ANIMAL EXPERIMENTATION

Effect of KN-62, a Selective Inhibitor of Calmodulin-Dependent Kinase II, on Mouse Oocyte Activation

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Purpose: Our purpose was to determine the association of calmodulin-dependent protein kinase II (CaMK_{II}) with oocyte activation and to explore the network of protein kinases during mammalian fertilization.

Methods: Mouse M-II oocytes were collected after superovulation induced by PMSG-hCG injection. The oocytes were inseminated or artificially activated by Ca ionophore (A23187) or 12-O-tetradecanoyl phorbol 13-acetate (TPA). The effects of KN-62, a specific and selective inhibitor of calmodulin-dependent protein kinase II, on second polar body emission (2PBE), pronuclear formation (PF), and cortical granule exocytosis (CGE) during fertilization or after artificial oocyte activation were investigated.

Results: KN-62 inhibited 2PBE and PF after sperm or Ca ionophore inducing activation. Additionally, PF was inhibited by KN-62 after TPA activation, whereas KN-62 did not inhibit CGE in any case. KN-04, an inactive form of KN-62, did not inhibit significantly 2PBE, CGE, or PF. When oocytes were exposed to KN-62 after Ca ionophore or TPA activation, no inhibitory effects on 2PBE or PF were observed.

Conclusions: The CaMK_{II} activation that occurs after fertilization or artificial activation of mouse oocytes is presumably secondary to increases in the intracellular free calcium concentration. As determined by the use of inhibitor, CaMK_{II} activity is associated with 2PBE and PF but not with CGE.

KEY WORDS: egg activation; oocyte activation; in vitro fertilization; calmodulin-dependent protein kinase II; KN-62.

INTRODUCTION

In mammalian fertilization, inositol trisphosphate and diacylglycerol are produced after the activation of phosphatidylinositol (PI) turnover (1–3). Inositol trisphosphate releases Ca²⁺ from storage in the cytoplasm (4–6). Treatment with Ca ionophore or microinjection of calcium ions induces parthenogenetic activation of mouse oocytes (7,8). Calcium chelators prevent cortical granule exocytosis (CGE), second polar body emission (2PBE), and pronuclei formation (PF) in several species including the mouse (9,10). These studies demonstrate that calcium ions are closely associated with morphological changes in mammalian oocytes during fertilization. Ca²⁺ binds to calmodulin and activates it in mammalian fertilization (11). The downstream portion of the calmodulin activation pathway remains to be clarified. In *Xenopus* oocytes, activation of calmodulin-dependent protein kinase II (CaMK_{II}) releases oocytes from metaphase arrest while inducing cyclin degradation and cdc2 kinase inactivation (12). Winston and Maro reported that in mouse oocytes, CaMK_{II} was activated transiently in ethanol-stimulated mouse oocytes (13). However, little is known about the relation between the activation of CaMK_{II} and morphological changes from metaphase to anaphase, including 2PBE, PF, and CGE in mammalian oocytes. In the present study, we investigated the effect of KN-62 (14–16), a specific inhibitor of CaMK_{II}, on 2PBE, PF, and CGE during fertilization and oocyte activation

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induced by Ca ionophore (A23187) or 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) in comparison with that of KN-04 (17–19), a structural analogue of KN-62 with no inhibitory activity toward CaMK_{II}. We also showed the association of CaMK_{II} activation with 2PBE and PF but not with CGE. This is the first report to demonstrate the relation between CaMK_{II} activation and morphological changes in mouse oocytes.

MATERIALS AND METHODS

Oocyte Collection and Culture

Female ICR mice (6 to 8 weeks old) were superovulated with 5 IU of pregnant mare's serum gonadotropin (Teikoku Zoki, Tokyo) and 5 IU of human chorionic gonadotropin (Teikoku Zoki). Fourteen to 16 hr after human chorionic gonadotropin, the oviducts were excised and ovulated oocytes were obtained. Cumulus cells were dispersed with 0.05% hyaluronidase (Sigma, St. Louis, MO) in modified Krebs Ringer bicarbonate buffer (mKRB) (20) containing 4 mg/ml bovine serum albumin (BSA) (Sigma).

