The extracellular calcium-sensing receptor is expressed in the cumulus–oocyte complex in mammals and modulates oocyte meiotic maturation

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Abstract

The extracellular calcium-sensing receptor (CASR) plays an important role in cells involved in calcium (Ca^{2+}) homeostasis by directly sensing changes in the extracellular Ca^{2+} ion concentration. We previously reported the localization and quantitative expression of CASR protein in human oocytes. In this study, we examined the expression and the functional role of CASR during oocyte meiotic maturation in a large mammal animal model, the horse. As in humans, CASR protein was found to be expressed in equine oocytes and cumulus cells. Western-blot analysis revealed a single 130 kDa band in denuded oocytes and a doublet of 130–120 kDa in cumulus cells. CASR labeling was observed by confocal microscopy in cumulus cells and in oocytes on the plasma membrane and within the cytoplasm at all examined stages of meiosis. Functionally, the CASR allosteric effector NPS R-467, in the presence of 2.92 mM external Ca^{2+} , increased oocyte maturation rate in a dose-dependent manner and its stimulatory effect was attenuated by pre-treatment with the CASR antagonist NPS 2390. NPS R-467 had no effect in suboptimal external Ca^{2+} (0.5 mM), indicating that it requires higher external Ca^{2+} to promote oocyte maturation. In oocytes treated with NPS R-467, CASR staining increased at the plasmalemma and was reduced in the cytosol. Moreover, NPS R-467 increased the activity of MAPK, also called ERK, in cumulus cells and oocytes. These results provide evidence of a novel signal transduction pathway modulating oocyte meiotic maturation in mammals in addition to the well-known systemic hormones.

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Introduction

The extracellular calcium-sensing receptor (CASR; also called CaSR, CaR) plays a key role in the regulation of whole-body calcium (Ca^{2+}) metabolism (for review, see Brown & MacLeod (2001), Chang & Shoback (2004) and Chen & Goodman (2004)). The molecular identification of CASR in bovine parathyroid cells by Brown *et al.* (1993) opened up the possibility that Ca^{2+} can be considered as a first messenger outside cells (Brown *et al.* 1993, 1995). The CASR was originally named for its ability to detect/transduce subtle but physiologically meaningful changes in external Ca^{2+} concentration. However, it responds to many other bivalent and trivalent cations, to changes in other physiological parameters such as L-amino acids, polyamines, ionic strength, pH, and to drugs such as aminoglycosidic antibiotics, calcimimetics, and calcilytics (review by Brown & MacLeod (2001), Hofer & Brown (2003) and Messa *et al.* (2006)). CASR-null mice exhibit loss of feedback control of parathyroid hormone

secretion, hyperparathyroidism, and metabolic bone diseases. Moreover, inactivating and activating mutations of the receptor in humans have been shown to induce various disorders of Ca^{2+} metabolism (for review see Hofer & Brown (2003)).

CASR has been reported to be widely expressed in several mammalian tissues, including tissues that are not clearly involved in Ca^{2+} metabolism such as brain, lens epithelial cells, pituitary gland, bone marrow, peripheral blood, breast ductal cells, pancreas, keratinocytes, ovarian surface epithelial cells, and the gastrointestinal system (reviewed by Brown *et al.* (1993), McNeil *et al.* (1998a), Hofer *et al.* (2000), Brown & MacLeod (2001) and Hofer & Brown (2003)). CASR localization in cells that seem to have no functional relationship with the maintenance of the whole-body Ca^{2+} balance has been linked to the regulation of different cellular processes, such as secretion, chemotaxis, apoptosis, cell proliferation, differentiation, and ion channel activity (for review see Hofer & Brown (2003)).

Although, in a previous study, we reported the subcellular localization and quantitative expression of CASR in human oocytes and how these change with meiotic maturation (Dell'Aquila *et al.* 2006), to date no data are available on the possible functional role of CASR in oocytes of any other species. The hypothesis of a possible involvement of CASR in oocyte maturation is supported by several studies reporting the Ca^{2+} ion as a fundamental messenger for oocyte maturation (Homa 1995, He *et al.* 1997). The rise in cytosolic Ca^{2+} in immature oocytes (germinal vesicle (GV) stage) following LH and/or growth factor exposure is well known (Mattioli *et al.* 1998, Hill *et al.* 1999). Moreover, the additional role of external Ca^{2+} in mobilization of intracellular Ca^{2+} during oocyte maturation, activation, and fertilization has been described (review by Tosti & Boni (2004)).

Large domestic animals such as cows, pigs, sheep, and horses are widely used as animal models for investigations in reproductive biology due to their economic interest and to the wide availability of their biological materials. Among these, the horse has been previously used as a model to investigate oocyte physiology due to its particularly large follicle size, which allows the possibility to relate oocyte meiotic and developmental competence with biochemical and molecular features of corresponding follicle cells (Goudet *et al.* 1997, 1999, Hinrichs & Schmidt 2000, Hinrichs *et al.* 2005, Dell'Aquila *et al.* 2003, 2004, 2008).

The purpose of the present study was to clarify the expression and the physiological function of CASR in the cumulus–oocyte complex (COC) of a large mammal animal model, the horse. The expression study was conducted at both the mRNA and protein levels. Functional studies were performed by testing the effects of a CASR agonist, NPS R-467, and an antagonist, NPS 2390, in experiments of *in vitro* maturation (IVM). The involvement of the MAPK 3/1 (ERK 1/2) kinases in the signaling induced by CASR activation has been also evaluated, given the fact that MAP kinase activation is a signal of intracytoplasmic maturation in the oocyte (Fissore *et al.* 1996, He *et al.* 1997, Motlik *et al.* 1998, Kubelka *et al.* 2000, Hengyu *et al.* 2002, Petrunewich *et al.* 2009) that is activated by calcium increases (He *et al.* 1997). We found that CASR is expressed and activates MAPK 3/1 in the COC and it modulates oocyte meiotic maturation.

Results

Expression of CASR in the equine COC

At the beginning of the study, the expression of CASR in equine COCs was investigated. The presence of the CASR transcript, the biochemical characterization, and the subcellular localization of CASR protein in

equine-denuded oocytes and cumulus cells were analyzed. For the molecular study, groups of 10 immature oocytes at collection and 30 cumuli oophori from COCs following IVM were used. For the protein study in samples analyzed just after retrieval, the ovaries of 12 mares were processed, 70 follicles were scraped, 44 COCs (20 compact (Cp) and 24 expanded (Exp)) were recovered (1.8 oocytes/ovary; 63%, no. of recovered oocytes/no. of scraped follicles) and analyzed, 20 of which (10 Cp and 10 Exp) were used for western-blot analysis and 20 (10 Cp and 10 Exp) underwent immunofluorescence and CLSM. The remaining four Exp oocytes were discarded for signs of degeneration.

