Quantified Analysis of Cortical Granule Distribution and Exocytosis of Porcine Oocytes during Meiotic Maturation and Activation

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ABSTRACT

Polyspermy is one of the unresolved problems that exist regarding pig oocytes matured and inseminated in vitro. Quantitative study of the changes in the cortical granule (CG) population in oocytes is essential for understanding the mechanism of how oocytes block polyspermic penetration and for developing the optimum conditions for in vitro maturation (IVM) and in vitro fertilization (IVF). The present study was conducted to quantify the CG distribution in pig oocytes during IVM and IVF by using fluorescein isothiocyanate-labeled peanut agglutinin with laser confocal microscopy. The results indicate that CGs are distributed in the cortex cytoplasm of oocytes at the germinal vesicle (GV) stage with a mean number of 33.8 ± 7.3 CGs/100 μm² of cortex. As nuclear maturation proceeded to metaphase I and metaphase II, CGs migrated to the cortex and formed a continuous monolayer under the oolemma. No distinct CG-free domain was observed in oocytes during maturation. The migration of CGs to the cortex continued during maturation, with an increased CG density after the GV stage. All oocytes penetrated by spermatozoa were activated and released CGs from ooplasm with an average residual number of 3.5 ± 4.6 CGs/100 μm² of cortex at 18 h after insemination. Complete CG exocytosis was observed in 45% of oocytes. Calcium ionophore did not induce oocyte nuclear activation, but CGs were released from oocytes with an average of 7.1 ± 4.5 CGs/100 μm² of cortex still present when examined 18 h after treatment. An electrical pulse induced 89% of nuclear activation in matured oocytes, and CG exocytosis was observed only in nuclear-activated oocytes with an average residual number of 6.4 ± 9.4 CGs/100 μm² of cortex. Complete CG exocytosis was induced by ionophore and electrical pulse in 10% and 25% of the oocytes, respectively. These results indicate that CGs migrate to the cortex in pig oocytes during IVM and that the matured oocytes obtained under these maturation conditions possess the ability to release CGs upon sperm penetration, ionophore treatment, and electrical pulse. However, a functional block to polyspermic penetration in oocytes after CG exocytosis was not fully established in these studies. The present methods and results provide the approach for further investigation of the reasons for polyspermy in pig oocytes matured and inseminated in vitro.

INTRODUCTION

Polyspermic penetration has been one of the unresolved problems related to pig oocytes matured and inseminated in vitro [1]. Polyspermic penetration results in early embryo death, which greatly limits the production of useful pig embryos. It is well known that cortical granules (CGs) play an important role(s) in the block to polyspermic penetration in mammalian oocytes [2]. Upon sperm penetration and activation, oocytes release CGs; these disperse in the perivitelline space, forming a CG envelope [3] that releases enzymes that induce protein changes in the zona pellucida [2]. By these modifications, the oocytes establish the functional block to polyspermic penetration.

The CGs, a specialized group of membrane-bound secretory granules composed of specialized enzymes and glycoproteins, are found mostly in the cortex of unpenetrated oocytes. In the time since the presence of CGs in mammalian oocytes was discovered by Austin [4], many electron microscopy studies have been done on the structure and function of these organelles [5, 6]. Although the results contributed to an understanding of the structure and function of CGs, the methods were laborious, and only a small number of oocytes and a limited portion of plasma membrane could be analyzed. Recently, a method that uses the Lens culinaris agglutinin as a molecular probe for detecting CGs in hamster [7], mouse [8], and bovine [9, 10] oocytes has been developed. Using this method, Ducibella and co-workers, under light microscopy, analyzed the detailed distribution of CGs in mouse oocytes during meiotic maturation [8, 11], fertilization [8, 12, 13], and activation [11-14], and provided important evidence for understanding the characteristics of CGs in mouse oocytes. Yoshida et al. [15] also reported a staining technique for CGs in pig oocytes and found that a lectin from arachis hypogaea (peanut agglutinin, PNA), which is specific for β-D-gal(1→3)-D-gal-NAc, could be used as the molecular probe for staining the contents of CGs in pigs. They provided evidence that the results obtained by lectin staining are not only comparable to, but also more effective than, those obtained by electron microscopy because the intact oocyte could easily be observed [15]. However, there has still been no systematic report that quantifies the CG population in pig oocytes matured and inseminated in vitro. Therefore, the present study was conducted to quantify the CG changes of pig oocytes during in vitro maturation (IVM) and in vitro fertilization (IVF). Calcium ionophore treatment and electrical stimulation were also used in the present study because it has been reported that these treatments could mimic sperm penetration in inducing CG exocytosis [2, 5, 6, 16].

