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Preparation of the Cortical Reaction: Maturation-Dependent Migration of SNARE Proteins, Clathrin, and Complexin to the Porcine Oocyte's Surface Blocks Membrane Traffic until Fertilization[1]

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ABSTRACT

The cortical reaction is a calcium-dependent exocytotic process in which the content of secretory granules is released into the perivitellin space immediately after fertilization, which serves to prevent polyspermic fertilization. In this study, we investigated the involvement and the organization of SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins in the docking and fusion of the cortical granule membrane with the oolemma in porcine oocytes. During meiotic maturation, secretory vesicles that were labeled with a granule-specific binding lectin, peanut agglutinin (PNA), migrated toward the oocyte's surface. This surface-orientated redistribution behavior was also observed for the oocyte-specific SNARE proteins SNAP23 and VAMP1 that colocalized with the PNA-labeled structures in the cortex area just under the oolemma and with the exclusive localization area of complexin (a *trans*-SNARE complex-stabilizing protein). The coming together of these proteins serves to prevent the spontaneous secretion of the docked cortical granules and to prepare the oocyte's surface for the cortical reaction, which should probably be immediately compensated for by a clathrin-mediated endocytosis. In vitro fertilization resulted in the secretion of the cortical granule content and the concomitant release of complexin and clathrin into the oocyte's cytosol, and this is considered to stimulate the observed endocytosis of SNARE-containing membrane vesicles.

calcium, clathrin, complexin, cortical reaction, fertilization, gamete biology, in vitro fertilization, oocyte, oocyte development, SNARE

INTRODUCTION

Cortical granules (CGs) are a specialized group of secretory vesicles that are randomly dispersed throughout the cytoplasm of immature oocytes and migrate toward the cortical cytoplasm during meiotic maturation [1]. This relocalization is mediated by specific association with components of the cytoskeleton, for example, microfilaments [2]. Once the granules are positioned at the cortical area of the oocyte, they dissociate from the cytoskeleton structures and remain in close proximity to the egg plasma membrane (oolemma) [3, 4]. The redistribution of secretory granules is a preparative step for the cortical reaction (CR), a calcium-dependent exocytotic process. The trigger for the CR is the fusion of the sperm plasma membrane with the oolemma. This fertilization fusion activates a series of intracellular signaling pathways [3]. In mammals, the sperm cell introduces phospholipase C zeta, a sperm-specific isoform, into the oocyte cytoplasm (ooplasm), which serves to produce inositol 1,4,5-triphosphate (IP_3) by breaking down phosphatidylinostol 4,5-bisphosphate [5, 6]. The produced IP_3 in turn binds to receptors on the endoplasmic reticulum (ER) of the oocyte, causing an oscillatory release of calcium into the ooplasm. The resulting calcium oscillations in the ooplasm induce the fusion of CGs with the oolemma over the entire oocyte's surface [7, 8]. These calcium oscillations can in vitro be mimicked by the addition of a calcium ionophore (A23187), by stimulation with electrical pulses, or by a more physiological approach, namely the incubation of matured meiosis-II (M-II) oocytes with sperm cells [9]. During the CR, the enzyme content of the CGs is secreted into the perivitelline space, and these released enzymes subsequently modify the zona pellucida (ZP) proteins, resulting in a structural change of the ZP (known as ''zona hardening'') and/or in forming a specific coating on the oolemma [10, 11]. Both changes are known to inhibit polyspermic fertilization, which is incompatible with normal development of the zygote. A further understanding of cellular dynamics of proteins that are involved in the CR is considered to be important to reveal the mechanisms that contribute to the regulation of CR and subsequent events that are relevant for the normal development of the zygote.

Several studies have shown that exocytosis in gametes is controlled by interactions between specific sets of proteins that belong to the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) protein family [12–14]. Functional studies in mouse oocytes have provided solid evidence that SNAP25 is involved in the CR [13]. However, scarce information is available on the (re)localization of SNARE proteins during specific stages of oocyte development, maturation, and fertilization. In the present study, we report on the relocalization of secretory granules and the spatial-temporal reorganization of proteins that are involved in the regulation of the pre- and postfertilization CR. Furthermore, complexin has been shown to associate with SNARE complexes in neurons and thus not only stabilizes the formed SNARE complexes but also prevents premature fusion of interacting membranes [15]. Here we provide data that SNARE protein interactions contribute to the docking process of CGs to the oolemma and that the subsequent SNARE-complexin interactions are

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responsible for the arresting of CGs at the oolemma. Temporarily inhibiting this secretion at the oocyte's surface prior to fertilization will also arrest endocytosis. We therefore investigated the cellular organization of proteins involved in clathrin-dependent (i.e., clathrin) and clathrin-independent (i.e., flotillin and caveolin) endocytosis during oocyte maturation. The possible functional implications of CG docking for the CR and for its role in the prevention of polyspermic fertilization are discussed. The involvement of clathrin and complexin in the reestablishment of membrane trafficking are also investigated and discussed.

MATERIALS AND METHODS

Reagents and Antibodies

All the chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. Affinity-purified mouse monoclonal antibody against syntaxin 1 (STX1), rabbit polyclonal antibodies against syntaxin 2 (STX2), SNAP23, VAMP1, VAMP2, and VAMP3 (specific for each isoform and with no cross-reactivity with the other two) as well as complexin 1/2 (binds to both isoforms) were purchased from Synaptic System (Göttingen, Germany). Rabbit polyclonal antibody against SNAP25 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse IgG1 monoclonal antibodies against raft marker protein flotillin 1 and against a marker protein for caveolae (caveolin 1) were obtained from BD Biosciences (San Jose, CA). Mouse IgG antibody against clathrin heavy chain (X22) was a kind gift from J.J. Hölzenspies (Utrecht University, Utrecht, the Netherlands). Control SNAP23, VAMP1, and complexin 1/2 peptides were purchased from Synaptic System, and control flotillin-1 peptide was purchased from Abcam (Cambridge, U.K.).

