The role of calcium/calmodulin-dependent protein kinase II on the inactivation of MAP kinase and p34^{cdc2} kinase during fertilization and activation in pig oocytes

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Abstract

The objective of this study was to investigate the role of calmodulin-dependent protein kinase II (CaMKII) during fertilization in the pig. Since it has been reported that CaMKII is involved in the capacitation and acrosome reaction of spermatozoa, we tested whether supplementation with the CaMKII inhibitor, KN-93, in the fertilization medium affected sperm penetration. The results showed that the addition of KN-93 in the fertilization medium significantly reduced the rate of sperm penetration into oocytes. However, pre-treatment with KN-93 before *in vitro* fertilization (IVF) did not significantly affect sperm penetration, but it did affect pronuclear formation in a dose-dependent manner. In the oocytes pre-treated with KN-93 before IVF and then co-cultured with spermatozoa without the drug, the decrease in p34^{cdc2} kinase and the cyclin B1 level were significantly suppressed as compared with those in penetrated oocytes without treatment with KN-93. However, the decrease in MAP kinase activity was not affected by KN-93. Additional treatment with KN-93 after Ca²⁺ ionophore treatment also inhibited the reduction in p34^{cdc2} kinase activity and the cyclin B1 level, but not MAP kinase activity. Treatment with KN-92, an inactive KN-93 analogue, did not significantly affect sperm penetration and pronuclear formation. In conclusion, the activation of CaMKII by artificial stimuli or sperm stimulated the disruption of cyclin B1 and the inactivation of p34^{cdc2} kinase, but did not affect MAP kinase inactivation during oocyte activation in pigs.

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Introduction

In most mammalian species, matured oocytes are arrested at the meiotic metaphase II (MII) stage until fertilization. This meiotic arrest is supported by a high activity of maturation-promoting factor, a heterodimer of a catalytic subunit, p34^{cdc2} kinase and a regulatory subunit, cyclin B in *Xenopus* (Dunphy *et al.* 1988, Gautier *et al.* 1988, Draetta *et al.* 1989), mouse (Moos *et al.* 1996) and porcine oocytes (Naito *et al.* 1995). We have previously reported that the high activity of p34^{cdc2} kinase is maintained by MAP kinase, in porcine oocytes, via the synthesis of cyclin B at the MII stage (Ito *et al.* 2004). After sperm penetration, MAP kinase activity was decreased followed by p34^{cdc2} kinase inactivation, corresponding to the pronuclear formation (Moos *et al.* 1995, Liu *et al.* 1998, Miyano *et al.* 2000). The rise in intracellular Ca²⁺ and the activation of protein kinase C (PKC) were observed in sperm-penetrated oocytes (Fan *et al.* 2002). When the matured oocytes were treated with the Ca²⁺ ionophore, p34^{cdc2}

kinase activity was reduced via the degradation of cyclin B in pig oocytes (Ito *et al.* 2004). PKC activated by the Ca²⁺-dependent pathway was involved in cortical granule exocytosis and release from arrest at the MII stage in *Xenopus* oocytes (Bement & Capco 1990). In our previous study (Ito *et al.* 2003), treatment with the PKC activator phorbol 12-myristate 13-acetate (PMA) induced a decrease in MAP kinase activity; however, the treatment failed to decrease the level of p34^{cdc2} kinase activity. These results showed that the inactivation of p34^{cdc2} kinase was induced by Ca²⁺ elevation, and MAP kinase activity was decreased by both Ca²⁺ and the PKC-dependent pathway in sperm-penetrated oocytes.