In Vitro Fertilization

Incubations were carried out under paraffin oil at 37°C in an atmosphere of 5% CO₂ in air. Caudal epididymal sperm of ICR mice (12 to 16 weeks old) were obtained by cutting the caudal epididymis and allowing the sperm to swim for 10 min into 500 µl of mKRB. Sperm were incubated under capacitating conditions for 2 hr prior to their incubation with oocytes. The final sperm concentration for in vitro fertilization was 1.0–1.5 × 10⁶/ml in a drop of mKRB. At 60 min after insemination, the oocytes were washed six times in mKRB to remove superfluous sperm, and at 70 min they were transferred to a medium containing 10 µM KN-62 (Seikagaku Corp., Tokyo) or KN-04 (Seikagaku Corp.). Because all oocytes bind and fuse with sperm and Ca²⁺ oscillation in mouse oocytes during fertilization continues for over 2 hr (21,22), 70 min after insemination was selected as the best time to confirm the effect of KN-62 on oocyte activation. 2PBE and PF were examined 8 hr after insemination. Additionally, oocytes were exposed to KN-62 at 2 hr after insemination to investigate the effect of KN-62 on oocytes after oocyte activation induced by fertilization.

Ca Ionophore-Induced Oocyte Activation (Ca Ionophore Activation)

Ca ionophore readily induces a constant elevation of intracellular Ca²⁺ and is useful for determining the function of Ca²⁺ elevation in mammalian oocytes (7,8,23,24). We used Ca ionophore A23187 to activate oocytes by inducing an intracellular Ca²⁺ elevation. Although Ca ionophore-inducing oocyte activation is different from fertilization, which causes gradual Ca²⁺ oscillation, we can investigate the effect of calcium ion on oocyte activation clearly. Oocytes were exposed to 20 µM A23187 for 5 min to achieve oocyte activation. To investigate the effect of KN-62 or KN-04, oocytes were preincubated in medium containing 10 µM KN-62 or KN-04 for 5 min before activation and then activated by A23187 in medium containing 10 µM KN-62 or KN-04. After Ca ionophore activation, oocytes were washed four times and subsequently incubated in medium containing 10 µM KN-62 or KN-04. 2PBE and PF were examined 6 hr after activation. CGE was examined 30 min and 6 hr after activation. In an additional experiment, oocytes were exposed to KN-62 at 15 min after activation to investigate the effect of KN-62 on oocytes after Ca ionophore activation.

TPA-Induced Oocyte Activation (TPA Activation)

TPA is a potent parthenogenetic agent that causes activation of protein kinase C and Ca oscillation in mouse oocytes (2). In the present experiment, 200 ng/ml TPA (Sigma) was used for only 5 min to achieve oocyte activation. To investigate the effect of KN-62 or KN-04, oocytes were preincubated in medium containing 10 µM KN-62 or KN-04 for 5 min before exposure to TPA in the same medium. After TPA activation, oocytes were washed four times and subsequently incubated in medium containing 10 µM KN-62 or KN-04. 2PBE and PF were examined 6 hr after activation. CGE was examined 30 min and 6 hr later. In an additional experiment, oocytes were exposed to KN-62 at 15 min after activation to investigate the effect of KN-62 on oocytes after TPA activation.

Hoechst 33258 Staining

Oocytes were fixed in 3% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min, washed extensively in a blocking solution (PBS containing 1 mg/ml BSA, 100 mM glycine, and 0.2% sodium azide)

(25), permeabilized with 0.05% Triton X-100 solution for 5 min, and incubated in 10 $\mu\text{g/ml}$ bis(benzimide). After being washed in blocking solution five times for 5 min each time, oocytes were transferred to PBS containing both 3 mg/ml polyvinylpyrrolidone and 1 mg/ml paraphenylenediamine and mounted on slides.