MATERIALS AND METHODS

Medium

The basic medium used for maturation and in vitro fertilization of oocytes, designated TCM-199B, was composed of tissue culture medium (TCM) 199 (with Earle's salts; Gibco, Grand Island, NY) supplemented with 3.05 mM glucose, 2.92 mM calcium lactate, 0.91 mM sodium pyruvate,
75 µg potassium penicillin G/ml, 50 µg streptomycin sulphate/ml, and 10% (v:v) heat-inactivated fetal calf serum (FCS; Gibco). This medium was exactly the same as that used by Wang et al. [17].

**Preparation and Culture of Oocytes**

The collection and culture of oocytes were based on the procedures reported by Wang et al. [17]. Briefly, oocytes were aspirated from antral follicles (2–5 mm in diameter) of ovaries collected from maturing gilts at a local slaughterhouse with an 18-gauge needle fixed to a 10-ml disposable syringe. After being washed 4 times with TCM-199B supplemented with 0.57 mM cysteine (Sigma Chemical Company, St. Louis, MO), 10 IU eCG/ml (Serotropin; Teikoku-Zoki Co., Tokyo, Japan), and 10 IU hCG/ml (Purbogen; Sankyo Co., Tokyo, Japan), 10 oocytes surrounded by compact cumulus were transferred to a 100-µl drop of the same medium, which had been previously covered with warm paraffin oil in a polystyrene culture dish (35 × 10 mm, Falcon 1008; Becton Dickinson Labware, Lincoln Park, NJ) and equilibrated in an atmosphere of 5% CO₂ in air for more than 3 h. The oocytes were cultured for 0, 26, and 46 h at 38.5°C under the same atmospheric conditions. In some experiments, aged oocytes cultured for 72 h and cumulus-free oocytes cultured for 46 h were also used. For the latter, follicular oocytes were completely denuded of cumulus cells by mechanical pipetting before culture. After culture, all oocytes were freed from cumulus and corona cells (if present) by treatment with maturation medium containing 0.1% hyaluronidase, obtained from bovine testis (Sigma), followed by repeated passage through a fine pipette. Such oocytes were used for assessment of CGs or for induction of exocytosis of CGs.

Because high proportions of matured oocytes (metaphase II [M-II]) could be obtained after culture of the oocytes for 46 h, these oocytes were used to evaluate CG exocytosis after IVF, calcium ionophore (A23187) treatment, and electrical pulse stimulation.

**IVF of Oocytes**

Sperm-rich fractions of ejaculates were obtained from large white boars using the gloved-hand method. After collection, 150 µg penicillin/ml and 100 µg streptomycin sulphate/ml were added to the semen, which was kept in a 16°C incubator for 16–24 h before use. For IVF, a 0.5-ml semen sample was washed 3 times with TCM-199B supplemented with 10% FCS; the concentrations of washed spermatozoa were subsequently adjusted to 2–3 × 10⁶ cells/ml in the fertilization medium, and 50 µl of the sample was added to the 50 µl of fertilization drops to which oocytes and 10 mM caffeine-sodium benzoate (Sigma) had been added immediately before insemination. This resulted in final concentrations of spermatozoa and caffeine of 1–1.5 × 10⁶ cells/ml and 5 mM, respectively. Six to 7 h after insemination, oocytes were removed from fertilization medium, washed 4 times, and cultured for another 11–12 h in TCM-199B for assessments of sperm penetration, nuclear activation, and CG exocytosis.