It has been reported that calmodulin-dependent protein kinase II (CaMKII) which is activated by Ca²⁺ and calmodulin, is required for the inactivation of p34^{cdc2} kinase during fertilization in *Xenopus* oocytes (Lorca *et al.* 1991). In the matured oocytes, intracellular Ca²⁺ elevation induced the destruction of cyclin B corresponding with

the decrease in p34^{cdc2} kinase activity, but injection of the CaMKII inhibitory peptide, autocamtide2 inhibitory peptide, into the oocytes failed to decrease histone H1 kinase activity which corresponded with p34^{cdc2} kinase activity (Tatone *et al.* 2002). Suppression of CaMKII activity also results in a reduction in the amount of MAP kinase as well as a decreased level of activity of MAP kinase in mice and pigs (Hatch & Capco 2001, Fan & Sun 2004). Fan *et al.* (2003) reported that electrical pulse-induced inactivation of p34^{cdc2} kinase was prevented by treatment with the CaMKII inhibitor, KN-93, in porcine oocytes. Moreover, they examined the effect of supplementation with the CaMKII inhibitor on fertilization, but the inhibitor suppressed the penetration rate. It has been reported that the antagonist of calmodulin prevents sperm capacitation in the mouse (Zeng & Tulsiani 2003). When treatment with the calmodulin inhibitor, calmidazolium, was added to insemination medium in mice, more than half the oocytes were unfertilized (Courtot *et al.* 1999). Since KN-93 down-regulated sperm capacitation and acrosome reaction in these reports the effects of KN-93 on meiotic resumption and pronuclear formation of porcine oocytes followed by sperm penetration remain unclear.

In the present study, we have investigated the role of CaMKII during fertilization in the pig. First, the effect on sperm penetration of supplementation with the CaMKII inhibitor, KN-93, into fertilization medium was elucidated. Secondly, we examined the effect of pre-treatment with KN-93 before fertilization on p34^{cdc2} kinase activity, MAP kinase activity, cyclin B1 level and pronuclear formation. Moreover, in order to investigate the role of CaMKII under artificial activation, the kinetics of these kinases, cyclin B1 level and the rate of pronuclear formation in porcine oocytes activated by the Ca²⁺ ionophore were also analyzed.

Materials and Methods

In vitro culture of porcine cumulus oocyte complexes (COCs)

The isolation of porcine COCs has been described previously (Ito *et al.* 2001). Briefly, porcine ovaries were collected from 5- to 7-month-old prepubertal gilts at a local slaughterhouse. Oocytes were collected from the surfaces of intact healthy antral follicles measuring from 3 to 5 mm in diameter. Oocytes having evenly granulated cytoplasm with at least four layers of unexpanded cumulus cells were selected and washed three times with maturation medium. The COCs were cultured (about 20/drop) for 48 h in 100 µl drops of the maturation medium supplemented with both 0.6 µg/ml porcine follicle-stimulating hormone (Sigma Chemical Co., St Louis, MO, USA) and 1.3 µg/ml equine luteinizing hormone (Sigma) at 39 °C in a humidified incubator (95% air, 5% CO₂). The maturation medium was modified NCSU37 (Petters & Reed 1991) supplemented with 10% (v/v) fetal calf serum (FCS; Gibco BRL, Grand Island, NY, USA) and 7 mM taurine

(Sigma), 2% (v/v) essential amino acids (Gibco) and 1% (v/v) non-essential amino acids (Gibco). The proportion of oocytes at the MII stage in our culture system was 85.0 ± 4.7%. To evaluate their chromatin configuration, the other oocytes were mounted on slides, fixed with acetic acid/ethanol (1:3) for 48 h and stained with acetolacmoid before being examined under a phase-contrast microscope (× 400).

Ca²⁺ ionophore treatment

After 48 h of maturation, COCs were denuded and washed three times in the basic medium. Cumulus-free oocytes were treated with 50 µM calcium ionophore A23187 (Sigma) in the basic medium for 5 min at 39 °C and washed with the basic medium to quench the action of the ionophore. Oocytes were exposed to Ca²⁺ ionophore three times at 5-min intervals as described above. The oocytes were then washed at least three times, and each group of 15 oocytes was cultured in 100 µl drops of the basic medium supplemented with 100 µM KN-93 (Sigma) covered with mineral oil for 12 h at 39 °C in a humidified atmosphere of 5% CO₂ in air. KN-93 (10 mM) was dissolved in dimethyl sulfoxide (Sigma) and the stock solution was stored frozen at -20 °C.