Staining Procedure for CGE

Cortical granule exocytosis was examined by fluorescein isothiocyanate-conjugated *Lens culinaris* agglutinin (FITC-LCA) staining as described by Cherr *et al.* (25). We counted only oocytes whose cortical granules were apparently reduced 30 min after Ca ionophore activation and TPA activation. Additionally, we visually examined the fluorescence intensity (520-nm wavelength) of the cortical granules 6 hr after Ca ionophore and TPA activation (26) (Fig. 1). The mean fluorescence intensity of inactivated oocytes was normalized at 100.

RESULTS

Effects of KN-62 on 2PBE and PF After Fertilization

The rates of both 2PBE and PF of oocytes exposed to 10 μM KN-62 were significantly lower than those

of control oocytes, whereas the same concentration of KN-04 did not significantly suppress 2PBE or PF (Fig. 2). When KN-62 was added at 2 hr after insemination, the rate of PF in the KN-62-free group was not significantly lower than that in the control group (Fig. 3).

Effects of KN-62 on 2PBE, PF, and CGE After Ca Ionophore Activation

The rates of both 2PBE and PF of oocytes exposed to 10 μM KN-62 were significantly lower than those of control oocytes and those of oocytes exposed to KN-04 (Fig. 4). KN-62 did not inhibit CGE. The rate of CGE of oocytes exposed to KN-62 was almost the same as that of control oocytes. The fluorescent intensity of cortical granules 6 hr after Ca ionophore activation did not differ significantly between the control group and the KN-62 exposed group (Table I). When oocytes were exposed to 10 μM KN-62 after Ca ionophore activation, the rate of PF in the KN-62-free group was comparable to that in the control group (Fig. 5).

Effects of KN-62 on 2PBE, PF, and CGE After TPA Activation

The rate of PF of oocytes exposed to KN-62 and activated by TPA was significantly lower than that of

Fig. 1. Oocytes stained with FITC-LCA 6 hr after Ca ionophore activation. Oocytes which release cortical granules are shown (small arrows). The large arrow shows a cortical granule-intact oocyte.

Fig. 2. Effects of KN-62 and KN-04 on second polar body emission (2PBE) and pronuclear formation (PF) after insemination. Oocytes were incubated in a medium containing 10 μ M KN-62 or KN-04 70 min after insemination. 2PBE and PF were examined 8 hr after insemination. A total of 280 oocytes was used in these experiments. * P and ** P < 0.05, one-tailed paired t test (compared with control).

control oocytes, whereas KN-04 did not significantly suppress PF (Fig. 6). In 84% of oocytes that had no pronucleus in their cytoplasm, female chromosome separation and chromosome decondensation did not occur. Because the rate of 2PBE was very low even in the control group with TPA activation, there was no significant difference in the rate of 2PBE between the control group and the KN-62-exposed group. KN-62 did not inhibit CGE after TPA activation or after Ca ionophore activation. The fluorescent intensity of cortical granules 6 hr after TPA activation did not differ significantly between the control group and the KN-62-exposed group (Table I). When oocytes were exposed to 10 μ M KN-62 only after TPA activation, the rates of 2PBE and PF in the KN-62-free group were comparable to those in the control group (Fig. 7).

DISCUSSION

In the present study, we showed that 2PBE and PF were suppressed by KN-62, a specific inhibitor of CaMK Π , whereas KN-04, an inactive form of KN-62, was unchanged. These results are consistent with the

Fig. 3. Effects of KN-62 on second polar body emission (2PBE) and pronuclear formation (PF) after insemination when KN-62 was added 2 hr after insemination. Oocytes were incubated in a medium containing 10 μ M KN-62 2 hr after insemination. 2PBE and PF were examined 8 hr after insemination. A total of 213 oocytes was used in these experiments. The experiments here were performed at a different time than those in Fig. 2.

hypothesis that CaMK Π is activated in mouse oocytes following an intracellular calcium elevation. Additionally, KN-62 inhibited even female chromosome separation, implying that female chromosome separation is associated with the activation of CaMK Π . When oocytes were exposed to KN-62 at 2 hr after insemination, the rate of PF in the KN-62-free group was not significantly lower than that in the control group. Similarly, when oocytes were exposed to KN-62 only after Ca ionophore and TPA activation, the inhibitory effects of KN-62 on 2PBE and PF were not observed in spite

