

## Effects of protein kinase C on parthenogenetic activation of pig oocytes using calcium ionophore or nitric oxide-donor

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**ABSTRACT:** Porcine oocytes matured *in vitro* were activated for parthenogenetic development using either calcium ionophore (50 μM for 10 min) or nitric oxide donor SNAP (2 mM for 23.5 hours). Protein kinase C (PKC) inhibitors, bisindolylmaleimide I or rottlerin, are able to inhibit parthenogenetic activation induced by calcium ionophore. The rate of activated oocytes decreased from 69% to 2% ( $P < 0.05$ ) under the effect of bisindolylmaleimide I at a concentration of 0 or 20 nM, respectively. The activation rate decreased from 68% to 0% ( $P < 0.05$ ) under the influence of 0 or 20 μM rottlerin, respectively. PKC inhibitors Go6976 or hispidin had no effect on the oocyte activation using calcium ionophore or on oocytes activated by a nitric oxide donor. The activation of oocytes by a nitric oxide donor is not significantly influenced even under the effects of bisindolylmaleimide I or rottlerin. Based on these data we can conclude that the oocyte activation induced by calcium ionophore depends on PKC, especially on PKC-δ. On the other hand, the oocyte activation induced by nitric oxide is independent of the tested isotypes of PKC.

**Keywords:** oocyte; pig; parthenogenesis; activation; calcium ionophore; nitric oxide

Parthenogenetic activation of mammalian oocytes using artificial stimuli is commonly used in various reproductive biotechniques. *In vitro* matured oocytes are often exploited for these purposes (Samiec et al., 2003; Samiec and Skrzyszowska 2005a,b). The maturing oocyte underwent the germinal vesicle breakdown and then it passed through the stages of metaphase I, anaphase I and telophase I. Maturation is completed at the stage of metaphase II when the oocyte meiosis is spontaneously blocked (Wassarman, 1988). Exit from this meiotic block depends on the activating stimulus which induces destruction or inactivation of the molecules responsible for the maintenance of meiotic blockage. During fertilization, this activat-

ing stimulus is brought to the oocyte by the sperm (Yanagimachi, 1988).

Induction of parthenogenetic activation using an artificial stimulus without sperm is a hot topic today (Wierzchos, 2006). In our previous study we demonstrated oocyte activation using nitric oxide (Petr et al., 2005a). This activation is calcium-dependent and its signalling cascade involves cyclic guanosine monophosphate (Petr et al., 2005b, 2006). However, NO-dependent activation of the oocyte differs in many aspects from oocyte activation induced directly by calcium ions (Petr et al., 2005a,b, 2006).

Intracellular signalling by calcium ions affects many target systems. One of them is protein kinase

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Supported by the Czech Science Foundation (Grant No. 523/06/0295), the Ministry of Agriculture of the Czech Republic (Projects MZe 0002701401, QG50052), the Ministry of Education, Youth and Sports of the Czech Republic (Project No. MSM 6046070901).

C (PKC), which is known as a transducer of the calcium ion signal. PKC is therefore considered to be one of the enzymes playing an important role in oocyte activation (Bement, 1992; Fan et al., 2003).

Protein kinase C belongs to serine/threonine kinase and its numerous isotypes can be classified into three groups. Conventional PKCs (cPKCs), represented by PKC- $\alpha$ , - $\beta_1$ , - $\beta_{II}$  and - $\gamma$  isotypes can be activated by free calcium ions and diacylglycerols (DAG). Novel PKCs (nPKCs), represented by PKC- $\delta$ , - $\epsilon$ , - $\eta$ , - $\mu$ , and - $\theta$  isotypes, are independent of calcium ions, but they can be activated by DAG. Atypical PKCs (aPKCs), represented by PKC- $\zeta$ , - $\lambda$ , and - $\tau$  isotypes, are independent of both the calcium ions and DAG (Liu and Heckman, 1998).