In vitro p34^{cdc2} kinase assay

The p34^{cdc2} kinase assay was performed according to the method described in our previous report (Ito *et al.* 2003). Five microliters of oocyte extract (containing ten oocytes) were mixed with 45 µl kinase assay buffer A composed of 25 mM Hepes buffer (pH 7.5; MBL, Nagoya, Japan), 10 mM MgCl₂ (MBL), 10% (v/v) mouse vimentin peptide solution (SLYSSPGAYC; MBL) and 0.1 mM ATP (Sigma). The mixture was incubated for 30 min at 30 °C. The reaction was terminated by the addition of 200 µl PBS containing 50 mM EGTA (MBL). The phosphorylation of mouse vimentin peptides was detected using an ELISA MESACUP cdc2 kinase assay kit (MBL; code no. 5234). Data are expressed in terms of the strength of p34^{cdc2} kinase activity in oocytes matured for 48 h.

In vitro MAP kinase assay

A p44/42 MAP kinase assay kit (Cell Signaling Technology, Beverly, MA, USA) was used for measuring MAP kinase activity. The methods used for the MAP kinase assay were based on those reported by Shimada & Terada (2001). Five microliters of oocyte extract (containing 20 oocytes) were mixed with 25 µl kinase assay buffer B, 25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM MgCl₂ with 0.1 mM ATP (Sigma) and 2 µg Elk 1 fusion protein (Cell Signaling Technology), and the mixture was incubated for 30 min at 30 °C. All chemicals except for ATP were purchased from New England Biolabs. The reaction was terminated by the addition of 10 µl 4 × Laemmli sample buffer; the samples

were boiled at 99.5°C for 5 min and then subjected to 12.5% SDS-PAGE. The phosphorylation of Elk 1 fusion protein was detected by immunoblot analysis and chemiluminescence detection using antiphospho-specific Elk 1 antibody. The data are expressed in terms (means $\pm$ S.E.M.) of the fold strength of MAP kinase activity in oocytes matured for 48 h.

Western blot analysis of cyclin B1

The procedure for cyclin B1 immunoblotting was carried out by the methods in our previous report (Ito *et al.* 2004). Twenty oocytes were put into plastic tubes containing 5 μ l Laemmli sample buffer. After denaturing by boiling at about 99.5°C for 5 min, 4 μ l protein sample were separated by SDS-PAGE on 12.5% polyacrylamide gel (Amersham Biosciences, Uppsala, Sweden), then transferred onto polyvinylidene difluoride membrane (Amersham Biosciences) using the PhastTransfer system (Amersham Biosciences). The membrane was blocked using blocking buffer (3% (w/v) non-fat dry milk (Amersham Biosciences) in PBS supplemented with 0.1% Tween20 (T-PBS)), then incubated with mouse monoclonal anti-cyclin B1 antibody (Upstate Biotechnology, New York, NY, USA) at 1:250 dilution overnight at 4°C in blocking buffer. After three washes in T-PBS, the membranes were treated with horseradish peroxidase-labeled anti-mouse IgG (1:1000; Amersham Biosciences) in 5% (v/v) bovine serum albumin (BSA; Sigma) in T-PBS for 1.5 h at room temperature. After five washes of 5 min each with T-PBS, peroxidase activity was visualized using the ECL Plus Western blotting detection system (Amersham Biosciences), according to the manufacturer's instructions. The intensity of the bands was analyzed using a Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD, USA).

In vitro fertilization (IVF)

After denudation, the matured oocytes were washed three times with the fertilization medium: modified Tris-buffered medium (mTBM) supplemented with 10% (v/v) FCS and 5 mM caffeine (Sigma). After washing, 20 oocytes were placed in 50 μ l drops of the fertilization medium which had been covered with mineral oil in a 35 $\times$ 10 mm² polystyrene culture dish (Falcon, Bedford, MA, USA). These dishes were kept in the incubator until spermatozoa were added for fertilization.