Table I. Effect of KN-62 and KN-04 on the Fluorescence Intensity of Cortical Granules After Ca Ionophore (A23187) Activation or TPA Activation^a

Oocytes	Ca ionophore (A23187) activation	TPA activation
Inactivated	100 $\pm$ 9.8	100 $\pm$ 14.5
Activated	69.4 $\pm$ 13.1*	73.9 $\pm$ 12.7*
Activated		
KN-62-exposed	70.2 $\pm$ 13.1*	71.6 $\pm$ 5.9*
KN-04-exposed	71.4 $\pm$ 12.3*	74.8 $\pm$ 8.2*

^a Ten oocytes were examined in each case. The experiment was performed three times. The result of one experiment is shown. Similar results were obtained in the other experiments.

* P < 0.05 (unpaired t test) compared with inactivated oocytes.

Fig. 4. Effects of KN-62 and KN-04 on second polar body emission (2PBE), pronuclear formation (PF), and cortical granule exocytosis (CGE) after Ca ionophore (A23187) activation. Oocytes were preincubated in medium containing 10 μ M KN-62 or KN-04 for 5 min before activation by A23187 in the same medium. After Ca ionophore activation, oocytes were incubated in medium containing 10 μ M KN-62 or KN-04. 2PBE and PF were examined 6 hr after activation. CGE was examined 30 min after activation. A total of 282 oocytes was used in these experiments. * P and ** P < 0.05, two-tailed paired t test (compared with control).

of 5.75 hr of exposure to KN-62. These results show that the inhibitory effect of KN-62 on 2PBE and PF is not toxic to KN-62, and the activation of CaMK Π occurs just after oocyte activation induced by an intracellular Ca $^{2+}$ elevation. In addition, KN-62 cannot inhibit CaMK Π after the activation of CaMK Π itself. Tokumitsu *et al.* demonstrated that KN-62 did not inhibit the activation of autophosphorylated Ca $^{2+}$ /CaMK Π in purified rat CaMK Π (16). The affinity of CaMK Π for dansylated calmodulin increases 1000 times after autophosphorylation of the threonine at position 286 of the protein and autophosphorylation markedly inhibits the release of bound calcium-calmodulin in purified rat CaMK Π (27). The failure of KN-62 to inhibit PF and 2PBE after oocyte activation shows the peculiarity of CaMK Π activation.

In contrast, CGE was not suppressed by KN-62, even when KN-62 was added before Ca ionophore activation and TPA activation, implying that the activation of CaMK Π is not associated with CGE. A value of about 70 may seem to be high for the residual fluorescence intensity of a reacted oocyte. But the level is not absolute. It depends on the nonspecific slight staining of the oocyte surface by FITC-LCA, residual granules, and extruded granule contents. Artificial

Fig. 5. Effects of KN-62 on second polar body emission (2PBE) and pronuclear formation (PF) after Ca ionophore (A23187) activation when KN-62 was added 15 min after activation. Oocytes were incubated in medium containing 10 μ M KN-62 15 min after the Ca ionophore exposure. 2PBE and PF were examined 6 hr after activation. Control: Oocytes were activated and incubated in KN-62-free medium. (A) Oocytes exposed to 10 μ M KN-62 15 min after activation; (B) oocytes exposed to 10 μ M KN-62 5 min before activation. A total of 302 oocytes was used in these experiments. The experiments here were performed at a different time than those in Fig. 4. * P and ** P < 0.05, two-tailed paired t test (compared with control).

oocyte activation including Ca ionophore activation induces irregular CGE on mouse oocytes (24) and cannot cause complete exocytosis, because the effect of Ca $^{2+}$ elevation on artificial oocyte activation is shorter than that on fertilization. Especially, residual cortical granules fused to an oocyte surface just before the finish of exocytosis have an intensity slightly higher than that of normal cortical granules, though the number of residual cortical granules in an oocyte is greatly reduced 6 hr after activation. Because we measured the whole intensity of an oocyte, the higher intensity of residual cortical granules influenced the residual intensity of an oocyte. However, the measurement of fluorescence intensity of the whole oocyte is a more objective method for evaluating CGE as compared to measurement of a portion of the whole oocyte only because cortical granules in an oocyte are not distributed uniformly. Because oocytes in the experimental groups were fixed, stained, and washed simultaneously with oocytes in the control group in each experiment, the statistical difference is reliable. In sea urchin eggs, the calmodulin antagonist trifluoperazine inhibited Ca $^{2+}$ -mediated glucanase release