The expression of individual PKC isotypes depends on the type of cells and their stage of development (Aderem, 1995). Several authors reported the presence of isotypes PKC- $\alpha$ , - $\beta_1$ , - $\beta_{II}$ , - $\gamma$ , - $\delta$ , - $\epsilon$ , - $\mu$ , - $\lambda$ , and - $\zeta$  in rodent oocytes at the stage of metaphase II (Gangeswaran and Jones, 1997; Raz et al., 1998; Luria et al., 2000; Pauken and Capco, 2000; Downs et al., 2001; Eliyahu et al., 2001; Eliyahu and Shalgi, 2002; Viveiros et al., 2003) and mRNA for PKC- $\alpha$ , - $\delta$ , and - $\lambda$  (Gangeswaran and Jones, 1997; Raz et al., 1998) although the data on the PKC isotype spectrum and their mRNA are different. Fan et al. (2002a,b) demonstrated PKC- $\alpha$ , - $\beta_1$ , - $\gamma$  isotypes in both immature and mature porcine oocytes.

The role of PKC in the activation of oocytes matured to MII was confirmed in experiments with *Xenopus* and mouse oocytes treated with PKC. Processes occurred there which are typical of this activation (Bement and Capco, 1991; Colonna et al., 1997; Gallicano et al., 1997). According to other authors, however, the PKC activation does not result in the complete spectrum of processes typical of oocyte activation (Moore et al., 1995; Ducibella and Lefevre, 1997). The effects of individual PKC isotypes were not studied in these experiments. DAG activating all isotypes of cPKC and nPKC are usually used for PKC activation.

In porcine oocytes, PKC was observed to participate in the regulation of the resumption of meiotic maturation (Jung et al., 1992; Coskun and Lin, 1995; Kim and Menino, 1995; Su et al., 1999; Shimada et al., 2001). Fan et al. (2002b) demonstrated a change in the localization of PKC- $\alpha$ , - $\beta_1$ , - $\gamma$  in maturing porcine oocytes after their activation. On the other hand, Sun et al. (1997) did not observe any metaphase II transition and meiosis resump-

tion in mature porcine oocytes after PKC stimulation, which indicates that PKC does not trigger these processes. Green et al. (1999) reported that PKC inhibition, together with protein kinase A and myosin light chain kinase inhibition, results in the activation of porcine oocyte. These results show that the role of PKC in the regulation of porcine oocyte activation has not been completely elucidated. Insufficient and contradictory information has also been obtained in the case of single types or isotypes of PKC.

The results of our previous study demonstrated that nPKC isotype PKC- $\delta$  significantly participated in the regulation of porcine oocyte activation induced by calcium ionophore. However, there are no data on the role of PKC isotypes during the activation of oocytes using nitric oxide.

In the present study, the effects of various PKC isotypes on the activation of porcine oocytes after treatment with calcium ionophore or nitric oxide donor were examined. Calcium ionophore (Sedmíková et al., 2006) or nitric oxide donor S-nitroso-N-acetylpenicillamine (SNAP) (Petr et al., 2005a) was used for the activation and specific inhibitors were applied for the inhibition of PKC in the oocytes: bisindolylmaleimide I, which inhibits cPKC (isotypes PKC- $\alpha$ , - $\beta_1$ , - $\beta_{II}$ , - $\gamma$ ) and some of nPKC (isotypes PKC- $\delta$ , - $\epsilon$ ). Go6976 was used for the inhibition of cPKC (isotypes PKC- $\alpha$ , - $\beta_1$ ). This inhibitor does not affect either nPKC (isotypes PKC- $\delta$ , - $\epsilon$ ) or some atypical PKC (isotype PKC- $\zeta$ ). Rottlerin was used for a specific inhibition of the PKC- $\delta$  isotype and hispidin for the inhibition of PKC- $\beta$ .

## MATERIAL AND METHODS

### Isolation and culture of oocytes

Pig ovaries were obtained from a local slaughterhouse from gilts at an unknown stage of the oestrous cycle and transported to the laboratory within 1 h in a saline solution (0.9% sodium chloride) at 39°C. Fully-grown oocytes were collected from follicles by aspirating those that were 2–5 mm in diameter with a 20-gauge needle. Only oocytes with compact cumuli were chosen for further study. Before culture, the oocytes were washed three times in a maturation culture medium.

