The effect of protein kinase C activator and nitric oxide donor on oocyte activation and cortical granule exocytosis in porcine eggs

L. Tůmova, R. Romar, J. Petr and M. Sedníková

1Department of Veterinary Sciences, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences in Prague, 165 21 Prague 6-Suchdol, Czech Republic; 2Department of Physiology, Faculty of Veterinary, University of Murcia, 30100 Murcia, Spain; 3Institute of Animal Science, Plátělství 815, 110 00 Prague 10 - Uhříněves, Czech Republic

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Nitric oxide (NO) and protein kinase C (PKC) are involved in the activation of mammalian oocytes, although their role in the exit from the metaphase II stage and cortical granule (CG) exocytosis is still not fully understood. The aim of this study was to verify whether the NO-donor together with specific PKC-activators induce the complete activation of porcine oocytes assessed as meiosis resumption and a cortical reaction. Pig maturated oocytes were treated with the NO-donor S-nitroso-N-acetylpenicillamine (SNAP, 2 mM) or PKC-activators such as phorbol-12-myristate-13-acetate (PMA, 100 nM), 1-oleoyl-2-acetyl-sn-glycerol (OAG, 400 µM) and L-α-phosphatidylinositol-3,4,5-trisphosphate dipalmitoyl heptammonium salt (DPAM, 2 µM). To study the combined effect of NO-donor and PKC-activators, aliquots of oocytes were also incubated with SNAP (0.5 mM) together with PKC-activators at the same concentration as above (SNAP–DPAM, SNAP–OAG and SNAP–PMA groups). After in vitro maturation, an aliquot of oocytes was placed in a fresh medium without NO-donor or PKC-activators (Control group). Another aliquot of oocytes was activated by calcium ionophore A23187 (25 μM, 5 min). The results showed that 0% of the control oocytes reassumed meiosis. However, both the PKC-activators (DPAM 44.0 ± 10.0%, OAG 63.3 ± 1.0% and PMA 45.0 ± 16.5%) as well as the NO-donor alone (48.7 ± 21.0%) significantly induced exit from MII. Interestingly, the combination of PKC-activators and SNAP mainly restrained to the meiosis resumption (SNAP–OAG 0, SNAP–DPAM 17.4 ± 2.5% and SNAP–PMA 38.4 ± 8.5%). Control oocytes did not show a cortical reaction and the area occupied by CG reached 25.9 ± 1.7%, whereas CGs were partially released after Ca²⁺ ionophore treatment (13.0 ± 3.2%). Treatment with PKC-activators induced a cortical reaction compared with the control group (8.6 ± 2.5, 6.7 ± 1.9 and 0.7 ± 0.4%, respectively, for DPAM, OAG and PMA groups). However, treatment with the NO-donor alone (SNAP group 17.2 ± 2.2%) or combined with any PKC-activator prevented cortical reaction (SNAP–DPAM 20.7 ± 2.6%, SNAP–OAG 16.7 ± 2.9% or SNAP–PMA 20.0 ± 2.4%). Besides, meiosis resumption was not always accompanied by a cortical reaction, indicating that these two activation events are independent. In conclusion, PKC-activators alone induce CG exocytosis to the same degree as calcium ionophore. However, an NO-donor alone or combined with PKC-activators is not able to induce a cortical reaction in pig oocytes.

Keywords: protein kinase C, nitric oxide, pig oocyte, activation, cortical granules

Implications

Parthenogenetic activation of mammalian oocytes is commonly used in reproductive biotechnologies, for example, cloning using nuclear transfer, and its success depends on an adequate artificial stimulus. Nitric oxide (NO) has been described as an oocyte activator but it does not induce a cortical reaction. Protein kinases C (PKC) play an important role in the cortical reaction but the PKC cascade is not triggered after oocyte activation by an NO-donor. There is a possibility of improving the results of oocyte activation by using an NO-donor together with PKC stimulation. We verified that this treatment induces meiosis resumption accompanied by a cortical reaction in pig oocytes. These results might lead to the optimization of oocyte activation using NO.

