Calreticulin from subolemmal vesicles affects membrane regulation of polyspermy

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Abstract

This study was designed to determine whether calreticulin (CRT), a chaperone protein, is present in *in vitro*-matured (IVM) pig oocytes and to study its potential role in the block to polyspermy. Western blot analysis, using an anti-CRT antibody, of oocyte lysate showed an immunoreactive band of ~60 kDa. Simultaneous labeling of IVM oocytes with anti-CRT antibody and peanut agglutinin lectin (PNA lectin, a porcine cortical granules (CG)-specific binding lectin) revealed localization of CRT in the subplasmalemmal region with a 27.7% colocalization with PNA staining. After IVF, PNA labeling was not observed and anti-CRT labeling decreased significantly in zygotes and disappeared in two-cell embryos. Western blot analysis of oocyte exudate obtained from zona pellucida (ZP)-free oocytes activated with calcium ionophore confirmed the presence of a band that reacted with an anti-CRT antibody. Anti-CRT antibody and PNA labeling were not observed in activated oocytes despite being detectable in non-activated oocytes. The presence of CRT in vesicles located under the oolemma was demonstrated using immunogold cytochemistry at the ultrastructural level. To study the role of CRT in fertilization, ZP-enclosed and ZP-free oocytes were incubated with exogenous CRT and then inseminated. Whereas ZP-free oocytes showed fewer penetrating sperm and lower polyspermy rates than untreated oocytes, the opposite effect was observed in ZP-enclosed oocytes. In conclusion, CRT is confined to subplasmalemmal vesicles partially overlapping with CG contents. Its exocytosis after the oocyte activation seems to participate in the membrane block to polyspermy in pigs but is not involved in the ZP block.

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Introduction

Following gamete membrane fusion at fertilization, the cortical granules (CG) undergo exocytosis releasing their contents into the perivitelline space, modifying the egg's zona pellucida (ZP), and preventing penetration by additional spermatozoa (reviewed by Wessel *et al.* (2001)). Although the specific CG components responsible for the zona block have not yet been identified, several proteins have been partially characterized and reported to be released from CG or present in the medium surrounding activated eggs (exudate). Mouse CG contain ovoperoxidase (Gulyas & Schmell 1980), *N*-acetylglucosaminidase (Miller *et al.* 1993), peptidyl-arginine deiminase (p75) (Pierce *et al.* 1992, Liu *et al.* 2005), a 32-kDa protein named P32 (Gross *et al.* 2000), several proteases (Moller & Wassarman 1989, Tawia & Lopata 1992, Burkart *et al.* 2012), and glycoconjugates (Lee *et al.* 1988). Carbohydrates are present in human CG

(Jiménez-Movilla *et al.* 2004); hamster CG contain two proteins (P56 and P62) immunologically related to sea urchin hyaline (Hoodbhoy *et al.* 2000); it has been suggested that rat CG contain tissue plasminogen activator (Zhang *et al.* 1992) and that this protein is also present in bovine CG (Rekkas *et al.* 2002), but ultrastructural studies have demonstrated its presence in the oolemma and not in the CG (Mondéjar *et al.* 2012). Functions of CG-derived proteins have been related to ZP modifications and further block to polyspermy (Moller & Wassarman 1989, Tawia & Lopata 1992, Zhang *et al.* 1992, Miller *et al.* 1993, Wessel *et al.* 2001), and regulation of preimplantation cleavage and early embryonic development (Hoodbhoy & Talbot 2001, Hoodbhoy *et al.* 2001). Studies concerning CG contents in pig oocytes are scarce and no specific granule protein has been described. Polyspermy remains an unsolved problem in the current IVF systems (Coy & Avilés 2010). Porcine CG contain glycoconjugates with terminal

β -D-galactosyl (1,3)-D-N-acetylgalactosamine (Gal β 1-3GalNAc) residues, and peanut agglutinin lectin (PNA lectin) is the only specific available tool for labeling pig CG contents (Yoshida *et al.* 1993).

More recently, attention has been drawn to calreticulin (CRT), an ancient highly conserved ubiquitous protein originally identified as an endoplasmic reticulum chaperone (Ostwald & MacLennan 1974). This protein contains an N-terminal cleavable signal sequence that directs it to the endoplasmic reticulum (ER), and an ER KDEL (Lys-Asp-Glu-Leu) retention/retrieval signal. When residing in the ER, CRT has important functions in directing proper folding of proteins and glycoproteins, as well as in homeostatic control of cytosolic and endoplasmic reticulum Ca^{2+} levels. There is also steadily accumulating evidence of diverse roles for CRT localized outside the ER, including data suggesting important roles for CRT localized on the outer cell surface of a variety of cell types, in the cytosol, and in the extracellular matrix (reviewed by Gold *et al.* (2010)).