The oocytes were cultured in a modified M199 medium (GibcoBRL, Life Technologies, Paisley, Scot-

land) containing sodium bicarbonate (0.039 ml of a 7.0% solution per millilitre of the medium), calcium lactate (0.6 mg/ml), gentamycin (0.025 mg per ml), HEPES (1.5 mg/ml), 13.5 IU eCG:6.6 IU hCG/ml (P.G.600 Intervet, Boxmeer, Netherlands) and 10% of foetal calf serum (GibcoBRL, Life Technologies, Germany, Lot No. 40F2190F).

The oocytes were cultured for 48 hours in 3.5 cm diameter Petri dishes (Nunc, Roskilde, Denmark) containing 3.0 ml of the culture medium at 39°C in a mixture of 5.0% CO<sub>2</sub> in air.

PKC inhibitors bisindolylmaleimide I, rottlerin, Go6976, and hispidin were dissolved in DMSO and were then added to the culture medium. The content of DMSO in the medium was not higher than 0.7%. Control experiments were conducted using a medium with a corresponding amount of DMSO but without PKC inhibitor.

### Activation of oocytes

The oocytes were cultivated *in vitro* for 48 hours. Then they were denuded of cumulus cells by repeated pipetting through a narrow glass pipette. The oocytes were placed for 30 min in a medium containing the respective PKC inhibitor or a medium enriched with DMSO without inhibitor to assure the effect of the inhibitor before activation.

For ionophore activation, the oocytes without cumulus cells were exposed to the ionophore A 23 187 (50µM for 10 min). Then they were cultivated for another 23.5 hours in a medium containing PKC inhibitor or a medium with the corresponding amount of DMSO without inhibitor.

For nitric oxide activation, the oocytes without cumulus cells were exposed to the nitric oxide do-

nor SNAP (2mM) for 23.5 hours in a medium also supplemented with the respective PKC inhibitor or supplemented with the respective amount of DMSO without PKC inhibitor.

### Evaluation of oocytes

At the end of the culture period, the oocytes were mounted on slides, fixed with acetic alcohol (1:3, v/v) for at least 24 h and stained with 1% orcein. The oocytes were examined under a phase contrast microscope. Only the oocytes at the pronuclear stage were evaluated as activated. The oocytes at the stage of anaphase II and telophase II were evaluated as non-activated.

### Statistical analysis

A statistical analysis was made on the basis of the results of all experiments. Each experiment was performed four times. The results were summarized for presentation and evaluated by chi-square analysis (Snedecor and Cochran, 1980). The mean percentage of oocytes that had reached the respective stage of nuclear maturation in all repeated experiments did not differ from the average of summarized data by more than 2.5%. The value  $P < 0.05$  was considered statistically significant.

## RESULTS

Under our culture conditions, 96% of the oocytes completed their maturation to the stage of metaphase II after 48-hour *in vitro* culture. We did not

Table 1. Effect of bisindolylmaleimide I on activation in oocytes treated with calcium ionophore A23187 or treated with nitric oxide donor SNAP. Oocytes ( $n = 120$  for each respective treatment) were cultured for 48 hours, denuded of cumulus cells and cultured for 30 minutes in the respective concentration of bisindolylmaleimide I. For treatment with calcium ionophore, the oocytes were exposed to ionophore A23187 (50µM) for 10 minutes. Subsequently, the oocytes were cultured in a medium supplemented with bisindolylmaleimide I for another 23.5 hours. For activation using nitric oxide, *in vitro* matured oocytes were cultured in a medium supplemented with the respective concentration of bisindolylmaleimide I for 30 minutes and then they were exposed to a medium supplemented with the nitric oxide donor SNAP (2mM) and the respective concentration of bisindolylmaleimide I for 23.5 hours

Concentration of bisindolylmaleimide I (nM)	0	1	2	5	10	20
Percentage of oocytes activated after ionophore treatment	69 <sup>a</sup>	56 <sup>b</sup>	31 <sup>c</sup>	25 <sup>c</sup>	8 <sup>d</sup>	2 <sup>d</sup>
Percentage of oocytes activated after SNAP treatment	75 <sup>a</sup>	77 <sup>a</sup>	72 <sup>a</sup>	68 <sup>a</sup>	70 <sup>a</sup>	67 <sup>a</sup>