CASR transcript detection in oocytes and cumulus cells

RT-PCR using primers targeting CASR amplified a fragment of expected length (202 bp) from denuded oocytes and cumulus cells (Fig. 1). The exogenous luciferase control was detected in both denuded oocytes and cumulus cells (data not shown).

Immuno-identification of CASR protein in oocytes and cumuli oophori

To determine whether CASR protein is expressed in both the germinal and somatic compartments of the equine COC, total cell extracts were prepared separately from denuded oocytes and their corresponding cumuli oophori ($n=10$). In denuded oocytes, a single 130 kDa protein band was found by immunoblotting (Fig. 2, lane A) that represents a glycosylated form of CASR protein (Bai *et al.* 1996) while in cumulus cells a doublet of 130–120 kDa was observed (Fig. 2, lane B), corresponding to a glycosylated and the non-glycosylated form of the CASR protein.

Figure 1 Detection of CASR transcript in denuded oocytes (lane B) and cumulus cells (lane C). Analysis onto agarose gel of fragments generated by RT-PCR. (lane A) Negative control.

Figure 2 Identification of CASR in equine oocytes (lane A) and cumuli oophori (lane B) by western-blot analysis. The protein equivalent of ten denuded oocytes and corresponding cumuli oophori were loaded. The proteins were subjected to SDS-PAGE on a linear gradient running gel of 4–12%. The CASR protein was detected with a primary antibody against a 20 amino acid peptide sequence near the C-terminus of human CASR. Denuded oocytes showed a single 130 kDa band corresponding to a glycosylated form of CASR. The doublet seen in cumulus cells corresponds to the non-glycosylated 120 kDa form and to the glycosylated 130 kDa form of CASR.

Subcellular localization of CASR protein in oocytes and cumuli oophori examined at recovery time

Twenty oocytes, ten with Cp cumulus and ten with Exp cumulus, were used for immunofluorescence detection of CASR expression in oocytes examined just after retrieval. As can be seen in Fig. 3, CASR was found to be expressed (green labeling) in oocytes at the GV stage and in corona radiata cells. Figure 3A, A1, and A2 show a representative GV stage oocyte originally recovered with Cp cumulus. In this sample (Fig. 3A2) at the equatorial plane, marked staining in light green can be seen with an ubiquitous distribution within the cytoplasm but lower intensity along the oolemma. Figure 3A2 also shows corona radiata cells on the oocyte surface that had still persisted after denuding and zona pellucida permeabilization. Notwithstanding the differences in size between the oocyte and corona cells, plasma membranes and cytoplasm of corona cells and their transzonal cytoplasmic processes were observed to be also stained in green (Supplementary Fig. 1, which can be viewed online at www.reproduction-online.org/supplemental/). No staining was detected in the negative controls (Fig. 3B2, D2, and F2).

Dose–response effect of the CASR agonist NPS R-467 on oocyte nuclear maturation in the presence of 2.92 mM external calcium

In six consecutive experimental trials, the ovaries of 29 mares were retrieved and processed. The scraping procedure was carried out on 300 follicles from which 180 oocytes were recovered (3.1 oocytes/ovary; 60%, no. of recovered oocytes/no. of scraped follicles). For this experiment, 176 oocytes (98%) were used, the other 4 oocytes recovered with partial or degenerated cumulus and/or degenerated cytoplasm were discarded. At the end of denuding, fixing, and staining procedures, 147 (84%, percentage of cultured) were evaluated for

nuclear chromatin configuration, 87 of which with Cp and 82 with Exp cumulus. The remaining seven oocytes were lost or damaged during handling, fixing and staining procedures. The effects of NPS R-467 were tested at the concentration of 0.5, 5, and 10 μ M. NPS R-467, when used at the concentration of 5 μ M, significantly increased the maturation rate of Exp oocytes ($P < 0.05$; Table 1) whereas no effects at 0.5 and 10 μ M

Figure 3 Detection of CASR expression in equine oocytes at different meiotic maturation stages by immunofluorescence with a primary antibody against a 20 amino acid peptide sequence near the C-terminus of human CASR. For each lane, corresponding bright-field, u.v. light, and confocal images of the same oocyte are shown. Confocal laser-scanning microscopy optical sections show the positive labeling for CASR in GV (A2), MI (C2), and MII (E2) oocytes and corona radiata cells. Negative controls (B, D, and F). Scale bar represents 60 μ m.

were noticed. In relation to this finding, next experiments were performed in the presence of 5 μ M NPS R-467. No effect was observed on Cp oocytes at any NPS R-467 concentration.

Effects of IVM culture and dose–response effects of NPS R-467 on CASR expression and subcellular localization in the COC

In three trials of the dose–response experiment, at the end of IVM culture, oocytes ($n=46$ Cp and 28 Exp) underwent immunofluorescence detection of CASR as described above in order to evaluate: 1) CASR expression in later stages of meiotic development (MI and MII + PB), and 2) whether there is any effect of increasing doses of the CASR agonist NPS R-467 on CASR protein expression and subcellular localization in the COC. In Fig. 3C2 and E2, at the equatorial plane, marked staining in light green can be seen with an ubiquitous distribution along the oolemma and within the cytoplasm in two Cp cumulus oocytes at MI and MII stages respectively. No staining was detected in the negative corresponding controls (Fig. 3D2 and F2). Out of 46 Cp oocytes, 26 (21 MII and 5 MI) were used for CASR analysis; the remaining 20 oocytes could not be analyzed since 7 of them were blocked at the GV stage and 13 had abnormal chromatin configuration. Out of 21 MII oocytes, 4, 2, 9, and 6 oocytes were obtained and analyzed after culture respectively in control conditions (no NPS R-467) or in the presence of 0.5, 5, or 10 μ M NPS R-467. Out of 28 Exp oocytes, 26 (21 MII and 5 MI) were used for CASR analysis; the remaining 2 oocytes had abnormal chromatin configuration and were not analyzed. Out of 21 MII oocytes, 1, 4, 7, and 9 oocytes were obtained and analyzed respectively after IVM in control conditions or in the presence of 0.5, 5, or 10 μ M NPS R-467. Figure 4 shows representative Cp (A, B, C, and D) and Exp (E, F, G, and H) MII oocytes observed after culture in the presence of increasing doses of NPS R-467. It can be seen that, in

Cp oocytes, CASR protein expression and localization were not dependent on NPS R-467 addition and concentration whereas, in Exp oocytes, the exposure to 0.5 and 5 μ M NPS R-467 induced marked CASR expression on the plasma membrane (Fig. 4 and Supplementary Fig. 2, which can be viewed online at www.reproduction-online.org/supplemental/).