Introduction

Mammalian oocyte activation involves a sequence of morphological and biochemical events including an increase in intracellular calcium levels, cortical granule (CG) exocytosis and release from the second meiotic arrest. Under physiological
conditions, oocyte activation is induced by sperm penetrating the oocyte (reviewed by Williams, 2002). The sperm contains a sperm-specific phospholipase C isoenzyme (PLC; Saunders et al., 2002), which stimulates the production of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃), a common Ca²⁺-releasing compound, from phosphatidyl-inositol 4,5-bisphosphate (PIP₂). Elevation in intracellular calcium causes the fusion of CGs with oolemma. The enzymes released from the CGs into the perivitelline space modify the zona pellucida to prevent additional sperm penetration (Bleil et al., 1981; reviewed by Williams, 2002). Calcium oscillations also induce cyclin B degradation and cytostatic factor inactivation, allowing the female chromatin entry into anaphase II (Lorca et al., 1993).

Under in vitro conditions, various artificial stimuli increase the intracellular calcium levels, for example, electric pulses (Sun et al., 1992; Koo et al., 2008), calcium ionophore (Jilek et al., 2000 and 2001; Che et al., 2007) and chemicals that modulate the intracellular turnover of Ca²⁺ (Petr et al., 2000 and 2002) and are used for the parthenogenetic activation of porcine oocytes.

Protein kinase C (PKC) is one of the transducers of the calcium signal system. Several studies have demonstrated the possible involvement of PKC in the oocyte activation process (Colonna et al., 1997; Green et al., 1999; Sedmiková et al., 2006) and the exocytosis of CGs (Bement and Capco, 1989; Fan et al., 2002). PKC belongs to the serine/threonine kinase, and 12 isoforms are known, being classified into three groups. Conventional isoforms (PKC-α, -βI, -βII and -γ) are activated by free Ca²⁺ ions and DAG. Novel PKCs (nPKCs), represented by PKC-δ, -ε, -η, -μ, -θ, -ι and -λ isoforms, are independent of Ca²⁺ but can be activated by DAG. Finally, the PKC-ζ, -η, -τ isoforms belong to the atypical PKCs (aPKCs) and are independent of both Ca²⁺ and DAG (Liu and Heckman, 1998). The role of PKC activation in the exit from metaphase II of porcine oocytes has been studied using PKC-activators such as phorbol-12-myristate-13-acetate (PMA; Calbiochem, Germany), an activator of all isotypes of PKC, and 1-oleoyl-2-acetyl-sn-glycerol (OAG; Calbiochem, Germany), within 30 min of slaughter, ovaries from gilts were transported to the laboratory in saline containing 100 mg/ml kanamycin sulfate at 38.5°C, washed once in a 0.04% cetrimide solution and twice in saline. Oocyte—cumulus cell complexes (COCs) were collected from the antral follicles (3 to 6 mm diameter) and washed twice with Dulbecco’s phosphate-buffered saline (DPBS) supplemented with 1 mg/ml polyvinyl alcohol (PVA) and 0.005 mg/ml red phenol and twice more in maturation medium previously equilibrated for a minimum of 3 h at 38.5°C under 5% CO₂ in the air. Only COCs with a complete and dense cumulus oophorus were used for the experiments. Groups of 50 to 55 COCs were cultured in 500 µl maturation medium for 22 h at 38.5°C under a 5% CO₂ atmosphere. After culture, COCs were washed twice in a fresh maturation medium without dbcAMP, eCG and hCG, and cultured for an additional 20 to 22 h (Funahashi et al., 1997).

**Cultivation of oocytes with PKC-activators and an NO-donor**

The PKC-activators used were phorbol-12-myristate-13-acetate (PMA; Calbiochem, Germany), an activator of all isoforms of PKC, 1-oleoyl-2-acetyl-sn-glycerol (OAG; Calbiochem, Germany), and nitroprusside (SNP) are able to induce parthenogenetic activation in the same oocyte and no data showing the combined effect of PKC-activators together with an NO-donor on the cortical reaction and meiosis resumption. In pig oocytes, a cortical reaction can be triggered by several artificial stimuli (Sun et al., 1992; Okada et al., 2003), although the observed reaction is weaker when induced by a calcium ionophore and an electrical pulse (75.7% and 76.9% of CG release, respectively) than the one triggered by sperm penetration (86.3% CG exocytosis; Wang et al., 1998). Although many studies have shown that CG exocytosis is a PKC-dependent event, phorbol esters do not mimic the release of CGs exactly as in a fertilized mouse egg and CG exocytosis shows an atypical pattern (Ducibella and LeFevre, 1997). Furthermore, there is evidence that a cortical reaction can be triggered independently either by an increase in Ca²⁺ (Sun et al., 1997; Elyahu et al., 2005) or by PMA (Elyahu et al., 2005).