<sup>a,b,c,d</sup>statistically significant differences ( $P < 0.05$ ) in the percentage of activated oocytes are indicated by different superscripts

Table 2. Effect of Go6976 on activation in oocytes treated with calcium ionophore A23187 or treated with nitric oxide donor SNAP. Oocytes ( $n = 120$  for each respective treatment) were cultured for 48 hours, denuded of cumulus cells and cultured for 30 minutes in the respective concentration of Go6976. For treatment with calcium ionophore, the oocytes were exposed to ionophore A23187 ( $50\mu\text{M}$ ) for 10 minutes. Subsequently, the oocytes were cultured in a medium supplemented with Go6976 for another 23.5 hours. For activation by nitric oxide, *in vitro* matured oocytes were cultured in a medium supplemented with the respective concentration of Go6976 for 30 minutes and then they were exposed to a medium supplemented with nitric oxide donor SNAP ( $2\text{mM}$ ) and the respective concentration of Go6976 for 23.5 hours

Concentration of Go6976 ( $\mu\text{M}$ )	0	1	2.5	5	10	25	50	100
Percentage of oocytes activated after ionophore treatment	70 <sup>a</sup>	68 <sup>a</sup>	74 <sup>a</sup>	68 <sup>a</sup>	70 <sup>a</sup>	70 <sup>a</sup>	65 <sup>a</sup>	67 <sup>a</sup>
Percentage of oocytes activated after SNAP treatment	73 <sup>a</sup>	69 <sup>a</sup>	67 <sup>a</sup>	75 <sup>a</sup>	71 <sup>a</sup>	74 <sup>a</sup>	68 <sup>a</sup>	75 <sup>a</sup>

<sup>a</sup>statistically significant differences ( $P < 0.05$ ) in the percentage of activated oocytes are indicated by different superscripts

observe any spontaneous parthenogenetic activation in oocytes cultured for 48 hours, then denuded and cultured for another 24 hours (0 activated oocytes out of 120 oocytes in culture). Neither did we observe spontaneous activation in oocytes matured *in vitro* for 48 hours, then denuded of cumulus cells and subsequently cultured for 10 minutes in a FCS-free medium and then cultured in a standard culture medium for another 24 hours (0 activated oocytes out of 120 oocytes in culture). The activation of the oocytes that were cultured for 48 hours, then denuded of cumulus cells, subsequently exposed to calcium ionophore ( $50\mu\text{M}$  ionophore A23187 for 10 min) and then cultured for another 24 hours amounted to 71% (85 activated oocytes out of 120 oocytes in culture). The activation of the oocytes cultured for 48 hours, then denuded and subsequently exposed to  $2\text{mM}$  SNAP

for another 24 hours amounted to 76% (91 activated oocytes out of 120 oocytes in culture).

The activation rate was significantly suppressed in oocytes which were cultured in bisindolylmaleimide I after activation using the calcium ionophore. This suppression occurred in a dose-dependent manner. The inhibitory effect of bisindolylmaleimide I was not observed in oocytes activated by the nitric oxide donor SNAP (Table 1).

PKC inhibitors Go6976 (Table 2) or hispidin (Table 3) had no effect on oocyte activation using either calcium ionophore or nitric oxide donor SNAP.

The specific inhibitor of PKC- $\delta$  rottlerin was able to suppress activation in oocytes treated with calcium ionophore but had no effect on oocytes activated by the nitric oxide donor SNAP (Table 4).

Table 3. Effect of hispidin on activation in oocytes treated with calcium ionophore A23187 or treated with nitric oxide donor SNAP. Oocytes ( $n = 120$  for each respective treatment) were cultured for 48 hours, denuded of cumulus cells and cultured for 30 minutes in the respective concentration of hispidin. For treatment with calcium ionophore, the oocytes were exposed to ionophore A23187 ( $50\mu\text{M}$ ) for 10 minutes. Subsequently, the oocytes were cultured in a medium supplemented with hispidin for another 23.5 hours. For activation by nitric oxide, *in vitro* matured oocytes were cultured in a medium supplemented with the respective concentration of hispidin for 30 minutes and were then exposed to a medium supplemented with nitric oxide donor SNAP ( $2\text{mM}$ ) and the respective concentration of hispidin for 23.5 hours