CRT is the major Ca^{2+} -binding chaperone and it has been linked to CG being localized in the cortical area of human eggs (Balakier *et al.* 2002), hamster CG (Muñoz-Gotera *et al.* 2001), and on the extracellular surface of the mouse egg oolemma remaining in the perivitelline space after egg activation (Tutuncu *et al.* 2004). Confocal studies in porcine oocytes by Zhang *et al.* (2010) identified CRT at a subcellular level accumulated primarily in the cortical/plasma membrane region of matured oocytes. They associated CRT with the regulation of oocyte meiotic maturation and maternal gene expression but did not compare CRT localization with that of CG or other subplasmalemmal vesicles. A specific function of CRT on fertilization or post-fertilization events in mammals has not been fully described, although it has been associated with the block to polyspermy by remaining in the perivitelline space after being exocytosed (Muñoz-Gotera *et al.* 2001). Recent studies show that topically applied CRT has diverse and profound biological effects that enhance cutaneous wound healing in animal models (Gold *et al.* 2010). This evidence for extracellular bioactivities of CRT has provided new insight into this classically ER-resident protein, despite a lack of knowledge on how CRT progresses from the ER to the cell surface or how it is released into the extracellular milieu. Nonetheless, it has become clear that CRT is a multi-compartmental protein that regulates a wide array of cellular responses (Gold *et al.* 2010).

Is CRT located in the pig CG and, if so, does it have a role in the block to polyspermy? Our first hypothesis is that CRT is contained in CG. To confirm this, oocyte lysate and exudate from *in vitro*-matured (IVM) oocytes was analyzed by western blot with an anti-CRT antibody. CRT protein was immunolocalized in IVM oocytes, zygotes, and two-cell embryos, and transmission electron microscopy (TEM)-immunogold showed the

detailed location of the protein. Our second hypothesis is that CRT participates somehow in the block to polyspermy; this was tested by inseminating ZP-free and ZP-enclosed oocytes previously treated with exogenous CRT.

Materials and methods

This study was developed following institutional approval from the Bioethical Committee in the University of Murcia and was performed in accordance with the Animal Welfare regulations of that institution (Permit for study number: AGL2009-12512-C02-01). All the chemicals and reagents were purchased from Sigma-Aldrich unless otherwise stated.

Oocyte collection and IVM

Cumulus-oocyte complexes (COCs) were collected by aspiration of antral follicles (3–6 mm diameter) of ovaries from slaughtered 6- to 7-month-old animals (Landrace X Large White), weighing 80–100 kg. Only COCs with a dark cytoplasm and a complete and dense cumulus oophorus were used for the experiments. Groups of 50–55 COCs were IVM for 44 h in NCSU-37 medium supplemented with 0.57 mM cysteine, 1 mM dibutyl cAMP, 5 $\mu\text{g}/\text{ml}$ insulin, 50 μM β -mercaptoethanol, 1 mM glutamine, 10 IU/ml eCG (Folligon, Intervet International B.V., Boxmeer, Holland), 10 IU/ml hCG (Veterin corion, Divasa Farmavic, Barcelona, Spain), and 10% (v/v) porcine follicular fluid (Funahashi *et al.* 1997, Romar *et al.* 2005).

Collection of oocyte lysate and exudate

Oocyte lysate was obtained from matured COCs as follows. After IVM, COCs were stripped of cumulus cells by pipetting in PBS and those showing extrusion of the first polar body were quickly washed three times in ultrapure water (MilliQ, Millipore, Billerica, MA, USA), transferred to a water droplet, at a ratio of 200 oocytes in 50 μl , and allowed to lyse until the full release of cytoplasmic content (Supplementary Figure 1, see section on supplementary data given at the end of this article). All processes were carried out at 4 °C and, when necessary, oocytes were passed through a narrow glass pipette of $\sim 80 \mu\text{m}$ diameter to accelerate the release of cytoplasmic content. Once lysed, the ZPs were removed from the water droplet and washed three times in fresh water droplets. All droplets containing the cytoplasmic content (lysate) and ZPs were quickly aspirated separately and stored at $-80 \text{ }^{\circ}\text{C}$ until use.