The aim of this study was to verify the hypothesis that an NO-donor together with a specific PKC-activator induces porcine oocyte activation accompanied by CG exocytosis.

**Material and methods**

**Culture media**

Unless otherwise indicated, all chemicals and reagents were purchased from Sigma-Aldrich Química S.A. (Madrid, Spain). The medium used for oocyte maturation was NCSU-37 (Petters and Wells, 1993) supplemented with 0.57 mM cysteine, 1 mM dibutyryl cAMP (dbcAMP), 5 mg/ml insulin, 50 mM β-mercaptoethanol, 10 IU/ml eCG (Foligon, Intervet International B.V., Boxmeer, Holland), 10 IU/ml hCG (Veterin Corion, Divisa Farmavic, Barcelona, Spain) and 10% porcine follicular liquid (v/v) (Romar et al., 2005).

**Oocyte collection and in vitro maturation**

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an activator of calcium-dependent PKC isotypes, and 1-cyano-2,6-diphenyl-1,3,6-piperidinetrione-3-carboxylate (CPI-11) as an NO-donor, 5-nitroso-N-acetylpenicillamine (SNAP; Axxora, Germany) was used. All PKC-activators were dissolved in dimethylsulfoxide (DMSO) in a stock solution (10 mM PMA, 40 mM OAG, 200 μM DPAM) and then diluted to the desired concentration for the experiments. The stock of SNAP (2 mM) was freshly prepared every day. The final DMSO concentration in all cases was 1%. After the in vitro maturation (IVM) period (42 to 44 h), cumulus cells were removed by pipetting, and denuded oocytes were treated for 10 h with an NO-donor or PKC-activators in fresh supplemented NCSU-37 without dbcAMP or hormones, at 35.5°C, under a 5% CO₂ atmosphere.

Assessment of oocyte activation

After treatment, the oocytes were fixed for 30 min (0.5% glutaraldehyde in DPBS), stained for 15 min (1% Hoechst 33342 in DPBS), washed in PBS containing 1 mg/ml polyvinylpyrrolidone and mounted on glass slides. The samples were examined under an epifluorescence microscope at 200× and 400× magnifications. The germinal vesicle and metaphase I oocytes were discarded from the study. Oocytes at metaphase II were not considered as activated. Activation was considered to have occurred when the oocytes reached the anaphase II, the telophase II or the pronuclear stage.

Assessment of CGs

The methods of CG visualization were based on those described by Yoshida et al. (1993), with modifications in the fluorescein isothiocyanate-lectin concentration (Coy et al., 2002; Romar et al., 2005). Briefly, after treatment with an NO-donor and PKC-activators, oocytes were washed three times in DPBS without calcium chloride (DPBS-w/o). Thereafter, the cells were fixed with freshly prepared 3.7% paraformaldehyde in DPBS-w/o (30 min, room temperature) and washed in DPBS-w/o. This was followed by treatment with freshly prepared 0.1% Triton X-100 in DPBS-w/o for 5 min and three washes in DPBS-w/o. Oocytes were then incubated in the dark with 10 μg/ml FITC-PNA (lectin from Arachis hypogaea conjugated to fluorescein isothiocyanate) in DPBS-w/o for 30 min, followed by three washes in DPBS-w/o. Finally, the oocytes were incubated with 5 μg/ml Hoechst 33342 for 10 min to stain DNA. After staining, the oocytes were gently washed in DPBS-w/o and an equilibration buffer medium (Slowfade® Antifade kit, Molecular Probes, Inc./Life Technologies Czech Republic s.r.o., Prague, Czech Republic). An adhesive ring was attached to a clean slide, 5 to 7 oocytes were transferred inside the ring with 4 μl of antifade mounting medium and finally protected with a coverslide sealed with nail polish. The samples were kept in the dark at 4°C until confocal assessment in <48 h.

