Separation of Female Chromosomes is Specifically Induced by Protein Kinase C Independent of the Calcium Pathway during Fertilization of Mouse Eggs

Noboru Inagaki[1], Shuetsu Suzuki[2], Norie Nakatogawa[2], Hirokatsu Kitai[1], Naoki Kuji[2] and Yasunori Yoshimura[2]*

[1]*Department of Obstetrics and Gynecology, Social Insurance Saitama Chuo Hospital, Urawa 336 and* [2]*Department of Obstetrics and Gynecology, School of Medicine, Keio University, Shinjyuku-ku, Tokyo 160, Japan*

Abstract: *To evaluate the function of protein kinase C (PKC) during oocyte activation following fertilization, the effects of activators and inhibitors of PKC on the second polar body emission, pronuclear formation and protein phosphorylation of mouse oocytes were investigated during in vitro fertilization and during artificial oocyte activation with Ca ionophore (A23187). One ng/ml of 12-O-tetradecanoyl phorbol 13-acetate (TPA), a PKC activator, accelerated PF without accelerating the second polar body emission. 5–20 ng/ml of TPA suppressed the second polar body emission significantly but did not inhibit female chromosome separation of second meiotic division. Staurosporine, a PKC inhibitor, at 1–5 nM, suppressed the second polar body emission. Female chromosome separation was also suppressed by staurosporine and H7, an another PKC inhibitor. One diploid female pronucleus was formed in an egg or an oocyte without the second polar body emission. TPA itself caused female chromosome separation even in the presence of 20 μM of BAPTA-AM, a selective membrane-permeable calcium chelator. These results show that PKC has supportive effect on pronuclear formation. The activation of PKC is indispensable for female chromosome separation during fertilization.*

Key words: *Oocyte activation, In vitro fertilization, Protein kinase C, Chromosome separation.*

In mammalian fertilization, inositol trisphosphate and the diacylglycerol are produced by the activation of phosphatidylinositol (PI) turn over after the fusion of a sperm and an oocyte [1–3]. Inositol trisphosphate releases calcium ion from storage in the cytoplasm whereas diacylglycerol activates protein kinase C (PKC) [4–6]. Exposure to Ca ionophore or microinjection of calcium ions' solution into cytoplasm induces parthenogenetic activation of mouse oocytes [7, 8]. Calcium chelators prevent cortical granule exocytosis (CGE), the second polar body emission and pronuclei formation in several species including the mouse [9, 10]. These studies demonstrate that calcium ions play important roles in morphological changes in mammalian eggs during fertilization, but little is known about the morphological and biological changes induced by PKC activation during fertilization. PKC is present in the cytosol of mouse M-II oocytes [11, 12]. Protein kinase C can increase the permeability of the oocyte membrane to Ca^{2+} ions and cause the Ca^{2+} influx [13, 14]. In species other than the mouse, phorbol 12-myristate 13-acetate (PMA), a specific stimulator of PKC, increases the oocyte pH [15]. Cytoplasmic alkalinization is negatively affected by selective PKC inhibitors in both PMA-treated and fertilized eggs [16]. In Xenopus oocytes, PMA and other PKC agonists stimulate CGE, sperm chromatin decondensation, and cortical contraction at resting [Ca^{2+}] levels in the first mitotic interphase [17–19]. These physiological changes are virtually prevented by exposure to a PKC antagonist [18, 19]. Some PKC activators have been known to induce oocyte activation in mice [2, 20]. Proteins of approximately 20-, 30-, 70- and 80 kd are phosphorylated in mouse M-II oocytes by some PKC activators [12, 13, 21]. Egg activation induced by fertilization increases in the phosphorylation of a set of proteins of approximately 43 kd in mouse eggs [21].

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*To whom correspondence should be addressed.

However, no study has clearly distinguished the function of PKC from that of intracellular Ca^{2+} ions or shown interactions between PKC and intracellular Ca^{2+} ions. 12-O-tetradecanoyl phorbol 13-acetate (TPA) and diC_8, a synthetic diacylglycerol, are selective PKC activators [22, 23]. Previous studies demonstrated that TPA induced PKC activation in mouse oocytes *in vivo* [11, 12]. Staurosporine and 1-(5-isoquinolinesulfonyl)-2-methyl piperazine dihydrochloride (H7) are often used as selective PKC inhibitor [24, 25]. In the present study, we investigated effects of PKC activation on morphological changes in oocytes, especially the second polar body emission (2PBE), pronuclear formation (PF), and protein phosphorylation of 33- and 45 kd proteins, during *in vitro* fertilization and oocyte activation with Ca ionophore (A23187) [7, 8, 26, 27] by means of these PKC activators and inhibitors. And TPA is known as a potent parthenogenesis activating agent that causes Ca oscillation in mouse oocytes [2]. PKC induces an influx of calcium ions [14]. Therefore, we investigated the effect of TPA on oocyte activation without intracellular and extracellular calcium ion in the presence of 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl ester (BAPTA-AM). BAPTA-AM is a well-known calcium chelator. It is reported that BAPTA-AM chelates cytoplasmic Ca^{2+} of mammalian oocytes [14, 10, 28].