In Vitro Maturation and Fertilization

Sow (*Sus scrofa*) ovaries were obtained from adult fertile sows at a special slaughterhouse (Groenlo, the Netherlands). This abattoir is special as it is unique in facilitating a continuous slaughter line exclusively for adult fertile sows. Routinely, prepubertal gilts are slaughtered for meat production. Prepubertal gilts are not yet sexually active and thus contain irrelevant ovaria for our studies. Cumulus-oocyte complexes (COCs) were collected by aspiration of 3–6 mm follicles and subsequently selected using well-established morphological criteria [16]. The yield per daily collection session was usually above 100 oocytes. In vitro maturation (IVM) was performed as previously described [16]. COCs were collected in HEPES buffered M199 (Gibco Laboratories Inc., Grand Island, NY) and washed in preequilibrated M199 supplemented with 2.2 mg/ml $NaHCO_3$, 0.1% (w/v) polyvinylpyrrolidone (PVP), 100 μM cysteamine, 75 μg/ml potassium penicillin G, and 50 μg/ml streptomycin sulfate (oocyte maturation medium, OMM). Selected COCs were cultured at 38°C with 5% CO_2 for 22 h in OMM supplemented with 0.05 international units/ml recombinant human follicle-stimulating hormone (rhFSH, a kind gift from Organon, Oss, the Netherlands). After 22 h IVM, COCs were transferred into OMM without rhFSH for another 22 h to reach the metaphase II stage. The CR was induced by in vitro fertilization (IVF) by coincubating M-II oocytes with freshly ejaculated porcine sperm cells from fertile boars (Cooperative Boar Artificial Insemination Center, Bunnik, the Netherlands). In short, the freshly ejaculated sperm cells were washed with IVF medium (113.1 mM NaCl, 3.0 mM KCl, 20 mM Tris, 11.0 mM glucose, 0.1% bovine serum albumin (BSA), 1.0 mM caffeine, 7.5 mM $CaCl_2$, 0.05 mM sodium pyruvate, 75 μg/ml potassium penicillin G, and 50 μg/ml streptomycin sulfate; pH 7.4) for 5 min and then centrifuged at $700 \times g$ at room temperature (RT). Washed sperm cells were resuspended in the IVF medium and adjusted to a concentration of 1×10^6 spermatozoa/ml. This sperm suspension was added to the oocytes in a ratio of 1000 sperm cells/oocyte and coincubated at 38°C with 5% CO_2 in air for 8 h. The maturation and fertilization competence of the oocytes were determined based on their overall morphology and DNA-staining pattern, which were assessed as described previously [17] by confocal laser scanning microscopy (Leica TCS SP2, Leica Microsystems GmbH, Wetzlar, Germany). Oocytes could be divided into three categories: 1) germinal vesicle (0 h, GV), as indicated by the presence of the germinal vesicle structure, 2) meiosis-II (44 h after IVM, M-II), as indicated by the presence of the metaphase plate and the first polar body, and 3) fertilized (52 h, IVF), as indicated by the presence of two pronuclei. Three independent experiments were conducted, and for each experiment, at least 20 oocytes per group were evaluated. Oocytes that could not be categorized according to the criteria described above were excluded from this study.

Immunoblotting

Protein concentrations from all the samples were standardized according to the Lowry method [18]. For both sperm and control brain samples, 15 μg of total protein extract was mixed with an appropriate amount of lithium dodecyl sulfate loading buffer (Invitrogen, Carlsbad, CA) in the presence of 0.1 M dithiothreitol. Cumulus-free oocytes were obtained as previously described [17], and 100 oocytes were used per sample, which corresponds to 10–12 μg of total protein. All the samples were heated for 10 min at 100°C prior to immunoblotting. Proteins were separated in a 4% stacking and 12% running SDS-PAGE gel and wet-blotted onto polyvinylidene difluoride membranes (GE Healthcare, Buckinghamshire, U.K.). After blocking for 1 h with ReliaBLOT (Bethyl Laboratories, Inc., Montgomery, TX) at RT, the blots were incubated overnight at 4°C with primary antibodies diluted in ReliaBLOT (a 1:250 dilution from a 1 mg/ml stock was used for all experiments except for syntaxin2 where a 1:1000 dilution was used). After washing the blots in TBST (50 mM Tris, pH 7.4, 250 mM sucrose, and 0.2% v/v Tween-20), secondary antibodies (dilutions of 1:500 from a 2 mg/ml stock) were added for 30 min. After rinsing with TBST, protein was visualized using chemiluminescence (ECL-detection kit; Supersignal West Pico, Pierce, Rockford, IL).

Labeling of CGs

Labeling of the CGs was performed as previously described [1, 19] with minor modifications. For efficient lectin binding, partially denuded oocytes were used. To remove the surrounding cumulus cells, oocytes of different maturation stages were gently pipetted in HEPES-buffered M199; partially denuded oocytes were separately collected and washed two times in HEPES-buffered M199. After washing, oocytes were fixed in freshly prepared 4% (v/v) para-formaldehyde (Electron Microscopy Sciences, Hatfield, PA) in phosphate-buffered saline (PBS; 137 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4, pH 7.4) for 1 h at RT. After fixation, oocytes were washed twice in PBS-PVP (0.1 M PBS, pH 7.4, containing 0.3% (w/v) PVP) and permeabilized in PBSS (0.1 M PBS, pH 7.4, containing 0.1% saponin) for 10 min. Nonspecific-binding sites were blocked using blocking buffer glycine (PBSS with 1% (w/v) BSA and 2% (v/v) normal goat serum (Vector Lab, Burlingame, CA) supplemented with 100 mM glycine) for 2 h at RT. After rinsing in PBS, oocytes were incubated in fluorescein isothiocyanate-conjugated peanut agglutinin (PNA-FITC; EY Laboratory, San Mateo, CA) diluted in PBSSc (PBSS containing 0.1g/L $CaCl_2$) for 1 h at RT in the dark. As a negative control, PNA-FITC was preincubated in PBSSc containing 100 mM lactose for 30 min at RT before use.

Immunofluorescent Staining for SNARE Proteins, Complexin, and Clathrin

Immunolabeling was performed as previously described [17]. Both primary and secondary antibodies were diluted in blocking buffer and centrifuged at $100\,000 \times g$ for 1 h before use to prevent aggregation of the antibody. Oocytes were incubated with primary antibodies overnight at 4°C. For negative controls, purified mouse or rabbit IgG (BD Biosciences) that matched the host species of the primary antibodies (a 1:250 dilution from a 1 mg/ml stock was used for all experiments except for syntaxin2 where a 1:1000 dilution was used) were used with the dilutions of the negative controls being identical to the dilution of the primary antibodies used in the same experiment. Oocytes were rinsed three times in PBS after primary antibodies incubation. SNARE-labeled oocytes were subsequently incubated with (dilutions of 1:500 from a 2 mg/ml stock) Alexa-568 conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Molecular Probes, Eugene, OR) for 1 h at RT. DNA was labeled with 10 μM ToPro-3 iodide (Molecular Probes, Leiden, the Netherlands) in PBSS for 20 min. After three rinses in PBSS, oocytes were mounted in a 0.12 mm, 8-well Secure-Seal Spacer (Molecular Probes) on a cover slip, covered in a drop of Vectashield (Vector Lab), and sealed with a microscope slide (Superfrost Plus; Menzel, Braunschweig, Germany). In control experiments, 1 μg/ml primary antibodies were blocked with 5 μg/ml synthetic peptides epitopes of the corresponding proteins the antibodies were raised against. The synthetic blocking peptides for anti-SNAP23 antibody (sequence: DRIDIANARAKKLIDS), anti-VAMP1 (sequence: SAPAQPPAEGTEG), anti-complexin 1/2 (sequence: KYLPGPLQDMFKK), and anti-flotillin-1 (sequence: SISQVNHKPLRTA) were coincubated with the corresponding antibodies for 2 h prior to their use in the immunofluorescent experiments. These conditions were recommended by the company that delivered the blocking peptides and antibodies and were effective in preventing antibody binding to oocyte structures (see Supplemental Fig. S1; all the supplemental data are available online at www.biolreprod.org).