Spermatozoa from a Gottingen miniature pig was washed in mTBM supplemented with 0.1% BSA (fraction V, A 7888; Sigma) (Kawano *et al.* 2004). The sperm sediment was resuspended in the fertilization medium to give 2 $\times$ 10⁵ cells/ml. Then 50 μ l sperm suspension was added to 50 μ l of the fertilization medium with oocytes (final concentration of sperm, 1 $\times$ 10⁵ cells/ml). Oocytes were co-cultured with spermatozoa for 6 h at 39°C in a humidified atmosphere of 5% CO₂ in air. The mTBM used for IVF was essentially the same as that used by Abeydeera & Day (1997).

Statistical analysis

Statistical analysis of the data from three or four replicates was carried out for comparison by ANOVA and Fisher's protected least significant difference test using the STATVIEW (Abacus Concepts, Inc., Berkeley, CA, USA) program. All percentage data were subjected to arcsine transformation before statistical analysis. Differences were considered significant at $P < 0.05$. Values are given as means $\pm$ S.E.M. of three replicates.

Treatment of oocytes with KN-93

(1) Pre-IVF group (Fig. 1); after denudation, oocytes were treated for 30 min with the fertilization medium supplemented with 50 μ M KN-93. After the oocytes were co-cultured with spermatozoa for 6 h without KN-93, the fertilized oocytes were cultured in the medium supplemented with 50 μ M KN-93 for 6 h. (2) Pre-IVF and IVF group (Fig. 1); after denudation, oocytes were treated for 30 min with the fertilization medium supplemented with 50 μ M KN-93. After the oocytes were co-cultured with spermatozoa for 6 h with 50 μ M KN-93, oocytes were further cultured in the medium with 50 μ M KN-93 for 6 h. (3) Post-IVF group (Fig. 1); after denudation, oocytes were cultured for 30 min and IVF was carried out without the inhibitor. These oocytes after IVF were cultured for 12 h in the medium supplemented with 50 μ M KN-93. (4) Control treatment (Fig. 1); pre-IVF, IVF and post-IVF were carried out without KN-93. (5) KN-92 (Fig. 1); 50 μ M KN-92 (Sigma), a negative analogue of KN-93, was added to the medium during the pre-IVF, IVF and post-IVF periods. KN-92 (5 mM) was dissolved in dimethyl sulfoxide and the stock solution was stored frozen at -20°C .

To investigate the dose-dependent effect of KN-93, denuded oocytes were placed in the fertilization medium supplemented with 1, 10, 25 or 50 μ M KN-93 for 30 min. After the pre-IVF treatment, oocytes were co-cultured with spermatozoa for 6 h without KN-93. After IVF, the oocytes were placed in the medium supplemented with different concentrations of KN-93 (same concentrations as pre-IVF treatment) in Nunc four-well dish (Nunc, Roskilde, Denmark). These dishes were incubated for 6 h at 39°C in a humidified atmosphere of 5% CO₂ in air. Oocytes with decondensed or condensed sperm heads in the ooplasm were considered to have been penetrated.

Results

The effects on sperm penetration of KN-93 treatment of matured oocytes before IVF and/or during co-culture with spermatozoa were examined. After pre-treatment with 50 μ M KN-93, oocytes were co-cultured with spermatozoa in the fertilization medium supplemented with 50 μ M KN-93 for 6 h and further cultured in the medium including 50 μ M KN-93 (see Fig. 1). These results are shown in Table 1. In these oocytes, the rate of sperm penetration

Figure 1 A schematic diagram with regard to the effects of KN-93 on supplementation with pre-treatment and IVF medium. *Final concentrations of KN-93 (KN) or KN-92 (92) were 50 μ M.