Fig. 6. Effects of KN-62 and KN-04 on second polar body emission (2PBE), pronuclear formation (PF), and cortical granules exocytosis (CGE) after TPA activation. Oocytes were preincubated in medium containing 10 μ M KN-62 or KN-04 for 5 min before activation by TPA in medium containing 10 μ M KN-62 or KN-04. After TPA activation, oocytes were incubated in medium containing 10 μ M KN-62 or KN-04. 2PBE and PF were examined 6 hr after TPA exposure. CGE was examined 30 min after activation. A total of 215 oocytes was used in these experiments. * $P < 0.05$, two-tailed paired t test (compared with control).

Fig. 7. Effects of KN-62 on second polar body emission (2PBE) and pronuclear formation (PF) after TPA activation when KN-62 was added 15 min after TPA exposure. Oocytes were incubated in medium containing 10 μ M KN-62 15 min after TPA exposure. 2PBE and PF were examined 6 hr later. Control: Oocytes were activated and incubated in KN-62-free medium. (A) Oocytes were exposed to 10 μ M KN-62 at 15 min after activation; (B) oocytes were exposed to 10 μ M KN-62 at 5 min before activation. A total of 231 oocytes was used in these experiments. The experiments here were performed at a different time than those in Fig. 6. * $P < 0.05$, two-tailed paired t test (compared with control).

from a cortical granule lawn (28), whereas Xu *et al.* demonstrated that W7, a calmodulin inhibitor, did not inhibit CGE induced by intracellular Ca^{2+} release after insemination in mouse oocytes (11). The present data demonstrate that CGE is not related to activation of CaMK_{II} in mouse oocytes. These results show that CGE is not associated with the calcium- CaMK_{II} pathway although 2PBE and PF are dependent on CaMK_{II} . An association between intracellular Ca^{2+} elevation and CGE has been demonstrated in mammalian oocytes. However, there is no report to show clearly what is activated by calcium ion and directly connected with CGE. Recently an association between Rabphilin-3A, a putative target protein for Rab3A, and Ca^{2+} -dependent CGE in mouse oocytes was demonstrated (29). Rab3A is a small GTP-binding protein that has been suggested to be involved in Ca^{2+} -regulated exocytosis such as neurotransmitter release from presynapses (30). In another report, a microfilament inhibitor, cytochalasin B, was shown to block sperm-induced CGE in mouse oocytes, although W7 did not inhibit CGE (31), implying that microfilaments on the oocyte surface are activated in CGE without calmodulin activation. These factors may not be associated with the calcium-calmodulin pathway. However, a mouse

strain with a knockout mutation of Rab3A shows normal fertility (32). More research is necessary for revealing the association between calcium ion and CGE.

The association of oocyte activation caused by fertilization with the activation of the Ca^{2+} -calmodulin pathway has been pointed out for the past decade. Winston *et al.* recently reported that CaMK_{II} functioned upstream of the spindle-dependent cyclin B degradation machinery in the enzymatic pathway leading to MPF inactivation as a result of the ability of CaMK_{II} to become activated in the absence of microtubules (13,33). A previous study also demonstrated that CaMK_{II} was located on the mitotic apparatus in mammalian cells (34). The disruption of the network of cytoskeletal intermediate filaments induced by okadaic acid, a protein phosphatase inhibitor, was inhibited by KN-62 in rat hepatocytes (35). These results demonstrate that CaMK_{II} is associated with the cytoskeletal function of cells. However, the mechanism of the linkage between CaMK_{II} and cytoskeletal elements including microfilament and tubulin remains to be clarified.

In conclusion, CaMK_{II} is activated in the activation and fertilization of mouse M-II oocytes. CaMK_{II} is associated with PF and the 2PBE but not with CGE.

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