The slides were examined under a 63× oil objective of a modular confocal microscope (Eclipse C1 Plus, Nikon, Tokyo, Japan) equipped with a 408 nm diode laser and a 488 nm Argon ion laser. All oocytes were observed under the same setting parameters (14% laser intensity, 87 gain and 1 μm thickness section) from the outer zona pellucida in one oocyte’s pole to the opposite pole. The nuclear stage was recorded by means of Hoechst fluorescence. The fluorescent emissions from the oocytes were recorded as TIFF files using the software attached to the microscope. The largest diameter section (equator of the oocyte) was used for further image analysis using MIP-4.5 Microm Image Processing software (Consulting de Imagen Digital S.L., Microm, Barcelona, Spain). The quantitative measurement of CG density (FITC fluorescence intensity) was performed by selecting the FITC-PNA-stained portion (area occupied by CG) in the space immediately below the oolemma, as we have previously described (Coy et al., 2002; Romar et al., 2005). Briefly, on each captured image, the oocyte’s cortical area was selected by drawing two concentric circles. The selected cortical area was processed for further image analysis and the area occupied by CG was calculated as the FITC-PNA-labeled area divided by the entire cortical selected area x 100. In each oocyte, the data of cortical area occupied by CG (%) were calculated and saved for further analysis. The degree of CG exocytosis was evaluated based on cortical area occupied by CG (%) and oocytes were classified into four different groups: complete exocytosis of CG (oocytes with 0% to 5% area occupied by CG), effective CG exocytosis (oocytes with 5% to 8% area), initial CG exocytosis (oocytes with 8% to 15% area) and no cortical reaction (oocytes over 15% area). This classification is based on previous studies with porcine oocytes showing that just after the IVM cortical area occupied by CG is around 20%, whereas in fertilized oocytes assessed 18 h post insemination, it is around 5% (Coy et al., 2002; Romar et al., 2005). Penetrated pig oocytes assessed at 4 h post insemination show 8% of the cortical area occupied by CG (Romar et al., 2012).

Experimental design

Experiment 1: effect of PKC-activators and an NO-donor on oocyte activation. Immediately after IVM, all the oocytes were denuded by pipetting and an aliquot (n = 10) was fixed to assess the nuclear status (IVM group). The remaining oocytes were randomly incubated for 10 h in a medium containing 2 μM DPAM (DPAM group), 400 μM OAG (OAG group), 100 nM PMA (PMA group), 2 mM SNAP (SNAP group) or the medium alone (Control group). To study the combined effect of an NO-donor and PKC-activators, some oocytes were also incubated for 10 h with SNAP (0.5 mM) together with PKC-activators at the same concentration as above: SNAP–PMA, SNAP–OAG and SNAP–DPAM groups. The concentrations of PKC-activators and the NO-donor were selected based on previous results (Petr et al., 2005 and 2011). Since DMSO has been reported to induce a loss of electrode density in pig CGs (Campagna et al., 2006), an aliquot of oocytes was also incubated for 10 h in a medium supplemented with 1% DMSO (DMSO group). As a positive control of oocyte activation, an aliquot of denuded IVM oocytes was exposed to calcium ionophore A23187 (25 μM in DMSO, 5 min; Jilek et al., 2001). After ionophore treatment, the oocytes were
washed and incubated for 10 h in NCSU-37 as with the rest of the groups (Ca-Io group). All oocytes in all the experimental groups were incubated for the same time. After treatment, oocytes from all groups were fixed and stained to observe the nuclear status and to evaluate oocyte activation. This experiment was replicated 4 times with 15 oocytes per group, except the IVM group (10 oocytes per replicate).