and pronuclear formation were at a lower level ($25.0 \pm 4.4\%$ and $25.0 \pm 4.4\%$ respectively) as compared with those in oocytes without treatment with the drug ($91.3 \pm 6.9\%$ and $91.3 \pm 6.9\%$ respectively). The penetration rate was significantly increased, but the rate of pronuclear formation was similar when KN-93 was removed from the fertilization medium and was $82.8 \pm 6.8\%$ and $29.3 \pm 3.1\%$. In this group, many oocytes with a meiotic spindle and decondensed sperm heads in the ooplasm were observed. On the other hand, KN-92 (the negative analogue of KN-93) did not affect sperm penetration and pronuclear formation ($78.0 \pm 9.1\%$ and $78.0 \pm 9.1\%$ respectively). Thus, since the pre- and post-treatment of KN-93 did not significantly affect sperm penetration but did inhibit pronuclear formation, this treatment was available as a tool for the analysis of the role of CaMKII in meiotic resumption from the MII stage and pronuclear formation in porcine oocytes.

To investigate the dose-dependent effect of KN-93, denuded oocytes were pre-cultured for 30 min in the fertilization medium supplemented with 1, 10, 25 and 50 μ M

KN-93. After treatment, oocytes were co-cultured with spermatozoa without KN-93. After IVF, oocytes were placed in the medium supplemented with different concentration of KN-93 (the same concentrations as pre-IVF treatment). The pronuclear formation rate in oocytes pre- and post-treated with KN-93 was significantly decreased in a dose-dependent manner (Fig. 2). The inhibitory effect reached a plateau at 25 μ M.

The $p34^{cdc2}$ kinase activity in fertilized oocytes pre- and post-treated with KN-93 is shown in Fig. 3. After being co-cultured with spermatozoa, the activity of $p34^{cdc2}$ kinase in oocytes was significantly lower than that of oocytes before IVF (matured oocytes). Pre-treatment with 1 or 10 μ M of KN-93 did not affect activity; however, the decrease in $p34^{cdc2}$ kinase activity was significantly suppressed by pre-treatment with 25 or 50 μ M KN-93. The rate was significantly lower than that in oocytes before IVF, but higher than that in fertilized oocytes without pre-treatment.

The level of cyclin B1 in fertilized oocytes pre- and post-treated with KN-93 was significantly decreased as

Table 1 The effect of supplementation with KN-93 to pre-treatment and IVF medium on sperm penetration and pronuclear formation in pig oocytes. Values are percentage means $\pm$ S.E.M.

Treatment			Number of oocytes	MII (no.)	Penetration of sperm (no.)	Pronuclear formation (no.)
Pre-IVF	IVF	Post-IVF				
+	-	+	58	17.2 ± 2.2 (10) ^a	82.8 ± 6.8 (48) ^d	29.3 ± 3.1 (17) ^f
+	+	+	48	66.6 ± 5.5 (32) ^b	25.0 ± 4.4 (12) ^e	25.0 ± 4.4 (12) ^f
-	-	+	46	8.7 ± 7.9 (4) ^c	91.3 ± 6.9 (42) ^d	91.3 ± 6.9 (42) ^g
-	-	-	51	9.8 ± 6.9 (5) ^c	86.2 ± 5.5 (44) ^d	84.3 ± 3.9 (43) ^g
KN-92(+)	KN-92(+)	KN-92(+)	41	22.0 ± 8.9 (9) ^c	78.0 ± 9.1 (32) ^d	78.0 ± 9.1 (32) ^g

Different superscripts show significant differences between treatment groups ($P < 0.05$).

Figure 2 The effect of KN-93 on pronuclear formation rate of penetrated oocytes. The matured oocytes were treated with KN-93 and then co-cultured with spermatozoa for 6 h. After insemination, oocytes were cultured for a further 6 h. Different superscripts show significant differences ($P < 0.05$).

compared with that in oocytes before IVF (Fig. 4). Pre-treatment with 1 μM KN-93 significantly reduced the level of cyclin B1 (Fig. 4). However, at concentrations of more than 10 μM KN-93, the addition of KN-93 overcame the decrease in the cyclin B1 level in a dose-dependent manner (Fig. 4). The level in oocytes treated with 50 μM KN-93 recovered to levels similar to those in oocytes before IVF.