**Calcium Ionophore Treatment of Oocytes**

Cumulus-free oocytes were transferred to TCM-199B containing 30 µM A23187 (Calbiochem Corporation, La Jolla, CA) and incubated in a CO₂ incubator for 1 h. After treatment, oocytes were washed 4 times and cultured for 18 h in TCM-199B in preparation for examination of CG exocytosis.

**Electrical Pulse of Oocytes**

The method used for oocyte activation by electrical pulse was essentially the same as that reported by Sun et al. [16]. Briefly, the oocytes, after being washed 3 times in the pulse medium (0.3 M mannitol, 100 µM CaCl₂, 100 µM MgCl₂, and 0.1% polyvinyl alcohol), were stimulated by one pulse of 10 sec at 5 V/cm AC, followed by two pulses of 60 µsec, separated by 0.2 sec pulse interval, at 1.0 KV/cm DC field strength, in an electron cell manipulator (BCM200; Biotechnologies & Experimental Research Inc. San Diego, CA). After pulses were delivered, the oocytes were washed 4 times and cultured for 18 h in the TCM-199B for examination of CG exocytosis.

**Staining of CGs**

The methods for staining CGs were based on those reported by Yoshida et al. [15] with a few modifications. For assessment of CG distribution in maturing oocytes, cumulus-free oocytes were used, and for assessment of cortical reaction induced by different stimulators, the zona pelliculidae of oocytes were removed by placing the oocytes in PBS (Sigma) containing 0.1% pronase (Sigma). After being washed 3 times, oocytes were fixed with 3.7% paraformaldehyde (Sigma) in PBS for at least 30 min at room temperature, followed by 3 washes in PBS containing 0.3% BSA (Sigma) and 100 mM glycine (Wako Pure Chemical Industry LTD, Osaka, Japan) for 5 min each. After treatment for 5 min in PBS containing 0.1% Triton X-100 (Sigma), oocytes were washed 2 additional times for 5 min. Oocytes were then cultured in 100 µg/ml fluorescein isothiocyanate (FITC)-labelled PNA (Sigma) in PBS for 30 min in a dark box. After staining, the oocytes were washed 3 times in PBS containing 0.3% BSA and 0.01% Triton X-100. Nuclear status of oocytes was evaluated by staining the oocytes with 10 µg/ml bis-benzamide (Hoechst 33342; Calbiochem) in PBS for 5 min. Finally, these oocytes were washed and mounted on nonfluorescence slides and observed under a laser confocal and fluorescent microscope.

**Nuclear Evaluation and Assessment of CGs**

Stained oocytes were first scored for nuclear stage and sperm penetration under ultraviolet illumination. As shown in Figure 1, a–c, assessment of maturation of oocytes was based on the report by Wang et al. [17]. Oocytes were considered to be activated when they had formed a pronucleus or pronuclei (Fig. 1, d and e). After the nuclear stages were recorded, oocytes were observed under a laser confocal microscope (LSM410; Carl Zeiss, Oberkochen, Germany) with an argon laser (488 nm wave length). Two sections in each oocyte were observed at magnifications of ×400 or ×1000; one section, the largest optical section, is referred to as the equator of the oocyte, and the other is the top surface, referred to as the cortex of the oocyte. In each section, the image was obtained by repeated laser scanning (8 times/8 sec) to improve the signal-to-noise ratio. The numbers of CGs at the cortex were counted at four different areas in a square of 100 µm²; then the average number/100 µm² in each oocyte was obtained. If the number of CGs was small (in nuclear-activated oocytes), all numbers in the plane were counted, and then the numbers were transformed to number/100 µm². Typical examples of CG distribution in oocytes are shown in Figures 2–4.
TABLE 1. Nuclear maturation and cortical granule distribution in porcine oocytes during culture.