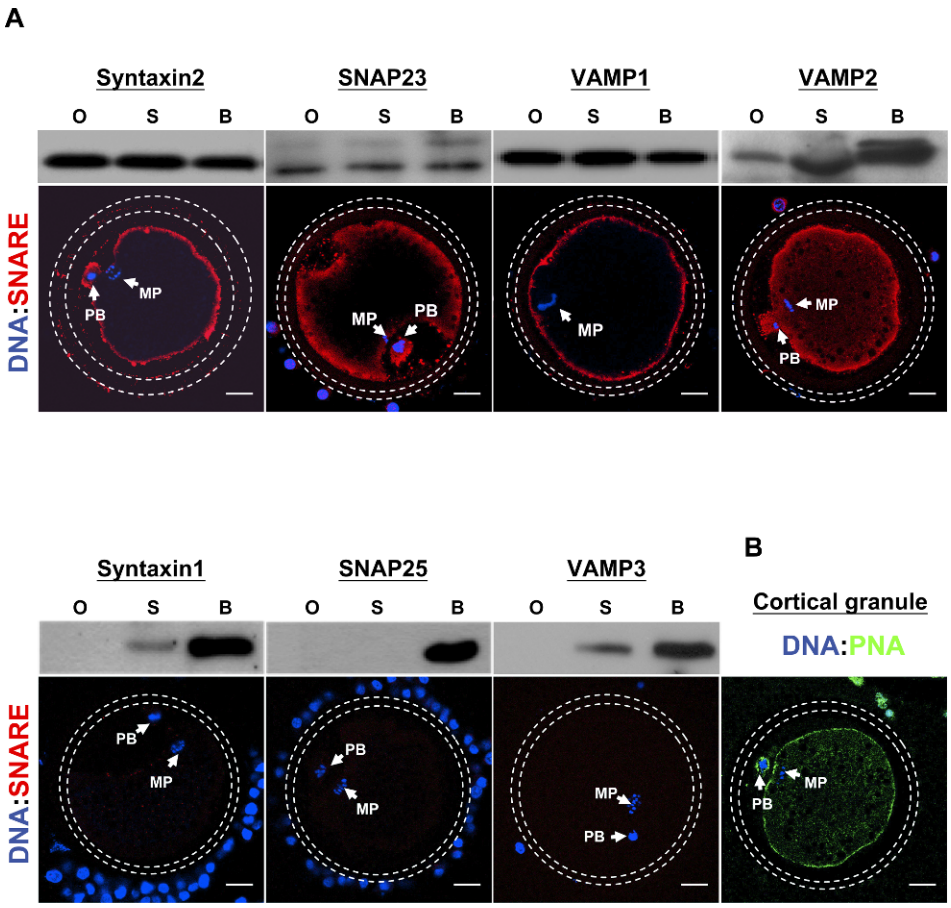


FIG. 1. Cortex orientation of SNARE proteins in M-II porcine oocytes. **A**) Immunofluorescent labeling of Q-SNARE proteins (red) syntaxin 2 and SNAP23 and of R-SNARE proteins (red) VAMP1 and VAMP2 in M-II porcine oocytes (O); absence of labeling for syntaxin 1, SNAP25, and VAMP3. Western blot analysis of SNARE proteins present in the M-II oocytes shows comparable molecular weights to that of control brain tissues (B) and of boar sperm cells (S). **B**) PNA-FITC staining (green) indicates a defined cortex arrangement of cortical granules adjacent to the oolemma at M-II arrested oocyte. PB, first polar body; MP, metaphase plate. The dashed circles indicate the area of the zona pellucida. Chromatin was stained with Topro-3 (blue). Bars = 20 μm.

Confocal Laser Scanning Microscopy and Image Acquisition

Images were obtained through a 40× oil immersion objective using a Leica TCS SP2 confocal system (Leica Microsystems GmbH), equipped with 488, 568, and 633 nm lasers. Dual and triple channel images were obtained by sequential scanning. ImageJ (National Institutes of Health, http://rsb.info.nih.gov/ij/) software was used for the analysis of the images. Laser power and acquisition settings were adjusted to produce submaximal pixel values in the oocyte, and the settings used to image control IgG stainings were matched to the highest settings used to image primary antibody staining in the same experiment. Images made at the equatorial region of the oocytes were used to discriminate on representative DNA structures for germinal vesicles for the GV stage or for metaphase plate/first polar body for the M-II stage. Background subtraction and contrast/brightness enhancement (up to ~20% enhancement using the maximum slider in Image J) were performed identically for all the images in the same experiment.

RESULTS

Distribution of SNARE Proteins in Porcine Oocytes

To determine how SNARE proteins are organized just prior to CR, we used M-II arrested oocytes (the final maturation stage of mammalian oocytes that leaves them ready to become fertilized by sperm). With indirect immunofluorescence and immunoblotting techniques, we demonstrated that the complementary SNARE proteins syntaxin 2, SNAP23, VAMP1, and VAMP2 were present in porcine M-II oocytes, whereas syntaxin 1, SNAP25, and VAMP3 were below the detection level (Fig. 1A and Supplemental Fig. S2). Therefore, all the SNARE members required to form a SNARE complex that enables intracellular membrane fusion are present in porcine oocytes.

In M-II oocytes, SNARE proteins were predominately observed in the cortical region of the oocyte and showed punctuate staining patterns that are characteristic for SNARE proteins (Fig. 1A). Note that at the light resolution limits of confocal microscopy the immunofluorescent signals at the cortex can originate either from membrane proteins that reside at the oolemma or from membrane proteins that reside in secretory vesicles that are associated with the oolemma. The fact that SNARE-staining patterns coincided with that of the PNA-FITC (Fig. 1B) could imply that the SNARE proteins are associated with the CGs. To our surprise, from the immunoblotting studies, SNAP25 was not detected in the porcine oocytes and only the low molecular weight form of VAMP1, which is similar to that found in porcine sperm, was seen (Fig. 1A immunoblot and Supplemental Fig. S2); this is in contrast to our observations in control pig brain tissues and the report on mouse oocytes [13]. Moreover, VAMP3 was below the detection level in our oocyte samples preparation, and a lower molecular weight form of SNAP23 was observed (Fig. 1A immunoblot and Supplemental Fig. S2).