In order to better determine the subcellular distribution of CASR, oocytes were examined in confocal microscopy in a series of focal planes, thus providing information on the three-dimensional distribution of CASR protein. Figure 5 and the Supplementary Video, which can be viewed online at www.reproduction-online.org/supplemental/, show a representative 25 optical plane analysis of a metaphase II Exp oocyte obtained after culture in the presence of NPS R-467 (0.5 μ M). In surface planes, in the lower (planes nos 1–7) and upper (planes nos 19–25) parts of the oocyte, CASR labeling in clumps/clusters can be seen while in planes nearer the equatorial position (planes nos 8–18), uniform distribution of CASR staining within the cytoplasm is observed together with an intense fluorescence beneath the oolemma evident in planes nos 12–18).

Antagonistic effect of NPS 2390 on NPS R-467-induced stimulation of oocyte maturation

For the functional study, we tested the effects of the synthetic CASR agonist NPS R-467. This compound was used at the concentration of 5 μ M as in previous studies performed in bovine parathyroid cells (Nemeth *et al.* 1998) and in human embryonic kidney (HEK 293) cells stably expressing CASR (Mun *et al.* 2004). The ovaries of 60 mares were retrieved and processed. The scraping procedure was carried out on 363 follicles from which 251 oocytes were recovered (two oocytes/ovary; 67%, nos recovered oocytes/nos scraped follicles). Two hundred and thirty-nine oocytes (96%, percentage of

Table 1 Dose–response effect of the calcium-sensing receptor (CASR) agonist NPS R-467 on oocyte nuclear maturation in the presence of 2.92 mM external Ca^{2+} .

NPS R-467 concentration (μM)	Cumulus morphology at retrieval	No. of examined oocytes	No. of oocytes showing (%)			
			Germinal vesicle	Metaphase I to telophase I	Metaphase II with 1° polar body	Abnormal chromatin
	Compact					
0		32	1 (3)	5 (16)	16 (50)	10 (31)
0.5		17	2 (12)	5 (29)	3 (18)	7 (41)
5		16	3 (19)	2 (12.5)	9 (56)	2 (12.5)
10		22	5 (23)	1 (4.5)	12 (54.5)	4 (18)
	Expanded					
0		16	1 (6)	3 (19)	6 (37.5)*	6 (37.5)
0.5		22	2 (9)	8 (36.5)	10 (45.5)	2 (9)
5		22	2 (9)	2 (9)	16 (73) [†]	2 (9)
10		22	2 (9)	5 (23)	14 (64)	1 (4)

χ^2 -test with the Yates correction for continuity and Fisher's exact test: *,[†] $P<0.05$.

Figure 4 Dose-response effects of the CASR agonist NPS R-467 on CASR subcellular localization in equine oocytes at the metaphase II stage after *in vitro* culture. In Cp oocytes, CASR protein fluorescent-labeling distribution and intensity does not change upon exposure of oocytes to increasing doses of NPS R-467. In Exp oocytes, marked expression of CASR in the pericortical subplasmalemmal region in samples exposed to 0.5 (F) and 5 μ M (G) NPS R-467 can be seen (arrows). Scale bar represents 60 μ m.

recovered) were selected and used for *in vitro* culture. The remaining 12 oocytes showing degenerated cumulus or cytoplasm were discarded. One hundred and seventy-four oocytes (73%, percentage of cultured) were evaluated. The remaining 65 oocytes were lost or damaged during handling, fixing, or staining procedures. NPS R-467 significantly increased the maturation rate of Exp oocytes (23 out of 31, 74% vs 8 out of 20, 40%, $P < 0.04$; Table 2). In the same experiment, we examined whether the CASR antagonist NPS 2390 could reverse

the stimulatory effect of NPS R-467 on IVM of equine oocytes. After 1 h pre-incubation in medium containing 10 μ M NPS 2390, oocytes were cultured for 26–28 h in the presence of 5 μ M NPS R-467. The pre-incubation with NPS 2390 significantly reduced the stimulatory effect of NPS R-467 on Exp oocytes (12/27 44% vs 23/31, 74%; $P < 0.04$). No effect of used compounds on Cp oocytes was observed (Table 1). These data suggest that CASR is functionally active in meiotically competent oocytes.

Effects of the CASR agonist NPS R-467 on oocyte meiotic maturation in the presence of suboptimal extracellular calcium concentration

After observing the agonist effect of NPS-R-467 on oocyte maturation in culture medium containing 2.92 mM Ca^{2+} , it has been considered of interest to investigate whether the stimulatory effect of NPS-R-467 is maintained at a suboptimal extracellular Ca^{2+} concentration (0.5 mM). For this purpose, the oocytes recovered from the ovaries of 33 mares were retrieved and processed in four consecutive trials. The scraping procedure was carried out on 366 follicles from which 142 oocytes were recovered (2.1 oocytes/ovary; 38%, no of recovered oocytes/no. of scraped follicles), selected,

Figure 5 CASR localization and distribution in 25 serial optical sections (step size of 0.45 μ m) in an equine Exp MII stage oocyte obtained after culture in the presence of 0.5 μ M NPS R-467. In surface planes, in the lower (planes nos 1–5) and upper (planes nos 19–25) parts of the oocyte, a labeling in clumps/clusters can be seen; while in planes nearer the equatorial position (planes nos 6–18), an intense fluorescence is observed beneath the oolemma together with a uniform distribution of CASR staining within the cytoplasm. Scale bar represents 60 μ m.

Table 2 Antagonistic effect of NPS 2390 on the NPS R-467-induced stimulation of oocyte nuclear maturation.

Culture conditions	Cumulus morphology	No. of examined oocytes	No. of oocytes showing (%)			
			Germinal vesicle	Metaphase I to telophase I	Metaphase II with 1° polar body	Abnormal chromatin
	Compact					
Controls		26	7 (27)	5 (19)	11 (42)	3 (12)
5 µM NPS R-467		40	11 (27)	3 (8)	17 (43)	9 (22)
10 µM NPS 2390 (1 h) + 5 µM NPS R-467		30	10 (33)	3 (10)	12 (40)	5 (17)
	Expanded					
Controls		20	5 (25)	3 (15)	8 (40)*	4 (20)
5 µM NPS R-467		31	4 (13)	2 (6)	23 (74)†	2 (7)
10 µM NPS 2390 (1 h) + 5 µM NPS R-467		27	4 (15)	4 (15)	12 (44)*	7 (26)

χ^2 -test with the Yates correction for continuity and Fisher's exact test: * $P < 0.05$

and used for IVM. One hundred and thirty-seven oocytes (96%, percentage of cultured) were evaluated, the remaining five oocytes were lost or damaged during handling, fixing, or staining procedures. Interestingly, it was observed that NPS R-467 at 5 µM had no effect on oocyte nuclear maturation when added in the presence of suboptimal external Ca^{2+} concentration (0.5 mM Ca^{2+} ; Table 3), indicating that NPS R-467 requires the presence of higher external calcium (2.92 mM, Table 1) to activate CASR. In the same trials, we tried to test whether increasing extracellular Ca^{2+} (4 mM) *per se* stimulates meiotic maturation. Interestingly, it was observed that increased Ca^{2+} concentrations had no effect on oocyte nuclear maturation (7 out of 17, 41% in the presence of high calcium versus 5 out of 14, 36% in controls: NS for Cp oocytes and 12 out of 15, 80% in high calcium versus 14 out of 16, 88% in controls: NS for Exp oocytes; Table 3). In relation to the observed effect of NPS R-467 (only when added at 5 µM and in the presence of 2.92 mM calcium) on Exp oocytes, the following experiment, performed to test the effects of NPS R-467 on MAPK 3/1 activation, has been conducted only with Exp oocytes incubated in medium containing 2.92 mM external Ca^{2+} and 5 µM NPS R-467.