Concentration of hispidin ( $\mu\text{M}$ )	0	2	20	200	2 000
Percentage of oocytes activated after ionophore treatment	71 <sup>a</sup>	68 <sup>a</sup>	75 <sup>a</sup>	69 <sup>a</sup>	72 <sup>a</sup>
Percentage of oocytes activated after SNAP treatment	76 <sup>a</sup>	72 <sup>a</sup>	77 <sup>a</sup>	74 <sup>a</sup>	77 <sup>a</sup>

<sup>a</sup>statistically significant differences ( $P < 0.05$ ) in the percentage of activated oocytes are indicated by different superscripts

Table 4. Effect of rottlerin on activation in oocytes treated with calcium ionophore A23187 or treated with nitric oxide donor SNAP. Oocytes ( $n = 120$  for each respective treatment) were cultured for 48 hours, denuded of cumulus cells and cultured for 30 minutes in the respective concentration of rottlerin. For treatment with calcium ionophore, the oocytes were exposed to ionophore A23187 ( $50\mu\text{M}$ ) for 10 min. Subsequently, the oocytes were cultured in a medium supplemented with rottlerin for another 23.5 hours. For activation by nitric oxide, *in vitro* matured oocytes were cultured in a medium supplemented with the respective concentration of rottlerin for 30 minutes and then they were exposed to a medium supplemented with the nitric oxide donor SNAP ( $2\text{mM}$ ) and the respective concentration of rottlerin for 23.5 hours

Concentration of rottlerin ( $\mu\text{M}$ )	0	1	2	5	10	20
Percentage of oocytes activated after ionophore treatment	68 <sup>a</sup>	48 <sup>b</sup>	24 <sup>c</sup>	18 <sup>c</sup>	0 <sup>d</sup>	0 <sup>d</sup>
Percentage of oocytes activated after SNAP treatment	75 <sup>a</sup>	73 <sup>a</sup>	71 <sup>a</sup>	70 <sup>a</sup>	74 <sup>a</sup>	69 <sup>a</sup>

<sup>a,b,c,d</sup>statistically significant differences ( $P < 0.05$ ) in the percentage of activated oocytes are indicated by different superscripts

## DISCUSSION

Our results clearly demonstrated a different role of PKC in the parthenogenetic activation of porcine oocytes between the activation using calcium ionophore and the activation induced by nitric oxide. In agreement with our previous results (Sedmíková et al., 2006) we proved that PKC was involved in the activation of porcine oocytes induced by calcium ionophore. This is evident from the fact that bisindolylmaleimide I, which is an inhibitor of both calcium-dependent cPKC and calcium-independent nPKC (isotypes PKC- $\alpha$ , - $\beta_1$ , - $\beta_{11}$ , - $\gamma$ , - $\delta$ , - $\epsilon$ ), effectively blocks the activation induced in porcine oocytes by the treatment with calcium ionophore A23187. The role of cPKC and nPKC in the activation was studied only indirectly by means of specific inhibitors. In our experiments, the effects of PKC- $\gamma$ , some of nPKC and aPKC were not studied by means of inhibitors. In spite of this, some specific inhibitors were used for detecting some key isotypes of PKC. The fact that Go6976 (a specific inhibitor of PKC- $\alpha$  and - $\beta_1$ ) is unable to block the activation of oocytes by ionophore suggested that these cPKC do not play a significant role in the activation of porcine oocytes induced in this way. The importance of PKC- $\beta$  isotype was not confirmed even in the experiments in which hispidin (a specific inhibitor of PKC- $\beta$ ) did not affect the oocyte activation by ionophore. On the other hand, the marked effect of rottlerin (a specific inhibitor of PKC- $\delta$ ) indicates that calcium-independent nPKC may play a significant role in the activation of porcine oocytes. However, it should be considered that all effects of rottlerin need not be due to the specific inhibition

of PKC- $\delta$ . PKC- $\delta$ -independent effect of rottlerin was demonstrated, e.g. in astrocytes (Susarla and Robinson, 2003). Based on *in vitro* studies it is concluded that rottlerin inhibits also other enzymes, e.g. calmodulin-dependent kinase III (Gschwendt et al., 1994) or p38-regulated/activated protein kinase (Davies et al., 2000).