MAP kinase activity in fertilized oocytes pre- and post-treated with KN-93 was significantly decreased compared with that in oocytes before IVF (Fig. 5). Supplementation with KN-93 further decreased the activity; however, there was no significant difference between the treatment groups (Fig. 5).

Figure 3 The effect of KN-93 on p34^{cdc2} kinase activity in penetrated oocytes. The matured oocytes were treated with KN-93 and then co-cultured with spermatozoa for 6 h. After insemination, oocytes were cultured for a further 6 h. Different superscripts show significant differences ($P < 0.05$). ¹Data are expressed in terms of the fold strength of p34^{cdc2} kinase activity in oocytes matured for 48 h.

Figure 4 The effect of KN-93 on cyclin B1 levels in penetrated oocytes. The matured oocytes were treated with KN-93 and then co-cultured with spermatozoa for 6 h. After insemination, oocytes were cultured for a further 6 h. Different superscripts show significant differences ($P < 0.05$). ¹Data are expressed in terms of the fold strength of cyclin B1 levels in oocytes matured for 48 h.

The effects of the CaMK II inhibitor on pig oocytes which were artificially activated by Ca²⁺ ionophore were investigated. The p34^{cdc2} kinase in oocytes which were treated with 100 μM KN-93 after 50 μM Ca²⁺ ionophore showed high activity compared with that of oocytes not treated with KN-93 after Ca²⁺ ionophore (Table 2). In the oocytes treated with KN-93, cyclin B1 also showed a significantly higher level compared with that of the oocytes cultivated for 48 h and not treated with KN-93 (Table 2).

Figure 5 The effect of KN-93 on MAP kinase activity in penetrated oocytes. The matured oocytes were treated with KN-93 and then co-cultured with spermatozoa for 6 h. After insemination, oocytes were cultured for a further 6 h. Different superscripts show significant differences ($P < 0.05$). ¹Data are expressed in terms of the fold strength of MAP kinase activity in oocytes matured for 48 h.

Table 2 The effect of 100 μM KN-93 on porcine oocytes cultured for 12 h after 50 μM Ca^{2+} ionophore treatment. Values are means $\pm$ S.E.M.

	Ca^{2+} ionophore	Ca^{2+} ionophore plus KN-93
p34 ^{cdc2} kinase activity ¹	55.2 $\pm$ 4.5	85.0 $\pm$ 4.3*
Cyclin B1 level ¹	52.9 $\pm$ 7.8	84.3 $\pm$ 2.2*
MAP kinase activity ¹	13.5 $\pm$ 3.3	8.7 $\pm$ 6.5
Pronuclear formation (%)	81.0 $\pm$ 5.7	7.9 $\pm$ 6.7*

¹ Data are expressed in terms of the fold strength of oocytes matured for 48 h, defined as 100.

* $P < 0.05$ between treatment groups.

However, MAP kinase in oocytes treated with KN-93 showed low activity and the value was not significantly different from that of oocytes treated with Ca^{2+} ionophore alone (Table 2). Moreover, treatment with KN-93 after 50 μM Ca^{2+} ionophore significantly reduced the proportion of pronuclear formation compared with that of oocytes treated with the Ca^{2+} ionophore (Table 2).