Activation of MAPK 3/1 by NPS R-467 in denuded oocytes and cumulus cells

Given the observed agonist activity of NPS R-467 on nuclear maturation of Exp oocytes, we next determined the effect of 5 µM NPS R-467 on MAPK 3/1 activity of Exp oocytes. The activity status of MAPK 3/1 was evaluated by measuring the effect of NPS R-467 on the phosphorylation state as described in Materials and Methods. For this purpose, the oocytes recovered from the ovaries of 33 mares were retrieved and processed in seven consecutive trials. The scraping procedure was carried out on 372 follicles from which 112 oocytes were recovered (1.7 oocytes/ovary; 30%, no. of recovered oocytes/no. of scraped follicles) and selected. Fifty Exp COCs (10 COCs per time point for a total of 50 COCs) were selected for this study and cultured in medium with 5 µM NPS R-467 for the following times: 0 min; 15 min; 2 h; 26 h; and 26 h without NPS R-467 as control. As shown in Fig. 6A and B, the ratio of phospho-MAPK 3/1 to total MAPK 3/1 increased already after 15 min incubation and, at 26 h, was still higher than that found in the non-treated 26 h controls. Importantly, MAPK 3/1 was observed to be expressed/activated only in the

Table 3 Effects of the calcium-sensing receptor (CASR) agonist NPS R-467 on oocyte meiotic maturation in the presence of suboptimal extracellular Ca^{2+} concentration.

Culture conditions	Cumulus morphology	No. of examined oocytes	No. of oocytes showing (%)			
			Germinal vesicle	Metaphase I to telophase I	Metaphase II with 1° polar body	Abnormal chromatin
	Compact					
Controls (2.92 mM Ca^{2+})		14	7 (50)	1 (7)	5 (36)*	1 (7)
0.5 mM Ca^{2+}		19	2 (11)	1 (5)	10 (53)	6 (32)
0.5 mM Ca^{2+} + 5 µM NPS R-467		20	5 (25)	5 (25)	8 (40)	2 (10)
4 mM Ca^{2+}		17	4 (24)	2 (11)	7 (41)	4 (24)
	Expanded					
Controls (2.92 mM Ca^{2+})		16	1 (6)	0 (0)	14 (88)†	1 (6)
0.5 mM Ca^{2+}		18	1 (5.5)	1 (5.5)	16 (89)	0 (0)
0.5 mM Ca^{2+} + 5 µM NPS R-467		18	1 (5.5)	1 (5.5)	13 (72)	3 (17)
4 mM Ca^{2+}		15	2 (13)	0 (0)	12 (80)	1 (7)

χ^2 -test with the Yates correction for continuity and Fisher's exact test: $P < 0.01$.

cumuli oophori and no phosphorylated MAPK 3/1 was observed in the denuded oocytes at any time point.

Given the observed lack of MAPK 3/1 in matured oocytes, we next performed immunofluorescence analysis of MAPK 3/1 expression and subcellular localization in MII stage oocytes obtained after IVM culture in the presence of NPS R-467. To this aim, the oocytes recovered from the ovaries of 28 mares were retrieved and processed in three consecutive trials. The scraping procedure was carried out on 208 follicles from which 99 oocytes were recovered (1.8 oocytes/ovary; 48%, no. of recovered oocytes/no. of scraped follicles) and selected. Fifty-four Exp COCs were selected for this study, cultured for 26 h in medium with ($n=32$) or without 5 μ M NPS R-467 ($n=22$), and evaluated. At the end of IVM culture time, 17 NPS-treated and 18 control oocytes, found at the MII stage, were examined for MAPK 3/1 expression and subcellular localization. Figure 7 shows representative oocytes labeled for total (A and C) or phospho-MAPK 3/1 (B and D) observed after culture in the presence (A and B) or absence (C and D) of the CASR agonist NPS R-467. Localized staining of total MAPK 3/1 was found around the two sets of chromosomes of the metaphase II plate and the first polar

Figure 6 (A) Effect of NPS R-467 on MAPK 3/1 phosphorylation status in equine cumuli oophori and oocytes from Exp COCs. Oocytes were treated with 5 μ M NPS R-467 and, at the indicated times, cumuli were carefully separated from the oocytes, and the phosphorylation status and total expression of MAPK 3/1 were measured in each cell fraction by western blotting using phosphospecific antibody (upper blot) or antibody against total MAPK 3/1 protein (lower blot). The left part of each blot contains extract from the cumuli and the right part from the denuded oocytes. (B) Graphic representation of phospho-MAPK 3/1 expressed as the phospho-MAPK 3/1/total ratio at different times of NPS R-467 treatment.

Figure 7 Detection of expression and subcellular localization of total and phospho-MAPK 3/1 in equine oocytes at the MII stage by immunofluorescence with primary polyclonal anti-MAPK 3/1 and anti-phospho-MAPK 3/1 antibodies respectively. For each lane, corresponding bright-field, u.v. light, and confocal images of the same oocyte are shown. Confocal laser-scanning microscopy optical sections show the positive labeling for total MAPK 3/1 in an NPS R-467-treated (A2) and control oocyte (C2) and for phospho-MAPK 3/1 in a treated (B2) and control oocyte (D2). Negative control (lane E). Scale bar represents 60 μ m.

body in all examined oocytes ($n=16$, eight NPS R-467-treated and eight control oocytes) and no effect of NPS R-467 was observed on total MAPK 3/1 localization or intensity. In NPS R-467-treated oocytes ($n=9$), phospho-MAPK 3/1 staining was clearly evident around the metaphase plate II and the first polar body whereas, in control oocytes ($n=10$), it was present only in tracks.

Discussion

Here we have analyzed the expression, localization, and function of CASR in equine COCs. This is the first study of the expression and function of CASR at the ovarian follicle level in mammals. A previous study performed in the female mammalian reproductive tract reported that CASR is spatio-temporally expressed and localized in the mammalian uterus at early pregnancy, suggesting that it might be important for blastocyst implantation and decidualization (Xiao *et al.* 2005). Up to now, only one previous study in the horse

(Toribio *et al.* 2003) reported CASR mRNA expression in parathyroid chief cells cultured *in vitro*. We detected the transcript in cumulus cells and at lower levels in the denuded oocytes. We hypothesize that the transcript is continuously synthesized in proliferating and differentiating cumulus cells, but not in fully grown oocytes. Indeed, transcription is believed to be inactivated at this stage in oocytes of large mammals (see review by Hyttel *et al.* (2001) and Bjerregaard & Maddox-Hyttel (2004)), and therefore there could be very little transcript present while the protein remains stable within the plasma membrane.