Peripheral Migration of Secretory Granules and VAMP During Oocyte Maturation Indicate SNARE-Mediated Docking of CGs to the Oolemma

Because we found that SNARE proteins are enriched in the cortex of M-II arrested oocytes, we further analyzed whether this localization pattern is caused by a redistribution of SNARE proteins during oocyte maturation. To this end, immature GV-stage oocytes were harvested from follicles and were stained for the SNARE proteins. Simultaneously, CGs were identified by labeling with secretory granule-specific binding lectin (i.e.,

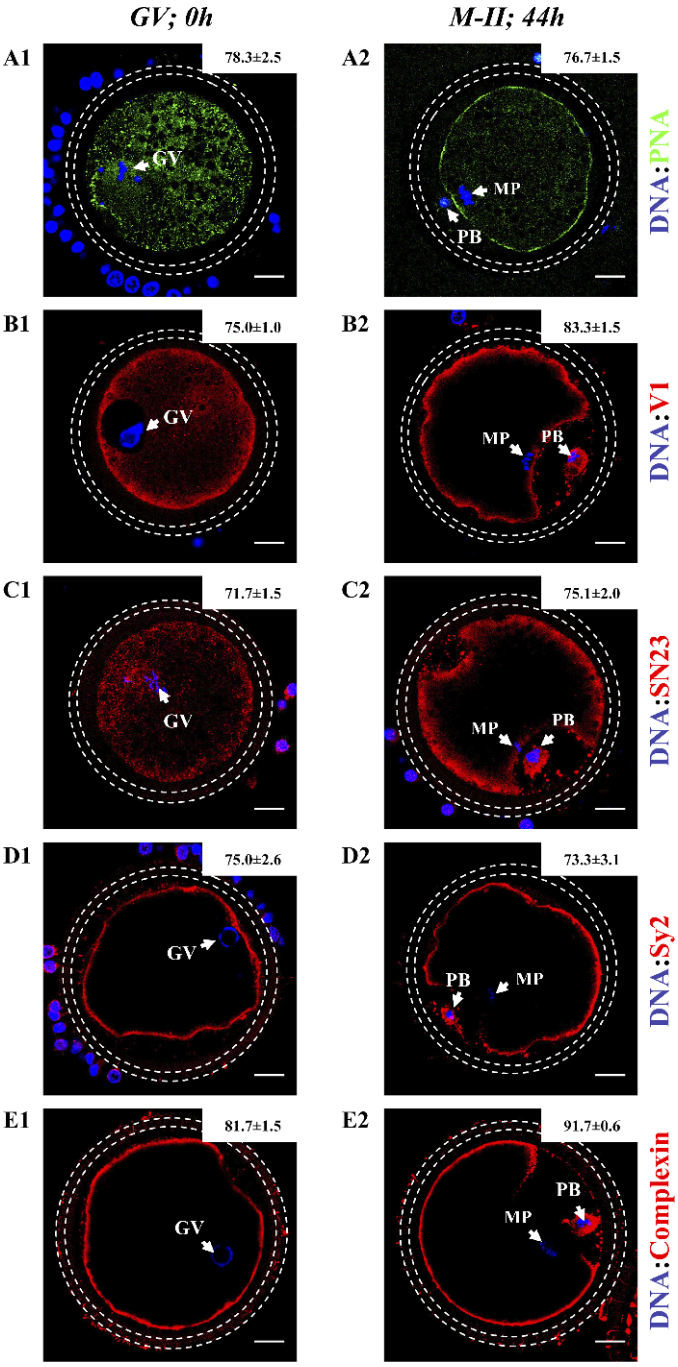


FIG. 2. Secretory granules and SNARE proteins migrate to the oocyte surface upon meiotic maturation. PNA-FITC was used to label secretory granules (green) and to demonstrate the migration of these granules upon oocyte maturation. Antibodies against different SNARE proteins and SNARE regulator (complexin) were used to indicate the localization of these proteins (red) at different maturation stages. DNA of the maturating oocytes was labeled with ToPro-3 (blue). Oocytes of two maturation stages (0 h: GV, and 44 h IVM: M-II) were used. PNA-positive secretory granules and SNARE-positive membrane vesicles showed dispersed punctuate pattern throughout the entire ooplasm at the GV stage (**A1**–**C1**), and syntaxin 2 was observed already in association with oolemma in GV oocyte (**D2**). Both PNA-positive secretory granules and SNARE-positive membrane showed either a pronounced oocyte surface-enriched (or oolemma-associated) staining pattern (**A2**–**B2**, **D2**) or they were concentrated to the cortex of the oocyte in M-II arrested oocytes (**C2**). Complexin in both GV and M-II stages showed a sharply plasma membrane-associated pattern (**E1**–**E2**). GV, germinal vesicle; PB, first polar body; MP, metaphase plate; V1, VAMP1; SN23, SNAP23; Sy2, syntaxin 2. Dashed circles indicate the relative position for the zona pellucida. Representative images of three independent experiments (n = 20 per group per experiment) are presented; the percent of oocytes with this labeling ± SD are indicated, while the other oocytes either had aberrant or very dim labeling. Bars = 20 μm.

PNA-FITC). During maturation, the CGs moved to a sharply defined region under the oolemma as was shown by changes in PNA labeling (Fig. 2, A1 and A2). A similar relocalization was observed for VAMP1-labeled structures (Fig. 2, B1 and B2). SNAP23-labeled structures, however, showed a similar peripheral redistribution but were not present under the oolemma (Fig. 2, C1 and C2). In contrast to the maturation-dependent redistribution of secretory granules and the above mentioned SNARE protein relocalization of VAMP1 and SNAP23, we observed that syntaxin 2 labeled structures did not show relocation upon meiotic maturation, being already associated with the oolemma at the GV stage and remaining there until the end stages of oocyte maturation (Fig. 2, D1 and D2).

The detected concentration of VAMP1- and PNA-positive structures just under the surface of the oolemma in M-II stage oocytes suggests that the CGs become docked at the oolemma by the use of SNARE proteins. SNARE-mediated membrane priming and docking requires additional components such as complexin to stabilize the formed SNARE complex [15]. Therefore, we subsequently analyzed maturing oocytes for the presence of complexin. Our results showed that the presence of complexin was confined to a sharply defined region underneath the oolemma in M-II oocytes (Fig. 2E2). However, in contrast to the redistribution of PNA- and VAMP1-positive structures, no redistribution of complexin was observed because it was already present near the oolemma in the GV-stage oocytes (Fig. 2E1) and remained at this location. This suggests that docking of secretory granules to the oolemma is mediated by SNARE proteins, and the subsequent involvement of complexin present in close proximity to the oolemma could contribute to the stabilization of this interaction and thus prevent exocytosis. To examine this possibility, we performed a series of colocalization studies in both immature (GV) and matured (M-II) unfertilized oocytes. We found in the majority of the immature oocytes that PNA-containing cortical granules and syntaxin 2 as well as VAMP1 to some extent colocalized at the oolemma but not at other areas in the oocyte (Fig. 3 arrows and Table 1). Moreover, in the matured M-II oocytes, the amount of colocalization between PNA-positive structures (i.e., CGs) and SNARE proteins was substantially increased at the oocyte surface (see Fig. 4, where the colocalization is indicated with arrows as well as in the enlarged panels, and Table 1), and this colocalization also matched the topology of complexin.