Oocyte exudate was obtained from ZP-free activated oocytes. IVM oocytes were mechanically denuded, ZP was digested with acidic Tyrode's solution (Sigma T1788), and ZP-free oocytes were washed three times in PBS without Ca^{2+} – Mg^{2+} . Then, ZP-free oocytes were incubated with calcium ionophore A23187 (6.5 μM , 2 min), washed three times in a medium consisting of 114.06 mM NaCl, 3.2 mM KCl, 0.5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 10 mM sodium lactate, 0.35 mM NaH_2PO_4 , 5 mM glucose, 25.07 mM NaHCO_3 , 8 mM calcium

lactate-5H₂O (pH 7.2, 320 mOsm), and transferred to a 50 µl droplet of fresh medium. Activated ZP-free oocytes were incubated at 38.5 °C and 5% CO₂ for 30 min to allow CG content release. After this time, ZP-free oocytes were carefully aspirated, a portion lysed as described previously, and the other portion processed to assess cortical reaction under confocal and TEMs. The medium containing the oocyte's exudate was collected, stored at -80 °C, and further used for electrophoresis and western blot.

Western blot analysis

CRT rabbit polyclonal antibody (synthetic peptide QAKDEL corresponding to C terminal amino acids of human CRT) was purchased from Abcam, Cardiff, UK (ab15607). The proteins in the oocyte lysate (both from IVM oocytes and from ZP-free activated oocytes) and exudate were dissolved in reducing SDS-PAGE loading buffer, separated by SDS/PAGE electrophoresis (RT, 1 h at 150 V) and electrotransferred to polyvinylidene fluoride membrane (4 °C, 1 h at 100 V). The membranes were washed and blocked in Tris-buffered saline (TBS) with 0.1% Tween 20 and 2% BSA at 37 °C for 1 h and incubated with anti-CRT antibody (1:1000) at 37 °C for 1 h. Then the membrane was washed three times with TBS with 0.1% Triton X-100 (TBST) and incubated with HRP-conjugated secondary antibody (Santa Cruz Biotechnology, sc2004; 1:20 000) at 37 °C for 1 h. After washing in TBST, the protein binding was detected using the ECL plus method and Typhoon 9410 (Amersham Biosciences). Briefly, the membrane was incubated for 5 min with the chemifluorescence ECL Plus substrate (40:1 mixture of component A and B) as instructed by the product manual. Then the membrane was drained, scanned with the appropriate filters for HRP (excitation 430 and 503 nm emission), and the obtained images saved as computer files.

To discard that CRT or cytoplasmic content had been aspirated with the ZPs, the samples ($n=200$) were solubilized (70 °C, 30 min), dissolved in SDS-PAGE loading buffer, and processed as described above. Human CRT full-length protein (ab91577) was used as control (0.1 µg). Anti-β-actin antibody (Abcam ab8226) was used as marker of cytoplasmic content using boar spermatozoa lysate (20 µg) as control.

IVF and embryo culture

The fertilization medium was modified TALP supplemented with 3 mg/ml fatty acid-free BSA and 1.10 mM sodium pyruvate (Rath & Niemann 1997). After IVM, COCs were denuded and transferred to a four-well multidish containing 250 µl IVF medium previously equilibrated at 38.5 °C under 5% CO₂. A sperm-rich fraction of semen from a mature, fertility-tested boar was collected and semen samples (0.5 ml) were centrifuged (700 *g*, 30 min) through a discontinuous Percoll (Pharmacia) gradient (45 and 90% v/v). Resultant sperm pellets were diluted in fertilization medium and centrifuged again (10 min, 100 *g*). Finally, the pellet was diluted in fertilization medium and 250 µl of this suspension were added to the wells containing the oocytes, giving a final concentration of 10⁵ cells/ml. An aliquot of zygotes was

processed for immunocytochemistry at 18 h post-insemination (hpi) and those remaining were cultured for an additional 24 h in NCSU-23 medium up to the two-cell embryo stage.

Immunofluorescence labeling of oocytes, zygotes, and embryo for CRT and protein disulphide isomerase localization