CASR protein in denuded oocytes and cumuli oophori was identified and localized. We found that CASR is present in both the germinal and somatic compartments of the COC and at all of the maturational stages investigated. This study provides the first characterization of CASR protein expression and localization in the oocyte of domestic mammals. Previous detection of CASR in isolated bovine parathyroid cells and in CASR-transfected HEK 293 cells described three immunoreactive bands of molecular masses ranging from 120 to 200 kDa and some additional bands of higher molecular mass (~350 kDa). The minor species at 120 kDa represents the non-glycosylated form of CASR and the two major species at 130–140 and at 150–160 kDa correspond to varying types and amount of glycosylation with mannose and complex carbohydrates (Bai *et al.* 1996, Brown & MacLeod 2001, Bai 2004). Moreover, Bai *et al.* (1998) demonstrated that CASR resides on the cell surface of transfected HEK 293 cells, mostly in a dimeric form. Our findings are in line with these previous studies; we found the 130 kDa glycosylated form in both compartments and the 120 kDa non-glycosylated form in cumulus cells. We did not observe any higher molecular mass bands, indicating that no dimeric forms were present in the investigated cells.

Immunofluorescence studies demonstrated that the CASR protein in equine oocytes is localized within the cytoplasm and on the plasma membrane in all stages examined. As previously observed in other cell systems, the receptor present on the cell surface was a small fraction of the total CASR and could represent the mature form of the receptor. The cytoplasmic CASR observed could simply represent the nascent receptor protein even if it cannot be excluded that it could have a distinct biological function, such as intracellular Ca^{2+} sensing (Bai *et al.* 1996, 1998). Interestingly, we observed that corona radiata cells were strongly labeled at their plasma membrane as well as on the transzonal cytoplasmic processes, which penetrate through the zona pellucida and abut the oocyte membrane. Our findings in equine oocytes are in line with our previous results in humans demonstrating, either by CLSM or by immunoblotting analysis, expression of this protein in the COC. Western-blot analysis of CASR protein in equine COCs revealed the same bands, 120 and

130 kDa, and immunocytochemical analysis revealed a similar labeling pattern as observed in human-denuded oocytes and cumuli oophori (Dell'Aquila *et al.* 2006). Confocal laser-scanning microscopy associated with image analysis allowed us to obtain detailed qualitative information on the localization of the protein in optical serial planes of specimens examined at different stages of meiotic progression. Interestingly, CASR labeling in surface planes was observed as organized in clumps/clusters. This distribution pattern could indicate CASR localization in caveolae, flask-shaped specialized microdomains in the plasma membrane enriched in signal-transducing molecules, as reported in other cell systems (reviewed by Brown & MacLeod (2001)). In the human oocyte, the presence of caveolae has been reported (Sathananthan 1997) leading to the hypothesis that included proteins could be involved in oocyte signal transduction.

These observations led us to determine the role of CASR activation in regulating oocyte maturation. We first determined the effect of NPS R-467, a calcimimetic with specific CASR agonist activity, on *in vitro* oocyte maturation. NPS R-467 is a phenylalkylamine compound, able to potentiate the effects of extracellular Ca^{2+} on CASR (Hammerland *et al.* 1998, Nemeth *et al.* 1998, Tu *et al.* 1999, Straub *et al.* 2000, Noeske *et al.* 2006) and is reported to act through the transmembrane domain of CASR by increasing the affinity of the receptor for its cognate G proteins or by enhancing the signal transduction from the 'head' of the CASR to its intracellular domains (Hauache *et al.* 2000, Zhang *et al.* 2002, Mun *et al.* 2004).

In the present study, the addition of NPS R-467 induced a stimulatory dose–response effect on the maturation rate of Exp oocytes. Our observations are in agreement with previous studies in which it was reported that NPS R-467 and the NPS R-467 precursor, NPS R-568, stimulate cell proliferation. Mailland *et al.* (1997) found that the increase in extracellular Ca^{2+} stimulated DNA synthesis in Chinese hamster lung fibroblasts transfected for CASR and that a strong potentiation of this response was obtained in the presence of NPS R-568. In additional studies in human myeloma cell lines expressing CASR, the exposure to NPS R-467 increased the sensitivity of the receptor to extracellular Ca^{2+} and increased cell proliferation (Yamaguchi *et al.* 2000). Tfelt-Hansen *et al.* (2004) reported that, in rat Leydig cancer cells, NPS R-467 induced cell proliferation comparable with that observed at high Ca^{2+} concentration, a response characteristic of a CASR-mediated effect.

As a confirmation to the observed CASR-mediated effect on meiosis progression of equine oocytes, we tried to revert the stimulatory effect observed in Exp oocytes in the presence of NPS R-467 by using a CASR antagonist, NPS 2390. NPS 2390 is a potent and selective non-competitive group I metabotropic glutamate receptor

(mGluR) antagonist (Lavreysen *et al.* 2003). In relation to CASR reported high structural homology with mGluR1 (review: Noeske *et al.* (2006)), NPS 2390 has been used as CASR antagonist in previous studies (Jung *et al.* 2005, Kwak *et al.* 2005). Indeed, the effect of NPS R-467 was significantly reduced by pre-incubation in the presence of NPS 2390. These data are in agreement with previous studies reporting its effects in reducing intracellular Ca^{2+} concentration (Jung *et al.* 2005) and cell proliferation (Kwak *et al.* 2005).

Our data confirm that the stimulatory effect on *in vitro* oocyte maturation rate is a specifically CASR-mediated effect. The different response to treatments between Cp and Exp oocytes suggests that CASR could be either differentially expressed or activated in Cp and Exp oocytes. It is possible, in fact, that CASR, in Cp and Exp oocytes, issuing from viable or slightly atretic follicles, may have different signal transduction pathways/mechanisms that may differentially regulate the response to CASR stimulation. Alternatively, the occurrence of morphological or functional modifications of the receptor during the process of COC maturation for cumulus expansion could be hypothesized. These hypotheses could be related to observations by Roussanne *et al.* (2001) who hypothesized that Ca^{2+} may regulate cell proliferation via two different pathways related to high or low CASR expression levels (Roussanne *et al.* 2001). Further studies are necessary to clarify whether different CASR expression levels could underlie the observed difference between oocytes surrounded by Cp or Exp cumulus, which are indicative of viable follicle/non-competent COC and initially atretic follicle/competent COC (Hinrichs *et al.* 2000, 2005).