Experiment 2: effect of PKC-activators and an NO-donor on cortical reaction. The experimental groups (IVM, Control, DMSO, Ca-Io, DPAM, OAG, PMA, SNAP, SNAP–DPAM, SNAP–OAG, SNAP–PMA) were the same as in Experiment 1. Both after IVM and after the different treatments, the oocytes were processed for a confocal study. In each oocyte, the nuclear status was recorded and the rate of area occupied by CG was quantified. To study CG exocytosis, three repetitions (5 oocytes/group) were performed.

Statistical analysis
The data are presented as mean ± s.d., and all rates were modeled according to the binominal model of parameters. The data were analyzed by a one-way ANOVA with the treatment as a fixed factor. When ANOVA revealed a significant effect, differences among the experimental groups were determined using the post hoc Tukey test. The P-value < 0.05 was considered to indicate statistical significance.

Results

Experiment 1: effect of PKC-activators and an NO-donor on oocyte activation
The results showed that both after IVM (IVM group) and after 10 h in NCSU-37 medium (Control group), 100% of oocytes remained at metaphase II and any oocyte exited from this stage (Table 1). The addition of 1% DMSO to the medium did not affect the activation of oocytes (only 14.3 ± 8.0% were activated), but treatment with calcium ionophore did, and all PKC-activators induced the exocytosis of CG (Figure 1). Similarly, PMA, an activator of all PKC isoforms, activated 45.0 ± 16.5% of the oocytes. Oleoyl (OAG), an activator of calcium-dependent PKC isoforms, was the most effective in the activation of oocytes, inducing exit from MII in 63.3 ± 1.0% of the oocytes. The NO-donor alone activated 48.7 ± 5.0% of the oocytes. A combination of PKC-activators and an NO-donor (SNAP–OAG, SNAP–PMA) activated 38.4 ± 8.5% of the oocytes, but the combined treatment of an NO-donor with OAG did not induce oocyte activation (0%).

Experiment 2: effect of PKC-activators and an NO-donor on cortical reaction
All oocytes in all the experimental groups were treated as in Experiment 1. The individual analysis of nuclear status and CG quantification revealed that after activation treatment, a cortical reaction had occurred in some oocytes at the metaphase II stage, and some oocytes that had reassumed meiosis still had their CGs under oolema (Table 2). These data confirm that nuclear progression and CG exocytosis can occur as independent events in pig oocytes. Because of this observation and in order to accurately analyze the likely differences in the cortical reaction among treatments, all oocytes from each group were considered together independent of their nuclear status. The results showed that after 42 to 44 h IVM, 17.8 ± 2.4% of the cortical area was occupied by CG. Matured oocytes incubated for 10 h in NCSU-37 medium (Control group) showed 25.9 ± 1.7% of their area occupied by PNA and the presence of 1% DMSO in the medium did not have any effect on the rate of CG area (21.2 ± 2.8%; Table 2). However, activation with a calcium ionophore-induced CG exocytosis, and the cortical area occupied by CG decreased significantly compared with the control group (25.4 ± 1.7% vs. 13.0 ± 3.2%). PKC-activators (DPAM, OAG and PMA) significantly reduced the cortical area occupied by CG (Table 2), and all PKC-activators induced the exocytosis of CG (Figure 1). The area occupied by CG decreased after incubation with PKC-activators compared with those oocytes incubated for 10 h in the same medium without activators (8.6 ± 2.5, 6.7 ± 1.9 and 0.7 ± 0.4 for DPAM, OAG and PMA groups vs. 25.9 ± 1.7% in the Control group). When oocytes were classified according to the degree of cortical reaction, the results showed that PMA, which activates all isoforms of PKC, induced the greatest cortical reaction of all the PKC-activators used, whereas the weakest cortical reaction was induced by dipalmitoyl (DPAM), an activator of calcium-independent PKC isoforms (Figure 1; Table 3).

Incubation with an NO-donor alone (SNAP group) or combined with any PKC-activator (SNAP–DPAM, SNAP–OAG...
and SNAP–PMA) prevented the occurrence of a cortical reaction. Treatment with SNAP alone did not induce a cortical reaction in the oocytes (Figure 1g), which reached an area stained by PNA–FITC similar to that of oocytes kept in culture (17.2 ± 2.2% vs. 25.9 ± 1.7; Table 2). The combination of an NO-donor with PKC-activators prevented CG exocytosis (Figure 1).