CRT antibody (Abcam ab15607) was used in combination with a secondary antibody conjugated with rhodamine isothiocyanate (anti-rabbit IgG-TRITC; 1:400) purchased from Sigma (T6778). The protein disulphide isomerase (PDI) mouse antibody (clone 1D3 corresponding to the sequence near the C-terminus of rat PDI) was purchased from Enzo Life Sciences, Lausanne, Switzerland (ADI-SPA-891) and was used in combination with a secondary antibody conjugated with FITC (anti-mouse IgG-FITC; 1:400) purchased from Millipore (12-506). Immunofluorescence staining of proteins was done as described previously (Mondéjar *et al.* 2012). Briefly, samples were first fixed in 3.7% freshly prepared paraformaldehyde in PBS (30 min) followed by permeabilization in 0.1% Triton X-100 (10 min) and blocking with 1% BSA and 100 mM glycine (30 min). Then, samples were incubated for 1 h in the dark at room temperature with CRT (1:10) or PDI (1:1000) primary antibodies followed by incubation with their corresponding secondary antibodies for 1 h. Finally, samples were labeled with PNA lectin conjugated to FITC (10 µg/ml, 30 min) to specifically label CG (Yoshida *et al.* 1993). After antibody and lectin incubations, samples were washed three times in equilibration buffer medium (Slow fade, Antifade kit, Molecular Probes, Eugene, OR, USA), mounted and kept in the dark at 4 °C until assessment within 24 h using confocal laser scanning microscopy (Nikon Eclipse). As negative controls, samples were incubated only with the secondary antibody. CRT protein was detected in oocytes ($n=20$), zygotes ($n=15$), and two-cell embryo ($n=10$) and PDI protein was detected in oocytes ($n=15$) and zygotes ($n=15$) from three independent experiments. Images were sequentially acquired in order to study the degree of overlapping between FITC (CG labeling) and TRITC (CRT labeling). Both equatorial and cortex images from each oocyte were used to calculate Pearson's correlation and Manders' colocalization coefficients using a colocalization software (NIS Elements Microscope Imaging Software, Tokyo, Japan). The degree of overlapping between two different fluorescent labels, each having a separate emission wavelength, is used to discriminate whether two different cellular targets are located in the same area or very near to each other (Zinchuk & Zinchuk 2008).

Images were also analyzed to quantify the intensity of anti-CRT labeling by means of the free software ImageJ (<http://rsbweb.nih.gov/ij/>). Briefly, images captured under RGB format were transformed into an eight-bit gray image and segmented to delimit the labeled area under a fixed threshold for gray color (values from 1 to 255). The generated binary mask of each image was used to measure the area (square micrometers) and the labeling intensity (mean gray color of the pixels in the area of interest) in the oocyte's cortical area and ZP. An example of the image analysis is presented in

Supplementary Figure 2, see section on supplementary data given at the end of this article.

CG quantification by TEM

IVM oocytes ($n=18$) and ZP-free activated oocytes ($n=12$) from three independent experiments were fixed in 2% glutaraldehyde in 0.1 M PIPES buffer (pH 7.2) for 1 h at 4 °C and post-fixed in 2% osmium tetroxide at room temperature. For preparation of ultrathin sections (50 nm thickness), oocytes were processed for embedding in Embed 812 (EMC, Washington, Pennsylvania 19034, USA), sections obtained with an ultramicrotome (Microm International GmbH, Walldorf, Germany), and mounted on formvar-coated nickel grids. After washing twice in distilled water, the grids were counterstained with uranyl acetate and lead citrate. The thin sections were observed under a Phillips Tecnai 12 electron microscope (Eindhoven, The Netherlands). For quantitative analysis of CG, four images at 2850 $\times$ were taken randomly in each oocyte and number of CG further counted.

TEM immunogold of oocytes and zygotes for CRT and PDI localization

For TEM, a rabbit polyclonal anti-CRT antibody tested for electron microscopy was purchased from Abcam (ab4) and used at 1:10 dilution. Anti-PDI antibody from Enzo Life Sciences (ADI-SPA-891) was used at 2 μ g/ml. Samples were fixed with 1% glutaraldehyde in cacodylate buffer (pH 7.4) for 2 h at 4 °C. After extensive washing in cacodylate buffer, samples were dehydrated through a graded series of ethanol and processed for embedding in LR-White resin. For immunocytochemistry, a two-step method was used. Ultrathin sections of oocytes and zygotes were incubated with anti-CRT and oocytes also with anti-PDI antibody. Grids were then washed and floated on a drop of Protein A gold (15 nm) conjugated antibody (Utrecht University; 1:60). After washing twice in distilled water, the grids were counterstained and observed as mentioned in the above section.

IVF of ZP-free and ZP-enclosed mature oocytes incubated with exogenous CRT

A recombinant full-length human CRT was purchased from Abcam (ab91577). Matured COCs were denuded by pipetting and half of the oocytes were subjected to ZP digestion in acidic Tyrode's solution. ZP-free and ZP-enclosed oocytes were incubated for 30 min in fertilization medium supplemented with 0, 100, 1000, and 5000 pg of CRT per oocyte. Protein incubation time was chosen based on preliminary trials from our laboratory and the literature (Tutuncu *et al.* 2004). After CRT treatment, oocytes were gently washed in fertilization medium and inseminated as described in the above section with a final sperm concentration of 10⁵ cells/ml (ZP-enclosed oocytes) or 2.500 cells/ml (ZP-free oocytes). At 18 hpi, putative zygotes were fixed, stained (Hoechst 33342), and sperm penetration, number of spermatozoa per penetrated oocyte, male pronucleus formation, and number of sperm bound to the ZP or oolemma were assessed.