<table>
<thead>
<tr>
<th>Hours of culture</th>
<th>No. of oocytes examined</th>
<th>No. of oocytes at the stage of</th>
<th>Degenerated</th>
<th>No. of oocytes with CG monolayer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Germinal vesicle (%)</td>
<td>Metaphase I</td>
<td>Telophase I</td>
</tr>
<tr>
<td>0</td>
<td>70</td>
<td>70 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>26</td>
<td>80</td>
<td>7 (9)</td>
<td>71 (89)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>46</td>
<td>78</td>
<td>4 (5)</td>
<td>4 (5)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>46 (no cumulus)</td>
<td>45</td>
<td>4 (9)</td>
<td>10 (22)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>72</td>
<td>60</td>
<td>0 (0)</td>
<td>2 (3)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

**Statistical Analysis**

Numbers of CGs in oocytes at different stages or treated with different stimulations were calculated as mean ± SD. Data were compared by ANOVA, and when the probability was lower than 0.05, the differences were considered to be significant.

**RESULTS**

As shown in Table 1, all cumulus-enclosed oocytes were at the germinal vesicle (GV) stage (Fig. 1a) at 0 h of culture (just after collection). Most oocytes (89%) had progressed to metaphase I (M-I, Fig. 1b) after 26-h culture, but only 1% had reached M-II. When oocytes were cultured for 46 h, 90% matured to M-II (Fig. 1c). When culture time was prolonged to 72 h, 83% of oocytes were still at the M-II stage, but some (13%) had degenerated. More than half (53%) of the cumulus-free oocytes cultured for 46 h reached M-II.

CGs were present in the cortex cytoplasm in the oocytes at the GV stage, but most were within the 6-10 μm of cortex cytoplasm beneath the oolemma (Fig. 2b). In the cortex, there were about 33.8 ± 7.3 CGs/100 μm². Some (15 of 70; 21%) oocytes possessed CGs throughout the cytoplasm, but the number was very low, with an average of 2.9 ± 3.6/100 μm². When the oocytes underwent GV breakdown and reached M-I to M-II, almost all CGs migrated to the cortex and were located just beneath the ooplasm membrane and formed a monolayer (Table 1; Fig. 2, d and f). In comparison with CG density in the oocytes at the GV stage (Fig. 2a), the number of CGs in the cortex had increased significantly, with mean numbers at 46 and 26 h of culture of 45.3 ± 4.5 and 49.6 ± 9.6/100 μm², respectively, at M-I (Fig. 2c), 45.3 ± 6.2 and 53.2/100 μm², respectively, at M-II (Fig. 2e) in cumulus-enclosed oocytes (Table 2). In all M-II oocytes, CGs existed in the first polar body (Fig. 2f).

As shown in Table 2, in comparison to CG densities in cumulus-enclosed oocytes cultured for 26–46 h, lower CG densities were observed in oocytes cultured for 72 h with cumulus or 46 h without cumulus. The 72-h oocytes were considered aged oocytes because they were cultured for another 26 h after they had reached M-II, although only 13% of oocytes showed nuclear degeneration (Table 1).

The migration of CGs was not observed in oocytes at the GV stage even when they were cultured for 46 h (Tables 1 and 2).