Functional Implications of CG Docking to the Oolemma for Endocytosis in Maturing Oocytes

Cortical reaction is a calcium-dependent exocytotic process, and in most of the cell types, exocytosis and endocytosis are indirectly coupled processes. Therefore, we extended our investigations with the immunolocalization of endocytotic proteins during oocyte maturation. Immunolocalization of clathrin (for clathrin-dependent endocytosis) showed that clathrin was not only present at the oolemma but also in intracellular vesicular structures in GV oocytes (Fig. 5A1). Clathrin migrated to the same sharply defined area just under the oolemma during oocyte maturation (Fig. 5A2). This indicated that clathrin was targeted to the oolemma (and/or oolemma-docked structures) in M-II oocytes and was not

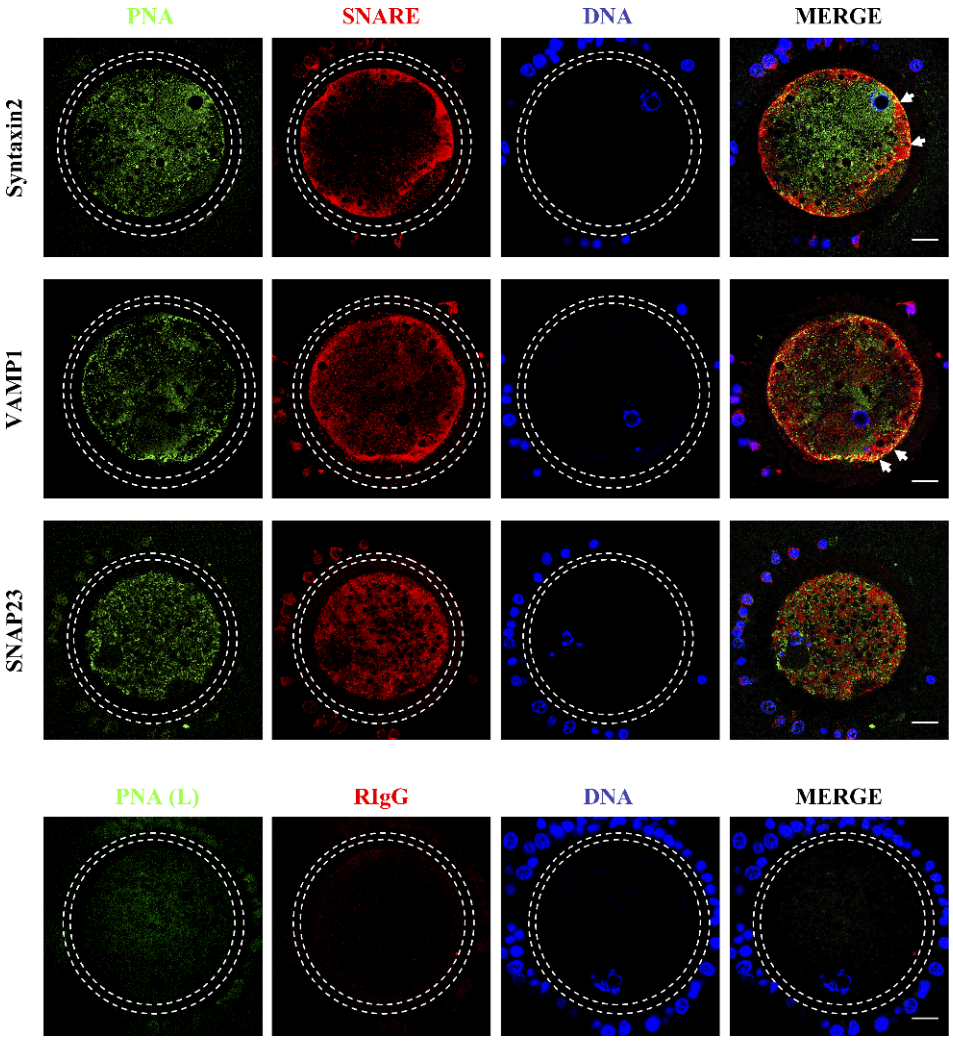


FIG. 3. Minimal colocalization of cortical granules and SNARE proteins in GV oocyte. Oocytes of germinal vesicle stage were used to demonstrate the distribution of cortical granules (labeled with PNA-FITC, green) and SNARE proteins (indirect immunolabeling, red). Chromatin was stained with ToPro-3 (blue). The majority of the secretory granules did not associate with SNARE proteins in GV-arrested oocytes; a small amount of colocalization between cortical granules and SNARE proteins can be observed just underneath the oolemma (arrows). Rabbit IgG (RIgG) was used as a negative control for SNARE proteins, demonstrating the absence of signal. PNA (L): PNA-binding sites were inhibited with 100 mM lactose, which was used as a negative control for PNA labeling. Dashed circles indicate the relative position for the zona pellucida. Representative images of three independent experiments (n = 20 per group per experiment) are presented. Bars = 20 μm.

sequestered further by endocytosis or released deeper in the oocyte. Another protein-mediated endocytotic pathway is initiated by the formation of membrane caveolae enriched with flotillin and caveolin. Both membrane raft marker proteins were present in oocytes, but they did not show obvious redistribution upon oocyte maturation (Fig. 5, B1–B2 and C1–C2).

Cortical Reaction Leads to Clathrin and Complexin Dissociation from the Oolemma

The fusion of a sperm cell with the oocyte leads to the initiation of the CR. During the CR, CGs fuse with the oolemma. Because this results in an enlargement of the oocyte surface, a compensatory reduction of this surface must be accomplished by an immediate induction of endocytosis. We tested whether fertilization induces the release of complexin and clathrin and as such reestablishes membrane traffic (exocytosis and endocytosis at the oolemma) in the fertilized oocyte. For this reason, we have analyzed the fate of SNARE proteins, complexin, and clathrin in fertilized oocytes until 8 h after IVF. At this stage, fertilized oocytes contain both a male and female pronucleus (Fig. 6, A–E). As expected, the intracellular PNA staining decreased below the detection level when compared to M-II oocytes as the CGs fused with the oolemma and the granule contents were released and diluted

TABLE 1. In vitro maturation coincides with colocalization of cortical granules and the SNARE proteins at the entire surface of the oocyte.[a]

	Partial colocalization[b,d]			Full colocalization[c,d]		
Oocyte stage	Syntaxin 2	VAMP1	SNAP23	Syntaxin 2	VAMP1	SNAP23
GV	70 ± 1.6	75 ± 2.6	75 ± 3.8	30 ± 0.9	25 ± 2.1	25 ± 3.0
M-II	17.5 ± 0.6[e]	15.0 ± 0.3[e]	22.5 ± 5.3[e]	82.5 ± 1.4[e]	85.0 ± 4.2[e]	77.5 ± 6.1[e]

[a] In total, 40–60 oocytes were counted from three independent experiments.
[b] Colocalization at restricted areas of the oocyte's surface: as shown in Figure 3 with yellow spots randomly present in the oocyte's cytosol and/or at its surface.
[c] Colocalization at the entire area of the oocyte's surface: as shown in Figure 4 with a yellow ring-like pattern at the entire oocyte's surface.
[d] Numbers shown are the percentage ± SD of the oocytes having the same immunostaining patterns as shown in the corresponding figures.
[e] A significant increase of colocalization at the surface of M-II oocytes was observed ($P < 0.005$).