Statistical analysis

Data are presented as the mean $\pm$ S.E.M. and all percentages were modeled according to the binomial model of variables. The IVF variables were analyzed by one-way ANOVA (percentage of oocyte penetration, mean number of sperm cells per penetrated oocyte, percentage of male pronucleus formation, percentage of monospermy, and number of sperm bound to the ZP or oolemma). When ANOVA revealed a significant effect, values were compared by Tukey's test. A P value <0.05 was considered to denote statistical significance.

Results

CRT is present in vesicles in the cortex of matured pig oocytes partially colocalizing with CG contents

Western blot of the lysate obtained from IVM oocytes showed an immunoreactive band of ~ 60 kDa, consistent with the expected size of CRT (Fig. 1A). In the samples from activated oocytes, no immunoreactive band was observed in the lysate but was present in the exudate, confirming that the band corresponds to the soluble isoform of the protein. The western blot of the ZPs showed that no cytoplasmic content was aspirated with them. Neither calretinin nor actin is detected in the ZPs aspirated from the lysis droplets (Fig. 1B).

Indirect immunofluorescence demonstrated that CRT protein is located within the cortical region of metaphase II oocytes (Fig. 2A) with a mean intensity labeling of 30.3 ± 0.2 units/area. The negative controls incubated with only the secondary antibody did not show any signal (image not shown). A defined CG band was present in IVM oocytes after labeling with PNA, which specifically binds to these organelles (Fig. 2B). Dual-channel images obtained by sequential scanning showed overlapping between FITC and TRITC fluorochromes, meaning that PNA and CRT colocalize to some extent in the same oocyte (Fig. 2C). Results showed a Mander's coefficient of 0.277 and a Pearson's correlation coefficient of 0.61. Mander's index indicates that 27.7% of the pixels in the image had overlapping of FITC and TRITC fluorophores whereas Pearson's coefficient ranges from -1.0 to 1.0 and values over 0.5 indicate that the colocalization is significant (Zinchuk & Zinchuk 2008).

At the light resolution limits of confocal microscopy, the immunofluorescent signals at the cortex can originate from either membrane proteins that reside at the oolemma or at the secretory vesicles that are close to the oolemma. To confirm that anti-CRT labeling did not originate from the oolemma itself, non-permeabilized oocytes were incubated with anti-CRT antibody. Results showed a very weak anti-CRT staining and not a full cortical labeling pattern (Supplementary Figure 3, see section on supplementary data given at the end of this article).

Figure 1 (A) Calreticulin (CRT) is present in the lysate and exudate collected from mature pig oocytes. Lysate is the whole cytoplasmic content of mature oocytes obtained after being lysed in water. Exudate corresponds to the content exocytosed after activation of mature oocytes. Western blots of lysate obtained from *in vitro*-matured oocytes ($n=150$), lysate from ZP-free oocytes activated with calcium ionophore ($n=250$), and the collected exudate. Experiment was replicated three times. (B) Cytoplasmic content is not detected in the ZPs. Neither calretinin nor actin is detected in the ZPs ($n=200$) aspirated from lysis droplets. Controls used for the ZP western blots are human CRT full-length protein (0.1 μ g) and boar spermatozoa lysate (20 μ g).

The specific localization of CRT in vesicles located immediately adjacent to the oolemma was demonstrated using immunogold cytochemistry at the ultrastructural level (Fig. 3). CRT localization was partially associated with CG and other cortical vesicles. The images obtained by TEM showed that CRT and CG are located close to each other in the egg cortex. After fertilization, CG and CRT molecules were exocytosed from the egg cortex and some CRT molecules were observed at the oolemma and ZP. These observations open the possibility that CRT, a classically ER-resident protein, was contained in secretory vesicles distinct from CG, which would be exocytosed after oocyte activation. To confirm this hypothesis, oocytes and zygotes were immunolabeled with a specific anti-PDI antibody, a classic ER-marker. Samples were observed under confocal microscopy and TEM to study the localization and likely exocytosis of ER-derived vesicles. Results showed that after IVM, PDI protein was present in vesicles located in the cortex of matured oocytes in a similar distribution pattern than CG (Supplementary Figure 4, see section on supplementary data given at the end of this article). After IVF, the anti-PDI labeling disappeared and was no longer

observed. These observations indicate a release into the extracellular milieu and might suggest extracellular bioactivities of CRT and PDI.