FIG. 1. Fluorescence micrographs of nuclear status of pig oocytes during meiotic maturation (a-c) and activation (d, e), stained by H33342. a-c) Oocytes at the GV (a), M-I (b), and M-II (c) stages, which were cultured for 0, 26, and 46 h, respectively, in vitro. Bar = 36 μm. d) A polyspermic oocyte with both male and female pronuclei and some additional penetrated sperm heads. This oocyte was examined 18 h after insemination. e) An oocyte stimulated by electrical pulse and examined 18 h after stimulation; two pronuclei have formed. Bar = 18 μm.
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CG exocytosis in matured cumulus-enclosed oocytes (cultured for 46 h), induced by sperm penetration, ionophore treatment, and electrical pulse, is shown in Table 3. All of the oocytes penetrated by spermatozoa (38 of 38), irrespective of monospermy (11 of 38; 29%) or polyspermy (27 of 38; 71%), were activated, forming one (4 of 38, 11%), two (20 of 38, 53%; Fig. 1d), and more than two (14 of 38, 37%) pronuclei. The mean number of intact CGs was 3.5 ± 4.6/100 μm², but no difference was observed between monospermic (3.2 ± 4.9/100 μm²) and polyspermic (3.6 ± 4.6/100 μm²) oocytes. However, as shown in Figures 3 and 4, even at 18 h after insemination there still existed CGs at different densities in most of the oocytes. The oocytes treated with A23187 (55) did not form a pronucleus, but CGs were released from these oocytes with an average residual number of 7.1 ± 4.5/100 μm². Of 54 oocytes treated by electrical pulse, 48 (89%) were nuclear-activated (Fig. 1e), with an average CG number of 6.4 ± 9.4/100 μm²; and 6 (11%) were not activated, with an average CG number of 41.2 ± 3.9/100 μm². It was observed that 45%, 25%, and 10%, respectively, of the oocytes penetrated by spermatozoa, treated by electrical pulse, or treated by A23187, released all of the CGs from the ooplasm. When oocytes were treated with A23187, as shown in Figure 4d, some of the CGs fused together to form clusters, but this was not observed in oocytes penetrated by spermatozoa (Fig. 4b) or treated by electrical pulse (Fig. 4c).

DISCUSSION

The present study quantified the CG population of pig oocytes during meiotic maturation in culture, IVF, and artificial activation by calcium ionophore and electrical pulse. The results indicate that CGs are distributed in the cortex cytoplasm of oocytes at the GV stage (just after collection) and, as maturation proceeds, migrate to the cortex and occupy the area just beneath the oolemma to form a monolayer after the nuclear status reaches M-I to M-II. The number of CGs in the cortex significantly increased at M-I but did not increase further as the oocytes finished meiotic division (M-II), indicating that the migration is completed by M-I. The number of CGs in matured oocytes decreased significantly after penetration by spermatozoa and treatment with A23187 or electrical pulse, indicating that the oocytes cultured under the present experimental conditions possess the ability to release CGs. This is the first quantification of the CG distribution and CG exocytosis in pig oocytes matured and inseminated in vitro.

The migration of CGs to the cortex is a common phenomenon in mammalian oocytes during meiotic maturation not only in vivo but also in vitro [2, 5, 6, 18]. It is generally believed that the formation of a more or less continuous CG monolayer beneath the plasma membrane is a continuous process until ovulation. In the present study, we found that in pig oocytes cultured in vitro, this migration was almost completed at 26 h after culture, and at this time most of the oocytes had reached M-I. This was supported by the results that the CG distribution (Fig. 2, d and f) and the number (Table 2) of CGs in oocytes cultured for 26 and 46 h were the same. In the latter period, i.e., during the transition from M-I to M-II, there is a transformation from light form to dark form [19] that may be more important than...
TABLE 3. Cortical granule exocytosis induced by sperm penetration (IVF), calcium ionophore (A23187), and electrical pulse.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of oocytes</th>
<th>Nuclear activated</th>
<th>Nuclear inactivated</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVF</td>
<td>38</td>
<td>38</td>
<td>0</td>
</tr>
<tr>
<td>A23187</td>
<td>55</td>
<td>0</td>
<td>55</td>
</tr>
<tr>
<td>Electrical pulse</td>
<td>54</td>
<td>48</td>
<td>6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CGs/100 μm² of cortex in oocytes (mean ± SD)</th>
<th>Nuclear activated</th>
<th>Nuclear inactivated</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVF</td>
<td>3.5 ± 4.6</td>
<td>7.1 ± 4.5</td>
</tr>
<tr>
<td>A23187</td>
<td>6.4 ± 9.4</td>
<td>41.2 ± 3.9</td>
</tr>
</tbody>
</table>