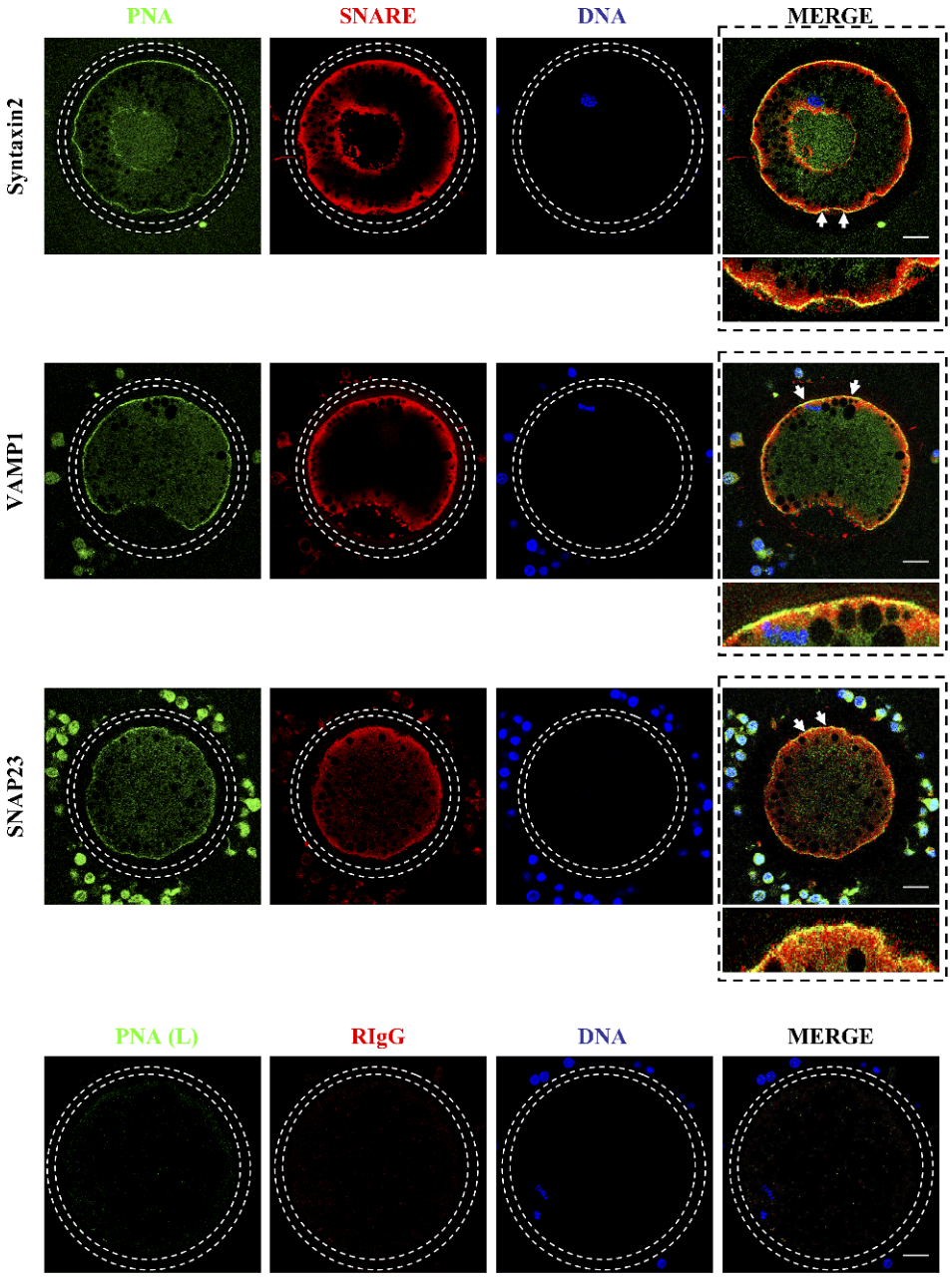


FIG. 4. Cortical granules are docked to the oolemma and colocalized with SNARE proteins in M-II-arrested oocytes. Oocytes of meiosis-II stage were used to demonstrate the colocalization of cortical granules (labeled with PNA-FITC, green) and SNARE proteins (indirect immunolabeling, red). Chromatin was stained with ToPro-3 (blue). Cortical granules are positioned in close proximity to the oolemma and show co-localization (indicated with arrows) with SNARE proteins over the entire oocyte surface. Areas indicated by the arrows are shown in the enlarged panels and clearly indicate colocalization. PNA (L): PNA-binding sites were inhibited with 100 mM lactose, which was used as a negative control for PNA labeling. Dashed circles indicate the relative position for the zona pellucida. Representative images of three independent experiments (n = 20 per group per experiment) are presented. Bars = 20 μm.

(Fig. 6A). In the fertilized oocytes, the CR coincided with the release of complexin from the oocyte's surface into the cytosol and surrounding lipid droplets (Fig. 6B), most likely a result of the dissociation of complexin from the SNARE protein complex. When SNARE distributions of fertilized and unfertilized mature M-II oocytes were compared, VAMP1 was found to be present at intracellular structures (Fig. 6C), indicating that VAMP1 was endocytosed or at least the VAMP1-containing membrane structure was transported away from the oolemma (where they were originally located, see Fig. 1A) after fertilization. The noted release of clathrin into the cytosol may be of importance for reestablishing endocytosis at the oolemma after the CR (Fig. 6D).

While the majority of the metaphase II-arrested oocytes were fertilized by one sperm (55% of all oocytes, Fig. 6), a relative low number of oocytes remained unfertilized (18%) and also some oocytes showed polyspermic fertilization (27% of all oocytes, Fig. 6, F–J), which is normal after porcine IVF. Both monospermic-fertilized and polyspermic-fertilized oocytes (Fig. 6, A–E) showed similar normal cortical secretion of PNA-containing vesicles (Fig. 6, A and F) and redistribution of complexin1/2 (Fig. 6, B and G). In contrast, VAMP1 and clathrin did not demonstrate redistribution into intracellular membrane structures in polyspermic-fertilized oocytes (Fig. 6, H and I) but did demonstrate redistribution in monospermic-fertilized oocytes (Fig. 6, C and D). Syntaxin 2 did not change its distribution after both types of fertilization (Fig. 6, E and J).

DISCUSSION

Precise regulation on the CR is crucial because premature or spontaneous CR blocks the entry of the sperm cell and belated CR leads to polyspermic fertilization. Both processes will result in the failure of fertilization. The CR is executed immediately after the sperm fuses with the oolemma [3] and involves fusion of a multitude of CGs with the oolemma. Most intracellular membrane fusion events are driven by a conformational change of ternary *trans*-SNARE complexes