CRT is exocytosed after the cortical reaction

Confocal visualization of zygotes showed that CG had been fully exocytosed as demonstrated by the disappearance of the CG band from the cortical region (Fig. 4). Anti-CRT staining of zygotes at the cortical level almost fully disappeared, reaching an intensity of 1.8 ± 0.2 units/area ($n=10$), a 94% reduction compared with unfertilized oocytes. At the resolution of confocal imaging, no anti-CRT antibody labeling was observed

Figure 2 Calreticulin (CRT) is located in the cortex region of ZP-enclosed mature pig oocytes. Confocal visualization of CRT and cortical granules (CG) in *in vitro*-matured pig oocytes. (A and A') Immunofluorescent labeling of CRT protein (red) showing intense fluorescence in the cortical region. (B and B') Staining with peanut agglutinin lectin conjugated to fluorescein (PNA-FITC) (green) indicates a defined cortex arrangement of CG adjacent to oolemma. (C and C') Colocalization (overlapping) between PNA-FITC and anti-CRT antibody with a secondary antibody conjugated to rhodamine (CRT-TRITC) in the entire cortical area of the oocytes. Bar represents 50 μ m.

Figure 3 Calreticulin (CRT) is present in IVM oocytes and exocytosed in zygotes. (Mature oocytes) Transmission electron microscopy-immunogold demonstration of CRT (black dots) in *in vitro*-matured pig oocytes. Arrows indicate CRT molecules close to or associated with cortical granules (CG) and other subplasmalemmal vesicles in the egg cortex. CRT is absent from the zona pellucida (ZP). Bar: 0.2 μ m. (Zygotes) CG and CRT molecules are exocytosed from egg cortex. A residual CRT is observed on the oolemma and ZP. m, mitochondrion. Bar represents 1 μ m.

in the perivitelline space of zygotes and labeling at ZP level was minimal with 1.5 ± 0.2 units/area (a 95% reduction compared with unfertilized oocytes) coinciding with previous observations by electron microscopy (Fig. 3). No anti-CRT antibody labeling was observed in the blastomeres of two-cell embryos whereas the ZP showed a residual intensity of 2.1 ± 0.1 units/area (Fig. 4). A positive PNA staining was observed on the outer ZP of zygotes and two-cell embryos due to the presence of acrosomal shrouds (Romar *et al.* 2012).

Cortical reaction induced in ZP-free IVM oocytes by calcium ionophore (6.5μ M for 2 min and further 30 min incubation) was confirmed by confocal microscopy and TEM since no literature was found about the CG exocytosis degree after chemical activation of porcine ZP-free oocytes. Results showed that ZP removal did not affect the integrity of the oolemma, and the CG remained tightly located near the plasma membrane (Fig. 5) with a mean number of 5.9 ± 1.8 CG per 5μ m of oolemma. This number significantly decreased to 0.7 ± 0.2 CG per 5μ m of oolemma in activated oocytes. Thus our activation protocol with a 30-min post-activation period was efficient enough to allow CG exocytosis and to collect CG-derived proteins in the exudate.

Cytochemical localization showed that PNA (CG) and anti-CRT antibody labeling were present in the cortical region just after IVM and ZP removal (Fig. 6); however, this staining disappeared after oocyte

activation. These observations are consistent with the appearance of an immunoreactive band for CRT in the immunoblot of exudate from activated oocytes as was shown in Fig. 1.

Taken together, these results strongly suggest that CRT is contained in secretory vesicles located within the oocyte's cortical area and is exocytosed during oocyte activation. The release into the extracellular milieu suggests an extracellular function for this protein, which was confirmed in the next experiment.

CRT blocks polyspermy at oolemma level

When ZP-enclosed oocytes were incubated with exogenous CRT before insemination, penetrability increased and monospermy was reduced (Table 1). Sperm penetration increased along with the number of sperm bound to the ZP compared with untreated ZP-enclosed oocytes. However, ZP-free oocytes incubated with CRT showed higher monospermy rates than untreated ZP-free oocytes. The increase in monospermy was not associated with a reduced penetration rate nor with a decrease in the number of sperm attached to the oolemma. Male pronucleus formation was not affected by CRT incubation and was over 95% in all groups of oocytes.

Discussion

CRT has been associated with human eggs (Balakier *et al.* 2002) and porcine oocytes (Ellederova *et al.* 2004), hamster CG (Muñoz-Gotera *et al.* 2001) and oolemma,

Figure 4 Calreticulin (CRT) and cortical granules labeling disappear in pig zygotes and embryos. Simultaneous labeling with anti-CRT antibody with a secondary antibody conjugated to rhodamine (CRT-TRITC) and peanut agglutinin lectin conjugated to fluorescein (PNA-FITC). Confocal visualization of zygotes obtained after IVF and assessed at 18 h post-insemination showed how PNA labeling disappeared from oolemma and anti-CRT labeling decreased significantly compared with oocytes. In the two-cell embryos assessed at 48 h post-insemination, both PNA and anti-CRT have completely disappeared. DIC, differential interference contrast. Bar represents 50μ m.