Discussion

CaMKII in spermatozoa plays the important roles in capacitation and acrosome reaction that are essential for penetration into mammalian oocytes (Courtot *et al.* 1999, Zeng & Tulsiani 2003). Since the addition of CaMKII inhibitor during co-culture of oocytes with spermatozoa suppresses sperm penetration, the effects of CaMKII inhibitor on intercellular events during fertilization and pronuclear formation in oocytes remain unclear (Fan *et al.* 2003). In this study, supplementation with KN-93 but not KN-92 in the fertilization medium markedly reduced the rate of penetration. However, when oocytes were treated with KN-93 before IVF, and then co-cultured with spermatozoa in the absence of the drug, sperm heads were observed in the majority of KN-93 pre-treated oocytes. The rate was comparable with that in oocytes fertilized without any treatment with the drug, whereas the pronuclear formation rate of the penetrated oocytes was significantly reduced by the pre-treatment. Suppression of pronuclear formation was also observed when matured oocytes parthenogenetically activated by Ca^{2+} ionophore were consecutively treated with KN-93. These results suggested that pre-treatment with KN-93 is a tool available for the analysis of the role of CaMKII during fertilization and pronuclear formation in oocytes.

Pre-treatment with KN-93 overcame the sperm penetration-induced decrease in p34^{cdc2} kinase activity and disruption of cyclin B1 in a dose-dependent manner. The effects of KN-93 on p34^{cdc2} kinase activity and cyclin B1 level were also observed in oocytes consecutively treated with KN-93 after parthenogenetic activation by the Ca^{2+} ionophore. It has been reported that in *Xenopus laevis* microinjection of an active form of CaMKII protein into MII-arrested eggs induces destruction of cyclin B and inactivation of p34^{cdc2} kinase (Lorca *et al.* 1993, 1994).

The inhibition of CaMKII by the inhibitory protein negatively affected histone H1 kinase activity which corresponds with p34^{cdc2} kinase activity during fertilization in the mouse (Tatone *et al.* 2002). It seems that the rise in intracellular Ca^{2+} induced by sperm penetration or Ca^{2+} ionophore activates CaMKII, which results in a decrease in cyclin B1 and then inactivation of p34^{cdc2} kinase during porcine oocyte activation.

Fan *et al.* (2003) demonstrated that MAP kinase was dephosphorylated after artificial activation and the amount was also dramatically decreased. Co-localization of MAP kinase and CaMKII in mouse (Hatch & Capco 2001) and pig (Fan *et al.* 2003) oocytes could directly interact in specialized areas in the cell. From these results, Fan & Sun (2004) hypothesized that CaMKII could serve to potentiate MAP kinase and p90 ribosomal S6 kinase activity after egg activation because the primary sequence of ERK2 indicates the consensus phosphorylation site for CaMKII at Thr92 (GeneBank Accession no. X58712) (Hatch & Capco 2001). Since our present study demonstrated that IVF oocytes treated with KN-93 showed significant lower levels of MAP kinase activity as compared with that in oocytes treated without KN-93, it is possible that CaMKII induces MAP kinase activation. Taken together, the treatment with KN-93 did not suppress the decrease in MAP kinase activity in oocytes activated by either Ca^{2+} ionophore or sperm penetration.

In the pathway of MAP kinase inactivation, our previous report (Ito & Shimada 2004) demonstrated that the PKC activator, PMA, triggered the decrease in MAP kinase activity. From these results, MAP kinase inactivation was required for the Ca^{2+} -dependent pathway, which would rather be via PKC than CaMKII, at least in pig oocytes. Moreover, since MAP kinase was involved in the synthesis of cyclin B1 (Ito *et al.* 2004), MAP kinase inactivation was not required for the inactivation of p34^{cdc2} kinase, but for inhibition of the reactivation of the kinase in pig oocytes. Therefore, the rise of intracellular Ca^{2+} induced by artificial stimuli or sperm was required for each PKC dependent- and CaMKII dependent-pathways, which induce inactivation of MAP kinase and p34^{cdc2} kinase respectively. Both Ca^{2+} -dependent pathways are required for oocyte activation and pronuclear formation in pig oocytes.

In conclusion, we have clarified the role of CaMKII during fertilization in pig oocytes using pre-treatment with the inhibitor. These data suggest that the activation of CaMKII by artificial stimuli or sperm stimulates the disruption of cyclin B1, resulting in inactivation of p34^{cdc2} kinase during oocyte activation in the pig.

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