Discussion

Our results demonstrated a different role of PKC and an NO-donor in the parthenogenetic activation of porcine oocytes. Despite oocyte activation using these chemicals, in which a high rate (over 78%) is achieved, a full cortical reaction pattern is not always observed (Su et al., 1999; Shimada et al., 2001; Petret al., 2005). It has been shown in pigs that different events in egg activation may be unrelated to each other (Sun et al., 1997), leading to some conflicting results in the literature. In the present study, both exit from MII and the cortical reaction were analyzed after the treatment of eggs with a calcium ionophore, an NO-donor and PKC-activators.

We observed the effect of individual PKC-activators on oocyte activation and CG exocytosis. PKC is involved in regulating the exit from metaphase II in porcine oocytes (Su et al., 1999; Shimada et al., 2001), but it is not entirely clear how PKC participates in the activation of oocytes. Our results show that oleyl was the most effective in the activation of oocytes among the PKC-activators. This PKC-activator was able to induce exit from MII in 63% of the oocytes. The treatment of oocytes by dipalmitoyl or PMA induced oocyte activation only in 44% and 45% of the oocytes, respectively. In contrast to our results, Sun et al. (1997) did not observe exit from the metaphase II stage and completion of meiosis after the stimulation of PKC in mature pig oocytes. Green et al. (1999) determined that PKC inhibition leads to the activation of porcine oocytes, but Sedmiková et al. (2006) found that inhibition of the calcium-independent isoform PKC-δ inhibits the activation of pig oocytes by a calcium ionophore. However, inhibition of PKC isoforms that are calcium-dependent is unable to suppress the activation of porcine oocytes. In our experiments, we observed 63% of activation of porcine oocytes by the activator of calcium-dependent PKC isoforms. The activator of calcium-independent PKC isoforms activated only 44% of the oocytes. The different results obtained can be attributed to the drug concentration, the duration of oocyte treatment and time for assessment of oocyte activation. The role of PKC in the activation of matured oocytes was also confirmed in Xenopus and mice oocytes, where there is a typical process for the activation of oocytes after treatment with PKC-activators (Bement and Capco, 1991; Colonna et al., 1997). However, PKC activation does not induce a complete spectrum of typical processes for the full activation of oocytes (Moore et al., 1995; Ducibella and LeFevre, 1997). In our study, we examined the influence of individual PKC-activators on the cortical reaction in porcine oocytes. The strongest effect on CG exocytosis was achieved with PMA (activator of all PKC isoforms), which is in agreement with the results of Sun et al. (1997), who recorded CG exocytosis in porcine oocytes treated 2 h with PKC-activators (OAG and PMA) independent of the Ca2+ concentration. In contrast, in our experiments, DPAM, an activator of calcium-independent PKC isoforms, showed the weakest influence on CG exocytosis. Incubation of oocytes for 10 h with 100 nM PMA led to a cortical reaction in 100% of pig oocytes. Wu et al. (2006) reported similar results using 3.2 μM PMA for 15 min, with considerable CG exocytosis in 80% of treated human oocytes. However, in pigs, Sun et al. (1997) reported a full cortical reaction only in 33% of the

### Table 2 Cortical area occupied by CG (%) based on nuclear status in in vitro matured pig oocytes after treatment with different PKC-activators and an NO-donor