Figure 5 Cortical granules (CG) released after activation of ZP-free mature pig oocytes. Transmission electron microscopy images of *in vitro*-matured pig oocytes after ZP digestion with acidic Tyrode's solution showing CG (arrows) and oolemma integrity. After chemical activation with 6.5 μ M calcium ionophore for 2 min followed by 30 min incubation, the number of CG significantly decreased. Bar represents 1 μ m.

and the perivitelline space of mouse eggs (Tutuncu *et al.* 2004) demonstrating several extracellular bioactivities (Gold *et al.* 2010) including the block to polyspermy in hamsters (Muñoz-Gotera *et al.* 2001). In this study, an anti-CRT antibody specifically recognized a single band of 60 kDa in the lysate from IVM porcine oocytes. Conversely, CRT was not found in the lysate of ZP-free activated oocytes, but it appeared in the exudate collected from these oocytes, indicating that CRT had been exocytosed after oocyte activation. Protein bands observed in the immunoblots were consistent with the expected size of this protein coinciding with the molecular weight described in studies with mouse oocytes (60 kDa) (Tutuncu *et al.* 2004), protein extracts from hamster oocytes (60 kDa) (Muñoz-Gotera *et al.* 2001), *Xenopus* oocytes (61 kDa) (Parys *et al.* 1994), and human oocytes (63 kDa) (Balakier *et al.* 2002).

The results from this study show that CRT is located in the cortical region of IVM porcine oocytes, associated with secretory vesicles and partially colocalizing with CG contents. CRT is released after induction of cortical reaction both by spermatozoa (physiological induction) and calcium ionophore (artificial induction). CRT signal

is then weakly observed on the oolemma of zygotes and is no longer observed in two-cell embryos. These results are consistent with a recent study in pigs reporting the accumulation of CRT in cortical/plasma membrane region of metaphase II oocytes with a weak fluorescence signal in the cytoplasm of pronuclear-stage zygotes and early cleaved embryos (Zhang *et al.* 2010). The CRT intracellular localization pattern is different in cows, where it is localized near the cortex of immature oocytes and absent from the cortical region in matured oocytes and two-cell embryos (Payne & Schatten 2003). Despite the fact that CRT contains the ER-retrieval sequence KDEL at its C-terminus, it can escape the ER and has been identified on the extracellular surface of a wide variety of cell types (Xiao *et al.* 1999, Goicoechea *et al.* 2000) including the surface of the egg plasma membrane and the perivitelline space in mice (Tutuncu *et al.* 2004). The variations observed in CRT localization might be related to the different antibodies employed in the cited studies detecting Ca^{2+} -bound or Ca^{2+} -free CRT (Balakier *et al.* 2002) and different CRT isoforms (Zhu *et al.* 1997). It is also important to bear in mind that the available information comes exclusively from confocal microscopy studies and not from TEM observations.

This study, where both confocal microscopy and TEM were used, shows that CRT is released from subcortical secretory vesicles after oocyte activation and fertilization. CRT colocalized to some extent with PNA lectin as 27.7% of the pixels in the oocyte's cortical area were concomitantly labeled with PNA (CG contents) and anti-CRT antibody. The TEM observations confirmed

Figure 6 Calreticulin (CRT) and cortical granules (CG) release after activation of ZP-free mature pig oocytes. Immunolocalization of CRT with an anti-CRT antibody with a secondary antibody conjugated to rhodamine (CRT-TRITC) (red) and CG labeled with peanut agglutinin lectin conjugated to fluorescein (PNA-FITC) (green). CRT and CG labeling appear in the cortical area of mature oocytes after ZP digestion by 0.5% pronase showing. After chemical activation with 6.5 μ M calcium ionophore for 2 min followed by 30-min incubation, both CRT and CG labeling disappeared. DIC, differential interference contrast. Bar represents 50 μ m.

Table 1 Calreticulin (CRT) blocks polyspermy at oolemma level. Effect of incubation of pig oocytes with exogenous CRT for 30 min at different concentrations before IVF on further fertilization parameters.