When oocytes were activated using the nitric oxide-donor SNAP, there was no significant effect of PKC inhibitors on oocyte activation. This indicates that signalling pathways in both modes of parthenogenetic activation are markedly different. Oocyte activation induced by nitric oxide donor depends on PKC to a very limited extent or is completely independent of PKC. This is also supported by our previous observation that, contrary to the activation by calcium ionophore, the activation using nitric oxide did not induce the exocytosis of cortical granules (Petr et al., 2005a). There is an agreement that PKC plays a key role in cortical granule exocytosis (Fan et al., 2002b). The inadequacy of oocyte activation after treatment with nitric oxide donor is also indicated by the very limited cleavage of resulting parthenogenetic embryos (Petr et al., 2005a). There are no data on the role of PKC in these events in oocytes treated with nitric oxide donor.

Quite a long activation (24 hours) of *in vitro* matured oocytes did not probably influence the resulting rate of parthenogenetic activation. It is known that a prolonged culture of oocytes reaching the stage of metaphase II triggers oocyte ageing and this is accompanied by higher susceptibility of oocytes to the spontaneous parthenogenetic activation. Under our culture conditions, however, the spontaneous parthenogenetic activation was

observed only rarely during the 24-hour ageing of porcine oocytes (Petrová et al., 2004, 2005).

The involvement of individual PKC isotypes in the activation of oocytes has not been studied in greater detail. Some authors assume that the activation processes are associated with  $Ca^{2+}$ -dependent isotypes of PKC (cPKC). Luria et al. (2000) detected the presence of cPKC isotypes  $\alpha$  and  $\beta$  in mouse oocytes and described the activation of PKC- $\alpha$  and PKC- $\beta_1$  in mouse oocytes after fertilization. Raz et al. (1998) demonstrated the relocalization of cPKC isotypes PKC- $\alpha$  and - $\beta$  during the activation of rat oocytes. The activation of isotypes PKC- $\alpha$  and - $\beta$  was also reported to occur during the activation of *Xenopus* oocytes (Stith et al., 1997). In our experiments, however, the activation of oocytes was not blocked either by hispidin (inhibitor of PKC- $\beta$ ) or by Go6976 (inhibitor of PKC- $\alpha$ , - $\beta_1$ ). Neither was the presence of PKC- $\alpha$  and - $\beta$  in porcine oocytes and of isotypes PKC- $\gamma$ , - $\epsilon$  in *in-vitro* matured porcine oocytes demonstrated. This is in contradiction with the results obtained by Fan et al. (2002b), who detected PKC- $\alpha$ , - $\beta_1$ , - $\gamma$  in mature porcine oocytes and described the translocation of these cPKC inside the oocyte after fertilization or parthenogenetic stimulation of these oocytes by ionophore A23187.

Some authors assume that the main role in maturation and activation of oocytes is played by  $Ca^{2+}$ -independent isotypes of PKC (nPKC). Gangeswaran and Jones (1997) consider nPKC isotype PKC- $\delta$  and aPKC isotype PKC- $\lambda$  to be dominant isotypes of PKC present in mouse oocytes. Like in our experiments on porcine oocytes, neither did these authors detect any of cPKC isotypes in mouse oocytes. Tatone et al. (2003) and Viveiros et al. (2003) described the role of PKC- $\delta$  in the activation of mouse oocytes. The significance of nPKC activation for exit from meiosis is also supported by the fact that PKC- $\delta$  also regulates transition from metaphase I to metaphase II in mouse oocytes (Viveiros et al., 2001).

The PKC activity itself is insufficient for the activation of porcine oocytes (Sun et al., 1997; Fan et al., 2002b). However, its activity is necessary for complete oocyte activation. For this reason we suggest testing the hypothesis that an artificial stimulus of PKC could improve the results of parthenogenetic activation in porcine oocytes treated with a nitric oxide donor. This treatment especially could improve the exocytosis of cortical granules and further parthenogenetic cleavage and development.

## Acknowledgements

We thank Mrs. Lucy Westcott for editorial assistance with this manuscript.

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Received: 2007–05–03

Accepted after corrections: 2007–05–30

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