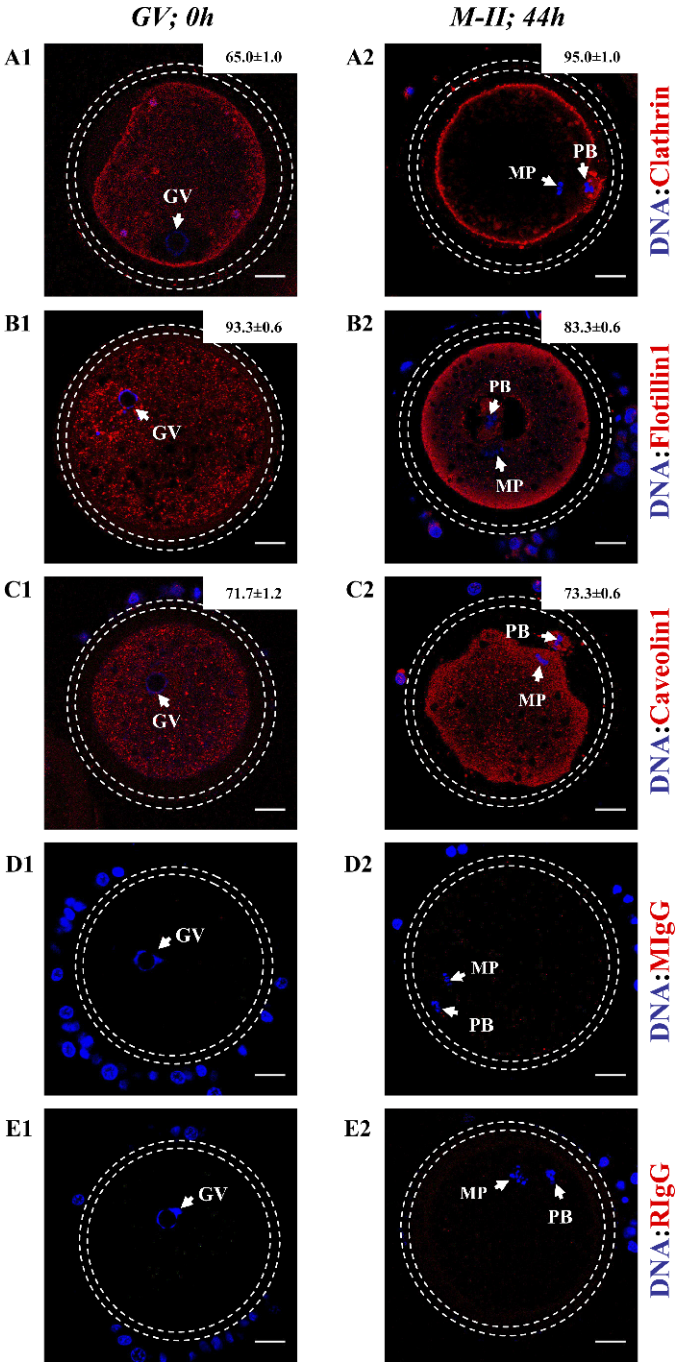
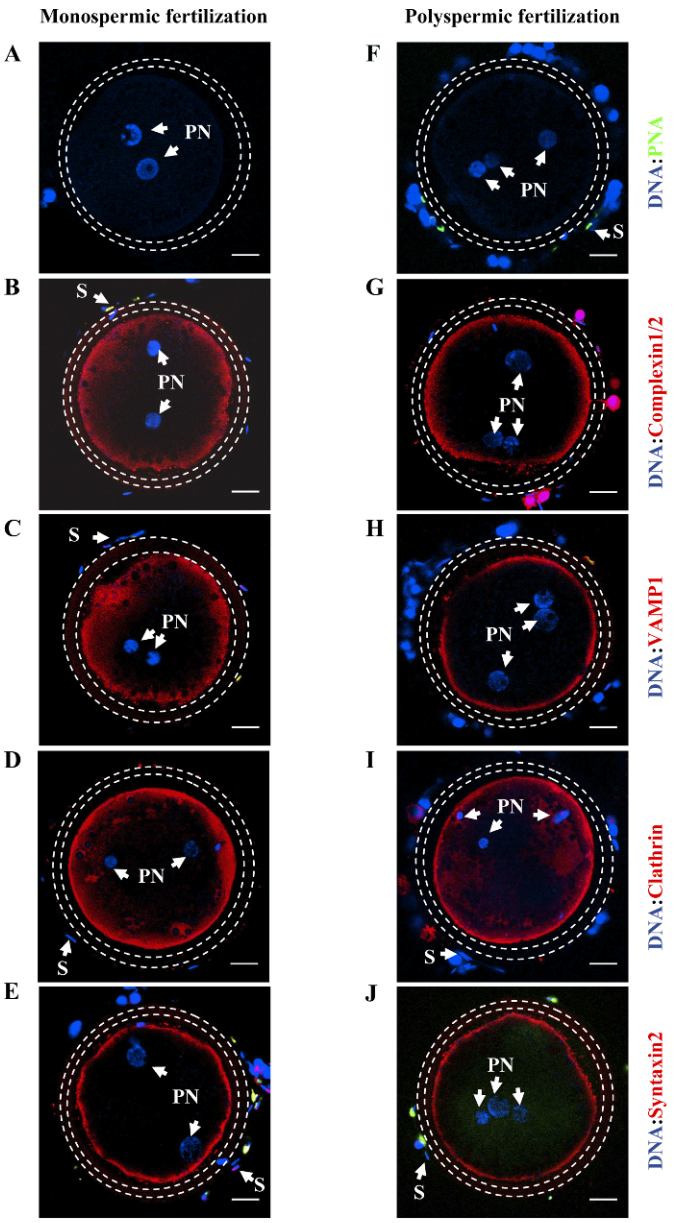


FIG. 5. Clathrin-dependent endocytosis is arrested in meiosis-II oocyte. Indirect immunolabeling of endocytosis-related proteins (red) and chromatin (blue) in two maturation stages (GV and M-II) oocytes. **A**) Clathrin migrated from the cytoplasm (**A1**) to the surface of the oocyte where it becomes arrested adjacent to the oolemma at the M-II stage (**A2**). **B1**–**B2**) Flotillin-1 concentrates to the cortex region of the oocyte but does not associate with or reside underneath the oolemma. **C1**–**C2**) Caveolin 1 does not show meiotic maturation-dependent redistribution. **D1**–**D2**, **E1**–**E2**) Purified mouse IgG (MIgG) and rabbit IgG (RIgG) were used as negative controls, demonstrating the absence of signal. GV, germinal vesicle; PB, first polar body; MP, metaphase plate. Dashed circles indicate the relative position for the zona pellucida. Representative images of three independent experiments (n = 20 per group per experiment) are presented; the percent of oocytes with this labeling ± SD are indicated, while the other oocytes either had aberrant or very dim labeling. Bars = 20 μm.

(which is formed by t- and v-SNARES of the two interacting membranes) into a *cis*-SNARE complex, merging the two fusing membranes (for a review, see [20]). Therefore, in this study, we have investigated matured oocytes that were ready to be fertilized and detected the presence and the spatial organization of SNARE proteins in relation to the secretary granules.