CRT (pg/oocyte)	ZP-enclosed oocytes						ZP-free oocytes					
	<i>n</i>	PEN (%)	S/O	MPN (%) ^a	MON (%) ^a	SPZ/ZP	<i>n</i>	PEN (%)	S/O	MPN (%) ^a	MON (%) ^a	SPZ/OL
0	117	82.1±3.6*	2.7±0.2*	96.9±1.8	37.5±5.0*	18.6±0.8*	118	77.1±3.8	3.0±0.2*	97.8±1.5	30.7±4.8*	11.4±1.1
100	107	83.2±3.6*	3.6±0.2 [†]	100	15.7±3.9 [†]	26.9±1.1 [†]	120	77.5±3.8	2.7±0.2*	98.9±1.1	39.7±5.1*	10.0±1.0
1000	115	96.5±1.7 [†]	3.6±0.1 [†]	98.2±1.3	6.4±2.3 [†]	37.5±0.5 [†]	122	77.0±3.8	2.3±0.1*	100	41.9±5.1*	12.8±1.2
5000	112	97.3±1.5 [†]	4.0±0.1 [†]	100	5.4±2.2 [†]	39.3±0.7 [†]	121	72.7±4.1	2.0±0.1 [†]	98.8±1.1	52.2±5.3 [†]	10.7±1.1

Data represent the mean±s.e.m. from four independent experiments. *n*, total number of evaluated oocytes; PEN, penetration; S/O, spermatozoa per penetrated oocyte; MPN, male pronucleus formation; MON, monospermy; SPZ/ZP, sperm bound to zona pellucida in penetrated oocytes; SPZ/OL, sperm attached to oolemma in penetrated oocytes. *,[†],[‡]Different symbols within each column indicate statistically significant differences ($P<0.05$).

^aFrom penetrated oocytes.

that CRT was not present in all CG, appearing mainly associated with other cortical vesicles. These PDI-containing vesicles were also released after fertilization. These results are consistent with the observation that mammalian CG contents are heterogeneous, existing as different subpopulations depending on electron density or lectin pattern labeling (Cran 1987, Liu *et al.* 2003, Jiménez-Movilla *et al.* 2004). Tutuncu *et al.* (2004) suggested that CRT is released from mouse egg vesicles distinct from CG as they did not observe colocalization between lens culinaris agglutinin (LCA) lectin and CRT, and the protein was detected in the exudate obtained after oocyte chemical activation. In hamster CG, colocalization between LCA and CRT has been reported (Muñoz-Gotera *et al.* 2001). The hypothesis that vesicles, distinct from CG and containing different proteins, might be released at fertilization was suggested in mice some time ago (Ducibella *et al.* 1988) and was suggested in mice with the release of tetraspanin CD9 from mouse eggs (Miyado *et al.* 2008). These findings open a new field of research and expand the possible mechanisms by which the oocyte modifies its extracellular surface to interact with the spermatozoa.

The exocytosis of CRT during oocyte activation leads us to consider that it may have a role in the block to polyspermy. The amount of CRT in a single egg is unknown, but a mouse egg releases 50–100 pg protein after calcium ionophore activation (Moller & Wassarman 1989), so our hypothesis was that exocytosed CRT might modify the oolemma, perivitelline space, or the inner part of the ZP due to its chaperoning function, oligosaccharide- and polypeptide-binding. The direct contact of exogenous CRT with the oolemma prevented polyspermy in pigs (ZP-free oocytes), whereas this effect was not observed in ZP-enclosed oocytes, where protein contacted directly with the outer ZP. Effect of exogenous CRT on sperm boar functionality was tested by incubation of sperm with the protein and no detrimental effect was observed (data not shown). Limited information is available about the molecular features of substrate binding to CRT (reviewed by Michalak *et al.* (2009)) and the protein (extracellular or secreted) adheres to cell surface integrins regulating cellular

adhesion (Coppolino *et al.* 1995, Johnson *et al.* 2001). Capacitated boar spermatozoa display integrins on their membrane (Barboni *et al.* 2001), so CRT might be modulating gamete membrane fusion. Furthermore, it is known that porcine oocytes establish a membrane block to polyspermy, which is among the Ca^{2+} -dependent egg activation events (Gardner & Evans 2006, Gardner *et al.* 2007). CRT is the major Ca^{2+} -binding chaperone and its fine-tuning together with other Ca^{2+} -binding proteins, such as calsequestrin, plays a crucial role in regulation of Ca^{2+} transience during oocyte maturation, fertilization, and early embryo development (Balakier *et al.* 2002). The likely role of CRT regulating porcine gamete fusion at the oolemma level needs further study.

In conclusion, we have demonstrated that CRT locates in cortical vesicles of IVM pig oocytes and that it is exocytosed after oocyte activation, contributing to the membrane block to polyspermy in pigs. Additional studies in different species are necessary to confirm whether this is an evolutionarily conserved mechanism or it is a particular consequence of porcine gamete interactions.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-13-0454>.

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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