Given the known role of NPS R-467 as an allosteric modulator of CASR, which sensitizes the CASR to Ca^{2+} without activating it in the absence of Ca^{2+} , it has been considered of interest to investigate the stimulatory effect of NPS R-467 at a suboptimal extracellular Ca^{2+} concentration (0.5 mM). Interestingly, it was observed that NPS R-467 at 5 μM had no effect on oocyte nuclear maturation when added in the presence of suboptimal external Ca^{2+} concentration (0.5 mM), confirming that NPS R-467 requires the presence of high external calcium to activate CASR as reported by Tfelt-Hansen *et al.* (2004). Moreover, we tried to test whether the natural agonist (Ca^{2+}), similarly to NPS R-467, can affect meiosis via CASR, i.e. whether increasing extracellular Ca^{2+} stimulates meiotic maturation. Interestingly, it was observed that increasing Ca^{2+} concentration (4 mM) had no effect on meiotic nuclear maturation. Thus, variations between 0.5 and 4 mM Ca^{2+} *per se* had no effects on nuclear maturation of equine oocytes cultured *in vitro*, but it cannot be excluded that higher or lower Ca^{2+} concentrations could be effective in increasing or reducing respectively the maturation rate of equine oocytes. Our data are in agreement with those reported by Hinrichs *et al.* (2007) who observed a similar lack of

calcium-dependent response, on oocyte maturation, in the range between 2 and 5.6 mM (75 vs 68% respectively, NS). Further studies are needed to investigate the effects of tested Ca^{2+} concentrations on other cytoplasmic (structural, biochemical, and molecular) parameters of oocyte competence. At the present time, it can be concluded that, at least in the range of tested Ca^{2+} concentrations, oocyte nuclear maturation of equine oocytes is not dependent on external Ca^{2+} and the stimulatory effect of NPS R-467 is evident only at the physiological Ca^{2+} concentration (2.92 mM).

The detection of CASR expression in the COC opens up the possibility that this receptor might function as an additional Ca^{2+} communication system between the somatic and germinal compartments of the female gamete. This hypothesis is in line with the results by Hofer *et al.* (2000) who reported that at physiological ambient $[\text{Ca}^{2+}]$, Ca^{2+} mobilization in one cell produces an extracellular signal that can be detected in nearby cells expressing CASR and that, due to its widespread tissue distribution, CASR may mediate a universal form of intercellular communication that allows cells to be informed of the Ca^{2+} signaling status of their neighbours. Indeed, it is well known that Ca^{2+} plays a fundamental role in the control of oocyte meiosis resumption and progression (Homa 1995, He *et al.* 1997, Tosti *et al.* 2000; review by Tosti & Boni (2004)). We can hypothesize that CASR expression in oocytes examined between GV and MII stages may be important for fine-tuning intracellular Ca^{2+} modifications induced following LH and growth factor exposure described in previous studies (Mattioli *et al.* 1998, Hill *et al.* 1999). Further studies aimed at analysing the role of CASR in the different stages of meiotic progression by following the dynamics of intracellular Ca^{2+} within the COC, upon CASR stimulation, will clarify this hypothesis.

We investigated the possible role of MAPK 3/1 in Exp COCs incubated with NPS R-467 at the concentration (5 μM) in which it increased the maturation rates, given the fact that MAPK 3/1, a member of the MAP kinase family involved in mitogenic signaling, has been reported to be activated by CASR agonists. In detail, it has been reported that the activation of CASR by its agonists or the calcimimetic NPS R568, which binds to the transmembrane domains (TMDs) of the receptor and activates it through an allosteric mechanism, stimulates the proliferation of several cell types, action that likely involves CASR-mediated activation of MAPK, as observed in rat fibroblasts and ovarian surface cells (McNeil *et al.* 1998b; review by Brown & MacLeod (2001) and Hobson *et al.* (2003)). Indeed, in bovine parathyroid and in CASR-transfected HEK cells, elevating Ca^{2+} or adding NPS R-467 elicited rapid, dose-dependent phosphorylation of MAPK3 and 1 (Kifor *et al.* 2001, Holstein *et al.* 2004). Our data showed

that incubation with NPS R-467 resulted in an increase in MAPK 3/1 activity at 15 min which still remained high at 26 h culture time compared with controls. To our knowledge, this is the first study demonstrating that NPS R-467 behaves as a CASR agonist in the COC in mammals by activating MAPK 3/1, as previously demonstrated in other cell systems. Our western-blot data also confirmed that MAPK 3/1 is only present in the cumuli oophori, as previously observed in bovine COCs upon opioid agent stimulation (Dell'Aquila *et al.* 2002). MAPK 3/1 was activated only in the cumuli oophori and no phosphorylated MAPK 3/1 was observed in the denuded oocytes at any time point, suggesting that if MAPK 3/1 activation has an effect on oocyte physiology the signal must be transduced by indirect mechanisms. The physiological meaning of the separation of MAPK 3/1 expression between the oocytes and cumulus cells has yet to be elucidated. In order to further investigate this point, additional immunofluorescence experiments were performed aimed to evaluate total and phospho-MAPK 3/1 expression and subcellular localization within NPS R-467-treated and control oocytes. In all examined equine Exp oocytes, total and phospho-MAPK 3/1 labeling were found to be exclusively localized around chromosomes' groups of MII and first PB whereas the cytoplasmic signal, in agreement with western-blot results, was very low. Total MAPK 3/1 localization and intensity were not modified by NPS R-467 treatment, whereas phospho-MAPK 3/1 labeling had higher intensity in NPS-treated oocytes. Observed distribution of MAPK 3/1 labeling was in line with data reported in previous studies. In mouse oocytes, MAP kinases were described as stably associated with the meiotic spindle to drive microtubule characteristics (review by Eichenlaub-Ritter *et al.* (2004)). MAP kinases are phosphorylated and activated downstream in the c-mos pathway at oogenesis and account for normal first meiotic metaphase I spindle dynamics, first PB formation, and activities of the 'cytostatic factor' that arrests oocytes at the MII stage until activation (Hengyu *et al.* 2002; review by Eichenlaub-Ritter *et al.* (2004) and Petrunewich *et al.* (2009)). Observed CASR-induced MAPK 3/1 phosphorylation could contribute to the regulation of cumulus cells survival/proliferation and oocyte maturation (see model in Fig. 8).