The maturation-dependent migration of secretory granules to the cortical area of the oocyte serves to prepare the oocyte surface for the CR. Studies on bovine oocytes suggest a positive role for ubiquitin C-terminal hydrolase-L1 (UCHL1) in the migration of secretory granules to the cortex area of the oocyte, which may contribute to the prevention of polyspermic fertilization [21]; however, whether or not UCHL1 plays a similar role in porcine oocytes and whether or not UCHL1 contributes to the SNARE-mediated cortical reaction is unclear. After their arrival at the oolemma in M-II oocyte, the CGs await for the proper signal—calcium oscillations in the ooplasm induced by sperm-specific phospholipase C zeta [5, 22]—without the occurrence of spontaneous exocytosis. It is known that SNARE and their associated proteins, for example, complexin and synaptotagmin, regulate acrosome reaction in the sperm cell [12, 23]. Thus, the interactions between SNARE proteins and their regulators as well as their importance in the regulation of the CR were studied. SNAP25 has been shown to be required for CG exocytosis in mouse oocytes as evidenced by significant inhibition of sperm-induced CG exocytosis in botulinum neurotoxin A-treated oocytes; the toxin has been shown to inhibit SNAP25-specific exocytosis [13]. However, the actual mechanism behind the regulation of the CR is essentially unknown. The low abundance of SNAP25 in the porcine oocyte—at least in this study, it was below the detection level—indicates that gametes from different mammals may employ distinct subsets of SNARE proteins for this highly specialized exocytosis process. Further support for the idea that SNARE proteins are involved in the CR was the detection of SNARE complex-composing proteins (syntaxin 2, VAMP1, and SNAP23) at the restricted CR upon meiotic maturation and the significantly increase in colocalization of SNARE proteins with CGs at the oocyte's surface. The consistent presence of complexin at the oolemma further supports the idea that SNARE protein complexes were stabilized by complexin and that this interaction could prevent premature or spontaneous CR.

Complexin has been shown to associate with ternary *trans*-SNARE complex and can subsequently inhibit the fusion reaction of the interacting membranes [15, 24], although such an involvement has not been studied for oocytes. We found that complexin 1/2 was released to the cytosol with a concomitant endocytosis of VAMP1, which was detected in intracellular membrane structures. This suggests that SNARE-complexin interactions may contribute to the temporary arresting of the docked CGs at the oolemma, thus further preventing the spontaneous fusion of granule membrane with the oolemma, which has been reported for other cell types [15]. The fertilization-dependent increase in cytosolic calcium levels probably releases complexin from the SNARE complex and thereby allows the onset of the SNARE-mediated CR. Most likely, the dissociation of complexin is mediated by calcium mobilization from the oocyte's ER by soluble signaling factors originating from the fertilizing sperm [22]. Consequently, during the CR, calcium-dependent dissociation of complexin from the *trans*-SNARE complex triggers the onset of massive exocytosis of granules. The CR-induced release of complexin into the cytosol can lead to interactions of complexin with SNARE proteins at intracellular membranes.



status	Exp. 1	Exp. 2	Exp. 3	Total
Monospermic	9	13	11	33 (55%)
Polyspermic	6	4	6	16 (27%)
Non-fertilized	5	3	3	11 (18%)
Total	20	20	20	60

FIG. 6. Cortical reaction releases enzymatic content and initiates the endocytosis-driven recycling of VAMP1 and the disassociation of complexin into the cytosol. Indirect immunolabeling of endocytosis-related proteins (red), chromatin (blue), and cortical granules (green, stained with PNA-FITC) in oocytes 8 h after IVF. Both monospermic-fertilized (**A**–**E**) and polyspermic-fertilized (**F**–**J**) porcine oocytes are shown. **A**, **F**) The cortical reaction has taken place as evidence from the absence of PNA labeling of cortical granules in the oocytes. **B**, **G**) Complexin 1/2 are released from the SNARE complex after sperm-induced fertilization and therefore have lost their oolemma-specific orientation. A portion of complexin appears to be liberated in the cytosol while another part is detected around lipid droplets. However, this redistribution of clathrin is less apparent in the polyspermic-fertilized oocytes. **C**, **H**) VAMP1 is endocytosed into an interior area of the monospermic-fertilized oocyte as the defined oolemma-specific pattern is lost and VAMP1 is detected at intracellular structures inside the ooplasm. More pronounced plasma membrane-orientated staining was observed for VAMP1 in polyspermic-fertilized oocyte. **D**, **I**) Clathrin is released from the oocytes surface after fertilization and diffuses into the oocyte's cytosol when compared to the oolemma-specific pattern in M-II oocytes. **E**, **J**) Syntaxin 2 showed consistent plasma membrane-specific staining in both types of fertilized oocytes. PN, pronuclei; S, sperm cell (indicative of IVF). Dashed circles indicate the relative position for the zona pellucida. Representative images of three independent experiments (n = 20 per group per experiment) were presented. In all cases, >85% of the monospermic- and polyspermic-fertilized oocytes showed the type of labeling presented here. Bars = 20 μm. The relative percent of unfertilized, monospermic-fertilized, and polyspermic-fertilized oocytes is given in the lowest panel.

Exocytosis and endocytosis are indirectly coupled processes that occur simultaneously in cells in order to maintain a cell surface area and volume. Therefore, if exocytosis is inhibited by complexin, then complexin also will inhibit endocytosis in M-II oocytes. It is interesting that clathrin concentrated in the same area where the CGs were docking and were being stabilized with complexin. Probably this serves to allow a rapid onset of endocytosis at the oocyte surface to compensate for the surface enlargement, which explains the accumulation of surface-associated clathrin in matured unfertilized (M-II) oocyte. Taken together, the oocyte undergoes preparative changes during meiotic maturation that prepare its surface for the CR and the subsequent compensatory endocytosis that is required to reestablish normal cellular processes. The less apparent migration of caveolin and flotillin during meiotic maturation may indicate that caveolin- and flotillin-mediated endocytosis are not directly involved in the compensatory reaction to the enlarged oolemma surface that results from the CR, but instead these proteins are more relevant to the later development of the embryo [25].

The absence of PNA-binding site in fertilized oocytes was due to the release of granule contents during the exocytosis of the CGs. Intriguingly, the released PNA was also not detected in the perivitelline space after CR which might be explained by 1) a dilution and diffusion of PNA-binding sites through the ZP and 2) a post-CR modification of the lectin-binding site that reduces or eliminates its affinity for PNA.

In conclusion, we showed that secretory granules required for the CR become associated with a SNARE complex containing syntaxin 2, VAMP1, and SNAP23 in M-II arrested porcine oocytes. We hypothesize that the docked CGs become stabilized and temporarily arrested by complexin in order toprevent prefertilization exocytosis, which would cause a premature fertilization block and which would result in a failure to fertilize. This then also indirectly shuts down membrane recycling by arresting clathrin-mediated endocytosis at the oocyte surface. Once these processes are resumed, namely, immediately following fertilization, the induced CR allows 1) secretion of PNA-binding sites, 2) compensatory recycling of VAMP1-containing membranes vesicles, and 3) dissociation of clathrin and complexin from the oocyte's surface into the cytosol and the subsequent reassociation of clathrin and complexin with intracellular membranes. Both VAMP1 recycling as well as clathrin and complexin redistribution are considered relevant for renewed membrane trafficking in the fertilized oocyte. Our study also showed that polyspermic-fertilized oocytes do not efficiently execute processes 2 and 3 but do execute process 1. This shows the possible relevance of the noted lack of postfertilization membrane recycling, as it may allow polyspermic fertilization of porcine oocytes. This is the first report showing the dynamics of SNARE proteins and complexin in forming an

arrested docking stage of CGs at the surface of the maturing oocyte. Furthermore, we have shown the fertilization-dependent removal of complexin and clathrin, which is needed to execute an immediate CR to achieve a polyspermic fertilization block.

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