a specific blocker of high conductance Ca^{2+} -activated K^+ channels (Candia *et al.*, 1992). In addition, a possible role of Ca^{2+} -activated Cl^- channels that are involved in the activation current of amphibians (Jaffe and Schlichter, 1985; Kline and Nuccitelli, 1985) and mammals (Eusebi and Siracusa, 1983) was excluded. On this basis, the first electrical event in bovine oocyte activation appears to be the gating of Ca^{2+} -activated K^+ channels. This finding may also explain why in matured oocytes ethanol induced activation events in a similar way to spermatozoa and sperm factor, as ethanol increases the activity of large conductance Ca^{2+} -activated K^+ channels (Dopico *et al.*, 1996).

In conclusion, the present study has shown that fertilization and parthenogenetic activation in bovine oocytes elicit plasma membrane outward currents and hyperpolarization of membrane potential. Both of these events are dependent on internal calcium release and are mediated by high conductance Ca^{2+} -activated K^+ channels. IP_3 and ryanodine receptors are active in both the immature and *in vitro* matured oocytes, but none of these factors is responsible for the activation current. Immature oocytes are sensitive to all

activators used, showing different responses with respect to matured oocytes. *In vitro* matured oocytes showed a higher electrical and calcium response after exposure to spermatozoa, sperm factor and ethanol compared with immature oocytes but a lower response to calcium releasers. This finding indicates that during maturation the oocyte acquires the competence to react to different activation stimuli with different dynamics.

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