In conclusion, we show that CASR is expressed at mRNA and protein levels in equine oocytes and cumulus cells, and is functionally active since the selective CASR agonist NPS R-467 induced a stimulatory effect on oocyte maturation and this effect was reversed by pre-incubation with the CASR antagonist NPS 2390. The CASR agonist improved maturation rate only in the presence of physiological calcium. In oocytes treated with NPS R-467, CASR immunostaining increased at membrane level and was reduced in the cytosol. Activation of this receptor is supported by the observation that cumulus cells and oocytes stimulated by NPS

Figure 8 Signaling pathways activated by CASR in the mammalian cumulus-oocyte complex. CASR is expressed in equine oocytes and cumulus cells. The CASR agonist, NPS R-467, induces a stimulatory effect on oocyte maturation and this effect is reversed by pre-incubation with the CASR antagonist, NPS 2390. In oocytes treated with NPS R-467, CASR immunostaining at the membrane level increases while being reduced in the cytosol. Cumulus cells stimulated by NPS R-467 show an increase in the activity of MAPK 3/1-type MAPK at the end of IVM. MAPK 3/1 phosphorylation in cumulus cells could have an effective role on cell survival/proliferation. A phosphorylated MAPK 3/1 downstream effector/product could move into or be effective in the oocyte, via transzonal cytoplasmic processes. The signaling pathway leading to MAPK 3/1 phosphorylation within the oocyte is hypothesized to be the same as represented for cumulus cells. Abbreviations: (Ca^{2+}), extracellular calcium ions; CASR, calcium-sensing receptor; NPS R-467, NPS R-467 CASR agonist; NPS 2390, NPS 2390 CASR antagonist; Gq11, α -subunit of the q subtype of the heterotrimeric G proteins; PLC, phospholipase C; DAG, diacylglycerol; PKC, phosphokinase C; MAPKKK, MAPK kinase. Please refer to the text for more detailed information.

R-467 showed an increase in activity of MAPK 3/1-type MAPK at the end of IVM. Taken together, our data firstly demonstrate that CASR plays a role in the control of oocyte maturation in mammals.

Materials and Methods

All chemicals were purchased from Sigma-Aldrich unless otherwise indicated. CASR transcript identification was performed at INRA (Nouzilly, France), whereas protein identification and functional studies were performed at University of Bari (Italy).

Oocyte recovery, selection, and processing/storage

In the part of the study performed at INRA, equine COCs were recovered by aspiration (Marchal *et al.* 2003) from follicles larger than 3 mm of ovaries collected at a nearby slaughterhouse. COCs were selected under a microscope and a subset was incubated for IVM during 30 h in TCM199 (Sigma) supplemented with 20% FCS (Sigma) and 50 ng/ml EGF (Sigma) under 5% CO₂ (Goudet *et al.* 2000). Oocytes and cumulus cells were separated by mechanical treatment then washed in Dulbecco's PBS solution (Dulbecco A, Paris, France). Groups of 10 immature oocytes and groups of 30 cumuli oophori from COCs following IVM were stored frozen (−80 °C) in RNAlater (Ambion, Applied Biosystems, Warrington, UK) until use.

Protein identification and functional studies were conducted at DPA (University of Bari, Bari, Italy). Ovaries from mares of unknown reproductive history, obtained at two local abattoirs located at a distance of 20 km (30 min) from the laboratory, were transported and processed as previously described (Dell'Aquila *et al.* 2003). COCs were identified in the mural granulosa cells using a dissection microscope and only those complexes, classified as having Cp or Exp cumulus investment, were used (Dell'Aquila *et al.* 2003). Oocytes for these studies (western-blot, IVM cultures, immunofluorescence before and after IVM, and MAPK 3/1 analysis) were used immediately and their further processing is detailed in related sections. Oocytes retrieved with a Cp or an Exp cumulus were separately cultured and analyzed in relation to their different meiotic competence as reported in previous studies (review Dell'Aquila *et al.* (1997), Hinrichs & Schmidt (2000) and Hinrichs *et al.* (2005)). The time between COCs collection and starting of oocyte analysis/culture was <1 h and total time between slaughter and oocyte processing ranged between 2 and 4 h.

RNA isolation and RT-PCR

After adding 1 pg of luciferase mRNA (Promega) per oocyte/cumulus as an exogenous control, total DNase-treated RNA was purified using Tripure isolation reagent (Roche Diagnostics). RT was performed at 37 °C for 50 min using oligo(dT)15 (Promega) by mouse Moloney leukemia virus reverse transcriptase (Invitrogen) in a final volume of 20 µl. One microliter was used as a substrate for PCR using iQ SYBR green supermix (Bio-Rad) with primers targeting the 3' untranslated region (5'-TGGCCACCACTAGGGTTAAG and 5'-TACTGGTTCAG-GAGGGATGC) based on the NW_001799677.1 sequence available in GenBank. A three-step protocol (95 °C for 30 s, 60 °C for 30 s, and 72 °C for 20 s) was repeated for 40 cycles. PCR products were analyzed onto an ethidium bromide containing agarose gel. Target identity was further confirmed by sequencing after cloning into pCRII vector using TA cloning kit (Invitrogen).

Western-blot analysis

Denuded oocytes and corresponding cumuli oophori were recovered from medium-sized follicles and examined just after retrieval. Cells were separately collected in SDS sample buffer

(50 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (w/v) glycerol, 50 mM dithiothreitol, and 0.1% (w/v) bromophenol blue), heated to 100 °C for 5 min, and extracted proteins were separated by means of SDS-PAGE on a 4–12% linear gradient running gel (Criterion XT Bis-Tris Precast gels, Bio-Rad). Separated proteins were electro-transferred to Immobilon P membrane (Millipore, Watford, UK) and the membrane was incubated with a primary rabbit polyclonal antibody against a 20 amino acid peptide sequence near the C-terminus of human CASR (antiCaSR C0117-15 US Biological, Swampscott, MA, USA; 1:1000 dilution). Immunocomplexes were detected with ECL reagent (Amersham Biosciences) and image processing was carried out by using Adobe Photoshop.

Immunofluorescence

After collection from medium-sized follicles, oocytes were either immediately incubated for 1 h in stabilizing medium (Medium M, Tremoleda *et al.* 2001, Dell'Aquila *et al.* 2006) or cultured for IVM, as described below, to obtain later meiotic stages and then incubated in the stabilizing medium. After zona pellucida permeabilization with medium M, oocytes were fixed in 2% (v/v) paraformaldehyde in PBS for 1 h, washed in 100 mM glycine in PBS, and incubated for 30 min in 1% (w/v) BSA in PBS (PBS-BSA). Oocytes were then incubated in a 1:2500 dilution of antihuman CASR for 90 min at room temperature. Oocytes incubated in PBS-BSA for 90 min were used as negative controls. For each experiment, one or two oocytes were used as minus primary controls. All oocytes were washed in PBS, then incubated for 2 h at room temperature with a FITC-conjugated rabbit IgG-secondary antibody, diluted 1:200 in PBS. Nuclear chromatin evaluation was performed as described below.

Confocal laser scanning microscopy and image analysis

Oocytes were observed at 600× magnification in oil immersion with a laser-scanning confocal microscope (C1/TE2000-U Nikon) equipped with the Argon Ions 488 laser and the 495–519 (B2-A) nm excitation/emission filter. For each oocyte, scanning was conducted with 25 optical series from the top to the bottom of the oocyte with a step size of 0.45 µm. Parameters related to fluorescence intensity were maintained at constant values for all measurements.