<table>
<thead>
<tr>
<th>Group</th>
<th>Metaphase II (n)</th>
<th>Activated oocytes (n)</th>
<th>Metaphase II and activated together (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>31.5 ± 2.9d (9)</td>
<td>22.4 ± 3.5e (6)</td>
<td>25.9 ± 1.7c (15)</td>
</tr>
<tr>
<td>DMSO</td>
<td>13.7 ± 2.4cd (4)</td>
<td>22.3 ± 2.7e (10)</td>
<td>21.2 ± 2.8b (14)</td>
</tr>
<tr>
<td>Ca-Io</td>
<td>11.7 ± 4.8cd (6)</td>
<td>6.8 ± 3.1bcde (9)</td>
<td>13.0 ± 3.2bc (15)</td>
</tr>
<tr>
<td>DPAM</td>
<td>14.1 ± 3.5bcde (10)</td>
<td>8.8 ± 4.0de (5)</td>
<td>8.6 ± 2.5bd (15)</td>
</tr>
<tr>
<td>OAG</td>
<td>3.9 ± 1.6cd (12)</td>
<td>7.7 ± 3.1bcd (3)</td>
<td>6.7 ± 1.9b (15)</td>
</tr>
<tr>
<td>PMA</td>
<td>0.2 ± 0.1d (9)</td>
<td>1.2 ± 0.7de (5)</td>
<td>0.7 ± 0.4d (14)</td>
</tr>
<tr>
<td>SNAP</td>
<td>20.1 ± 2.9abc (8)</td>
<td>15.0 ± 2.0abc (7)</td>
<td>17.2 ± 2.2abc (15)</td>
</tr>
<tr>
<td>SNAP–DPAM</td>
<td>28.6 ± 2.9b (11)</td>
<td>15.3 ± 4.2abc (4)</td>
<td>20.7 ± 2.6ac (15)</td>
</tr>
<tr>
<td>SNAP–OAG</td>
<td>18.5 ± 2.4abc (10)</td>
<td>16.5 ± 5.8bc (5)</td>
<td>16.7 ± 2.9abc (15)</td>
</tr>
<tr>
<td>SNAP–PMA</td>
<td>21.3 ± 2.9abc (7)</td>
<td>18.6 ± 3.8bc (8)</td>
<td>20.0 ± 2.4ac (15)</td>
</tr>
</tbody>
</table>

**Notes:**
- **CG** = cortical granule; **PKC** = protein kinase C; **NO** = nitric oxide; **DMSO** = dimethylsulfoxide; **Ca-Io** = calcium ionophore A23187; **DPAM** = 1-oleoyl-2-acetyl-sn-glycerol; **OAG** = 1-oleoyl-2-acetyl-sn-glycerol; **PMA** = phorbol-12-myristate-13-acetate; **SNAP** = S-nitroso-N-acetylpenicillamine; n = number of assessed oocytes.
- The oocytes were treated with DMSO, calcium ionophore, PKC-activators (DPAM, OAG or PMA) or NO-donor (SNAP) for 10 h in NCSU-37 medium.
- The area occupied by CG was calculated from images of the cortical area at the equatorial level using image analysis. The data are presented as mean ± s.e.m.
- Different letters a, b, c, d, e in the same column indicate differences among groups (P < 0.05).
oocytes after treatment with 100 nM PMA for 2 h. Even in this case, similar factors such as the different results of activation could have played a role.

In agreement with our previous results (Petr et al., 2005), we demonstrated that the activation of pig oocytes by an NO-donor did not induce CG exocytosis. However, exit from the metaphase II stage was achieved in 48% of the oocytes. It was shown that a relatively long exposure (10 h) is needed for the activation of pig oocytes with 2.0 mM SNAP (Petr et al., 2005), but activation cannot be considered complete, since this treatment does not induce a full cortical reaction. We did not observe CG exocytosis in those oocytes treated with SNAP alone, not even in combination with individual PKC-activators. The participation of individual PKC isoforms in the activation of porcine oocytes significantly differs in calcium ionophore-induced activation and activation induced by NO. When oocytes were activated by the NO-donor SNAP, there was no significant effect of PKC inhibitors on oocyte activation and CG exocytosis. This suggests that the oocyte activation events induced by the NO-donor depend on PKC to a very limited extent or are completely independent of PKC, as it has been previously suggested (Petr et al., 2007). Despite this, we hypothesize that the combined effects of an NO-donor and PKC-activator lead to an inhibition in the