- Oocytes were examined 18 h after treatments; experiments were repeated for three (IVF) or four times.
- Oocytes that had formed at least one pronucleus were considered activated.

a Oocytes were examined 18 h after treatments; experiments were repeated for three (IVF) or four times.

The density in number of CGs varies not only from species to species, but also from oocyte to oocyte. It has been reported that matured oocytes contained 34 CGs/100 μm² of cortex in mice [8], 95/100 μm of oolemma in rats [20], and 60/100 μm of oolemma in rabbits [21]. According to a study by Cran and Cheng [19] with in vivo matured pig oocytes, there were about 10–13 CGs per 10 μm of the plasm membrane. In the present study, we observed 46 CGs/100 μm² of cortex in IVM oocytes. This number is lower than that obtained in oocytes matured in vivo [19]. Whether this difference is the result of different oocyte resources or different examining methods is unclear. The CG populations in pig oocytes matured in vivo and in vitro are being conducted in our laboratory. In contrast to the situation in the IVM oocytes, the numbers of CGs in oocytes before sperm penetration varied, with a range of 31–67/100 μm² of cortex. After sperm penetration, there was also a great variation (0–16/100 μm²) in CG density. We did not find any difference in CG exocytosis between polyspermic and monospermic oocytes. However, this may be due to the time of examination after insemination in this experiment if there was a delay from normal time in exocytosis of CGs. It is unknown whether the CG number after oocyte maturation reflects the degree of cytoplasmic maturation or the ability of CGs to block polyspermy after initial sperm penetration.

A polarity of CG distribution is found in mammalian eggs because of the absence of granules in the area immediately overlying the second meiotic spindle [22–24]. This area has been called the CG-free domain (CGFD) [8, 22, 24–26]. In matured mouse eggs, the CGFD is more distinct than in eggs of other mammals since CGs are absent in 39–41% of the egg hemisphere where the meiotic spindle is located [8]. Development of the CGFD in hamster and mouse oocytes is the result of local exocytosis and redistribution of CGs [8, 26]. However, no distinct CGFD has been observed in cat [27], bovine [10], or porcine (present experiment) oocytes during meiotic maturation, indicating that premature CG exocytosis may not occur in these species.

CGs decreased significantly under prolonged culture of oocytes (72 h) or culture in the absence of cumulus cells (Table 2). It has been reported that this decline of granules was a result of their slow migration into the deeper portions of the cytoplasm in rabbit [28] and hamster [23] eggs, but apparently not in mouse eggs [22]; nor does it seem to be the case in pig oocytes (present experiment) since we did not observe any migration of CGs to the deeper portion of cytoplasm. The CG monolayer in aged oocytes did become discontinuous, which is consistent with observations
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FIG. 4. Confocal microscopic images of oocytes cultured for 46 h and then stained with FITC-PNA 18 h after insemination (a: unpenetrated oocyte and b: penetrated oocyte), electrical pulse (c), and A23187 treatment (d), showing the densities of CGs in the oocytes. The evaluated CG numbers/100 \( \mu m^2 \) in each oocyte were 40 \( \pm \) 9.4 (a), 0.9 (b), 1 (c), and 10 \( \pm \) 3.6 (d). In a and d, five areas of 100 \( \mu m^2 \) were counted and the mean number was obtained, but in b and c, all CGs in the cortex were counted and the number per 100 \( \mu m^2 \) was obtained. Some fluorescent clusters were observed in the A23187 treated oocyte. Bar = 25 \( \mu m \).

in aged rabbit eggs [28]. When ovulation of rat oocytes is delayed for 48 h, the number of CGs per 100 \( \mu m \) of plasma membrane is reduced to half [20], but migration of CGs into the deeper portions of the ooplasm was not reported. The reason for CG loss during aging is not clear. It would appear that the decline of CGs in aged pig oocytes is the result of the empty granules [29], which could not be stained by lectin.