In vitro maturation

IVM experiments were performed in medium TCM199 with Earle's salts buffered with 4.43 mM HEPES, 33.9 mM sodium bicarbonate and supplemented with 0.1 g/l L-glutamine, 2 mM sodium pyruvate, 2.92 mM calcium lactate (Serva Feinbiochem GmbH & Co., Heidelberg, Germany No. 29760), and 50 µg/ml gentamicin. The 2.92 mM calcium lactate concentration is referred in the text as control condition. After preparation, pH was adjusted to 7.18, and the medium was filtered through 0.22 µm filters (No. 5003-6, Lida Manufacturing Corp., Kenosha, WI, USA). Then, gonadotrophins (10 µg/ml ovine FSH, Sigma and 20 µg/ml ovine LH, NIADDK, NIH, Bethesda, MD, USA) and 17β estradiol (1 µg/ml) were added.

The medium was further supplemented with 20% (v/v) FCS. The medium was filtered again and allowed to equilibrate for 1 h under 5% CO₂ in air before being used. Cp and Exp COCs were washed three times in the culture medium and groups of COCs with the same cumulus morphology were placed in 400 µl of medium/well of a four-well dish, covered with pre-equilibrated lightweight paraffin oil and cultured for 28–30 h at 38.5 °C under 5% CO₂ in air. The effects of NPS R-467 ((R)-N-(3-phenylpropyl)-α-methyl-3-methoxybenzylamide hydrochloride; NPS Pharmaceuticals, Salt Lake City, UT, USA) and NPS 2390 (Quinoxaline-2-carboxylic acid adamantan-1-ylamide; Sigma N 4786) were tested at concentrations previously reported in dose–response curve experiments in other cell systems. The effects of NPS R-467 were tested at the concentration of 0.5, 5, and 10 µM. In previous studies, NPS R-467 was used at concentrations ranging between 1 and 10 µM and it was observed that maximal increase in intracellular Ca²⁺ concentration occurred after incubation with 1 and 5 µM NPS R-467 (Nemeth *et al.* 1998, Mun *et al.* 2004). The effects of NPS 2390 were tested at the concentration of 10 µM (Jung *et al.* 2005, Smajilovich *et al.* 2006). Control group oocytes were cultured in the absence of these substances.

Nuclear chromatin evaluation

Cumulus cells were removed by incubation in TCM199 with 20% FCS containing 80 IU/ml hyaluronidase and by careful aspiration in and out of finely drawn glass pipettes. To evaluate nuclear chromatin, totally denuded oocytes were fixed in 3.8% (v/v) buffered formaldehyde solution (J T Baker; No. 7385) in PBS, stained with 2.5 µg/ml Hoechst 33258 in 3:1 (v/v) glycerol/PBS, and observed under an E-600 Nikon fluorescent microscope equipped with a 365 nm excitation filter. Nuclear chromatin status was classified as follows: GV including those oocytes with fluorescent nucleus and those with a condensed chromatin as described by Hinrichs *et al.* (1993), metaphase to telophase I (MI to TI) and complete maturation at metaphase II with the first polar body extruded (MII+PB). Oocytes showing either irregular chromatin clumps or no chromatin were considered to be abnormal. Oocytes with fragmented or shrunken cytoplasm were classified as degenerated.

Immunoblotting analysis of MAP kinase

Oocytes were collected as above and incubated at 38.5 °C in the same culture medium described above. Particular attention was paid to select cumuli oophori of the same size by observation under a Nikon SMZ 1500 stereomicroscope (7.5–112.5× magnification) equipped with micrometric eyepiece. The number of oocytes for each group and culture times is detailed in the Results. Oocytes were cultured in the presence of 5 µM NPS R-467 for 15 min, 2 h and 26 h and control oocytes in the absence of NPS R-467. At the end of culture time, COCs underwent mechanical removal of cumulus cells by pipetting, and pooled denuded oocytes and cumuli oophori were separately frozen at –80 °C until processing. Cells were collected in SDS sample buffer, heated to 100 °C for 5 min. Extracted proteins were separated by means of SDS-PAGE on

a 4–12% linear gradient running gel and electroblotted to polyvinylidene difluoride membranes as described above (see CASR western-blot analysis). Activated MAPK 3/1 was detected by using anti-phospho-MAPK 3/1 polyclonal antibody (dilution 1:2000) (Cell Signaling Technology, Inc., Danvers, MA, USA) and total kinase by using anti-MAPK 3/1 polyclonal antibody (dilution 1:1000) (New England Biolabs, Inc., Beverly, MA, USA). Anti-rabbit IgG HRP-linked antibody (Cell Signaling Technology, Celbio, Milano, Italy) was used as secondary antibody. Immunocomplexes were detected with ECL reagent (Amersham Biosciences) and densitometric quantification and image processing were carried out using Adobe Photoshop and the NIH Image software package (Version 1.61, National Institute of Health).

Immunofluorescence analysis of MAP kinase

After collection from medium-sized follicles, oocytes were cultured for IVM, as described below, and incubated for 1 h in stabilizing medium (Medium M, Tremoleda *et al.* 2001, Dell'Aquila *et al.* 2006). After zona pellucida permeabilization with medium M, oocytes were fixed in 2% (v/v) paraformaldehyde in PBS for 1 h, washed in 100 mM glycine in PBS, and incubated for 30 min in 1% (w/v) BSA in PBS (PBS–BSA). For total MAP kinase analysis, oocytes were incubated in a 1:250 dilution of anti-MAPK 3/1 polyclonal antibody for 90 min at room temperature. For activated MAP kinase analysis, oocytes were incubated in a 1:500 dilution of anti-phospho-MAPK 3/1 polyclonal antibody for 90 min at room temperature. Primary antibodies were the same as used for immunoblotting analysis of MAP kinase. Oocytes incubated in PBS–BSA for 90 min were used as negative controls. For each experiment, two oocytes were used as minus primary controls. All oocytes were washed in PBS, then incubated for 2 h at room temperature with Alexa fluor 488 goat anti-rabbit IgG-secondary antibody (Molecular Probes, Invitrogen) diluted 1:1000 in PBS. Nuclear chromatin evaluation was performed as described above.

Data analysis

For IVM experiments, due to the limited number of oocytes available on a single day (~15–40), no more than four experimental groups (controls and treatments) were tested in four to seven replicates. In each replicate (day=replicate), equivalent numbers of COCs of each morphological category (Cp or Exp) were randomly assigned to groups. The proportions of oocytes having different chromatin configurations after culture were compared between treated and control groups by χ^2 -analysis with the Yates correction for continuity. Fisher's exact test was used when a value of <5 was expected in any cell. Differences were considered statistically significant at $P < 0.05$.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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