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**Figure 1** Cortical granule (CG) distribution in in vitro matured porcine oocytes after treatment with different protein kinase C (PKC)-activators and a nitric oxide (NO)-donor. The matured oocytes were treated with dimethylsulfoxide (DMSO), calcium ionophore, PKC-activators ([l-α-phosphatidylinositol-3,4,5-trisphosphate dipalmitoyl heptammonium salt (DPAM), 1-oleyl-2-acetyl-sn-glycerol (OAG) or phorbol-12-myristate-13-acetate (PMA)] and an NO-donor (S-nitroso-N-acetylpenicillamine (SNAP)) for 10 h in NCSU-37 medium. The images show the oocyte equatorial level under a confocal microscope. (a) Oocyte after 10 h in medium – Control. (b) Oocyte incubated with DMSO. (c) Oocyte activated by a calcium ionophore. (d) Oocyte treated with DPAM. (e) Oocyte treated with OAG. (f) Oocyte treated with PMA. (g) Oocyte treated with SNAP. (h) Oocyte treated with SNAP–DPAM. (i) Oocyte treated with SNAP–OAG. (j) Oocyte treated with SNAP–PMA. The scale bar represents 50 μm.
cortical reaction, suppressing the individual effect of PKC-activators on CG exocytosis. In pigs, CGs are exocytosed within 6 h after insemination (Wang et al., 1997; Coy et al., 2002). However, we could not observe CG exocytosis even after 10 h of the combined treatment with NO-donor + PKC-activator. These results indicate for the first time that combined treatment is unable to induce CG exocytosis. It is possible that functional PKC pathways and NO block each other. Perhaps the activation of porcine oocytes by an NO-donor proceeds at least partly through the activation of soluble guanylyl cyclase (sGC). The role of the cyclic guanosine monophosphate (cGMP)-dependent signaling cascade is apparently specific for the parthenogenetic activation of oocytes by NO-donors (Petret al., 2006). The linkage of NO-apparently specific for the parthenogenetic activation of monophosphate (cGMP)-dependent signaling cascade is guanylyl cyclase (sGC). The role of the cyclic guanosine monophosphate (cGMP)-dependent cascades may be mediated by cGMP and cGMP-dependent protein kinase G (PKG). PKG may regulate the amount of endogenous agonists of IP3 receptors, thus inhibiting the activity of phospholipase C, which is part of the activation cascade of PKC (Ruth et al., 1993). Intracellular Ca2+, which the oocyte utilizes during the activation, is mobilized through the inositol trisphosphate or ryanodine receptors. In sea urchin eggs, the increase in the intracellular Ca2+ ions through de novo synthesis of cyclic ADP ribose (Sethi et al., 1996; Willmott et al., 1996), which is a known agonist of ryanodine receptors (Sitsapesan and Williams, 1995). Cyclic ADP ribose alone is able to induce parthenogenetic activation in porcine oocytes. However, other mechanisms may be involved in the NO-dependent activation of pig oocytes. NO activates many molecules through their nitrosylation, for example ryanodine receptors, which, after activation, are able to induce parthenogenesis in matured pig oocytes (Petret al., 2002).

In conclusion, PKC-activators induce CG exocytosis to the same degree as a calcium ionophore. However, the combination of PKC-activators with NO-donors inhibits the stimulus, leading to exit from the metaphase II stage and a cortical reaction in pig oocytes.

Acknowledgments

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References


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PKC = protein kinase C; NO = nitric oxide; DMSO = dimethylsulfoxide; Ca-Lo = calcium ionophore A23187; DPAM = L-α-phosphatidylinositol-3,4,5-trisphosphate dipalmitoyl hexaammonium salt; OAG = 1-oleoyl-2-acetyl-sn-glycerol; PMA = phorbol-12-myristate-13-acetate; SNAP = S-nitroso-N-acetylpenicillamine; n = number of assessed oocytes; CG = cortical granule.

The degree of CG exocytosis was evaluated based on the cortical area occupied by CG. Complete exocytosis (oocytes with a 0% to 5% area occupied by CG), effective CG exocytosis (oocytes with a 5% to 8% area), initial CG exocytosis (oocytes with an 8% to 15% area) and no cortical reaction (oocytes over the 15% area). The data are presented as % oocytes of the total number of oocytes in the group, which occurred at a given level of CG exocytosis.

Different lettersa,b,c,d,e in the same column indicate differences among groups (P ≤ 0.05).

Table 3 Degree of CG exocytosis in in vitro matured pig oocytes after treatment with different PKC-activators and an NO-donor.