Fertilization in mammals is monospermic under normal conditions. When it occurs, polyspermy results in abnormal development and early embryonic death. It is believed that cortical reaction plays a critical role(s) in blocking polyspermy in mammalian oocytes [2]. However, when pig oocytes are inseminated in vitro under conditions, a high proportion of the penetrated oocytes are polyspermic [1]. According to a study in the mouse, CG exocytosis during IVM in medium containing serum does not result in zona modification [11] and thus not result in a premature block to polyspermy. It has been reported that dimethylsulfoxide could induce modification of zona pellucida glycoprotein and inhibition of sperm binding in mouse oocytes due to CG exocytosis, but the addition of serum was found to inhibit these effects without inhibiting CG exocytosis [30]. Thus in the present study, the failure of some oocytes penetrated by spermatozoa to exhibit a block to polyspermic penetration may be due to the fact that FCS inhibited the CG contents from acting on the zona pellucida modification since the CG exocytosis occurred after sperm penetration. The fertilization medium used in the present study was very effective for boar sperm capacitation [1], but it may not be suitable for the CG contents to complete their role(s) in zona pellucida modification, thus resulting in high polyspermic penetration. On the other hand, delayed CG exocytosis was also observed in pig oocytes inseminated in vitro [31]. Inclusion of FCS in fertilization medium also resulted in high polyspermy in bovine oocytes [32]. Sathanathan and Trounson [33] reported a delay in CG release in human oocytes fertilized in vitro, especially in polyspermic oocytes. Putting these results together, it would appear that it is the fertilization condition that resulted in this abnormal cortical reaction in pig oocytes. Some progress has been made by preincubation of boar sperm with follicular fluid [34] and oviduct cells [35] before insemination; however, a satisfactory medium for inseminating pig oocytes has not yet been developed.

Electrical stimulation can be used to activate mammalian ova. The activation rates of pig oocytes cultured in vitro were very high when stimulated by electrical pulse [16, 36]. The methods used in the present study can activate 89% of the matured oocytes to form a pronucleus or pronuclei and can induce most of the oocytes to release their CGs. Electrically stimulated pig oocytes were penetrable by spermatozoa in vitro [37], indicating that the role of CGs after exocytosis were not brought into play in that study. Calcium ionophore will induce CGs exocytosis [31] and nuclear activation [36] in the pig. However, the media and conditions for the treatments greatly affect the results. In the present study, when we used the insemination medium as an ionophore activation medium, CG exocytosis, but not nuclear activation, was observed. This result is consistent
with those observed in IVM pig oocytes treated in almost the same medium by Cran and Cheng [31]. Exposure of mouse eggs to calcium ionophore resulted in partial loss of CGs [12, 38]; the remaining CGs released upon sperm penetration [38]. Whether this would happen in pig oocytes needs to be examined.

In conclusion, the present results indicate that CGs migrate to the cortex during IVM and are released after IVF and artificial activation in pig oocytes under the present experimental conditions. It is necessary to quantify the CG distribution of pig oocytes matured in vivo, so we can compare those data with CG distribution of pig oocytes matured under in vitro conditions. The present IVF system can induce pig oocytes matured in vitro to release CGs after sperm penetration, but the modification in the zona pellucida and/or the CG fate after exocytosis to the perivitelline space, and to develop an effective medium for IVF of porcine oocytes. The methods used in the present study provide an approach for achieving this purpose.

ACKNOWLEDGMENTS

We thank Dr. T. Nagai for giving important advice during this study and B. Nichols for secretarial assistance in preparation of the manuscript.

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