Materials and Methods

Oocyte collection and culture

Female mice (ICR, 6 to 8 weeks old) were superovulated with 5 I.U. of pregnant mare's serum gonadotropin (Teikoku Zoki, Tokyo, Japan) and 5 I.U. of human chorionic gonadotropin (Teikoku Zoki). At fourteen to 16 h after human chorionic gonadotropin, the oviducts were excised and ovulated oocytes were obtained. Cumulus cells were dispersed with 0.05% hyaluronidase (Sigma, Mont., USA) in modified Krebs Ringer bicarbonate buffer (mKRB) 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% CO_2 in air. Caudal epididymal sperm of mice (ICR, 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 h prior to their incubation with oocytes. The final sperm concentration for *in vitro* fertilization was $1.0–1.5 \times 10^6$/ml. After a 60 min insemination, the oocytes were washed six times in mKRB and transferred into a medium containing PKC activatores or PKC inhibitors described below 70 min after insemination and subsequently incubated. If the percentage of PF in the controls was not above 70%, the data were omitted.

Artificial activation (Ca ionophore activation)

We used Ca ionophore (A23187) (Sigma) for activation of oocytes to evaluate the function of the calcium ion and compared the results on Ca ionophore activation with those on *in vitro* fertilization. Oocytes were exposed to 20 μM A23187 for 5 min to achieve oocyte activation. After activation with Ca ionophore, oocytes were washed four times and incubated in medium containing PKC activators or PKC inhibitors described below.

PKC activators and PKC inhibitors

TPA (Sigma) and diC_8 (Sigma) were used as PKC activators in the present study. Staurosporine (Sigma) and 1-(5-isoquinolinesulfonyl)-2-methyl piperazine dihydrochloride (H7) (Sigma) were used as selective PKC inhibitors.

TPA-inducing oocyte activation (TPA activation)

In the present experiment, oocytes were exposed to 200 ng/ml of TPA for only 5 min to achieve oocyte activation. After activation with TPA, the oocytes were washed four times and incubated in mKRB.

Calcium chelator (BAPTA-AM)

BAPTA-AM has high membrane permeability and, at a high concentration, completely chelates intracellular Ca^{2+}. In an experiment on TPA activation, oocytes were preincubated in Ca^{2+}-free medium containing 20 μM BAPTA-AM (Sigma) before activation to remove the effect of intracellular Ca^{2+}. After the TPA activation was performed in Ca^{2+}-free medium containing 20 μM BAPTA-AM, the oocytes were incubated in Ca^{2+}-free medium containing 20 μM BAPTA-AM again.

Hoechst 33258 staining

Oocytes were fixed in 3% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min. They were thoroughly washed extensively in a blocking solution (PBS containing 1 mg/ml BSA, 100 mM glycine, and 0.2% sodium azide) [29], permeabilized with 0.05% Triton X-100 solution for 5 min, and incubated in 10 μg/ml bis benzimide. After washed in blocking solution five times for 5 min each time, the oocytes were transferred to PBS containing both 3 mg/ml polyvinylpyrolidone and 1 mg/ml

paraphenylendiamine and mounted on glass slides.

Staining of microfilaments

Maro's method was used to stain the microfilaments [30]. After the oocytes had been fixed in 3% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min, they were permeabilized with 0.25% Triton X-100 solution for 5 min and incubated in 5 IU/ml FITC-phalloidin (Molecular Probes, Inc) for 15 min. After being extensively washed in blocking solution five times for 5 min each time, the oocytes were transferred to PBS containing both 3 mg/ml polyvinylpyrolidone and 1 mg/ml paraphenylenediamine and mounted on glass slides.

One-dimensional gel electrophoresis of [^{32}P]orthophosphate-radiolabeled proteins of mouse oocytes or eggs

In the experiment on *in vitro* fertilization, eggs were washed in phosphate-free mKRB after insemination and incubated for 2 h in mKRB containing 0.5–1 mCi/ml [^{32}P]orthophosphate and each reagent. Following radiolabeling, they were washed thoroughly four times with PBS (Ca^{2+}- and Mg^{2+}-free) containing 4 mg/ml BSA and transferred to an eppendorf tube with about 5 μl of medium to protect them from the effect of colloidal osmotic pressure change. One-dimensional gel electrophoresis was performed with 4% stacking gel and 10% separating gel in the presence of sodiumdodecyl sulfate according to the method of Laemmli [31]. When Ca ionophore or TPA activation was performed, oocytes were preincubated in mKRB containing 0.5–1 mCi/ml [^{32}P]orthophosphate for 2 h before activation and they were activated with each reagent in medium containing staurosporine or BAPTA-AM, to search for any momentary change in protein phosphorylation. Each examination was performed two or three times.

Results

Effects of PKC activators and inhibitors on PF and 2PBE during fertilization

TPA facilitated PF at lower concentrations (Fig. 1a). The rate of PF 6 h after the insemination of oocytes that were then incubated in medium containing 1 ng/ml of TPA was significantly higher than that of oocytes incubated in TPA-free medium. In contrast, PF was significantly suppressed by 20 ng/ml of TPA. TPA, 5–20 ng/ml, significantly suppressed 2PBE (Fig. 1b). In most eggs that were fertilized and had pronuclei without 2PBE, two female pronuclei were formed in the cytoplasm of each egg and, as a result, three pronuclei including a male pronucleus were formed (Fig. 2a). TPA, 20 ng/ml, suppressed the uptake of [^{32}P]orthophosphate

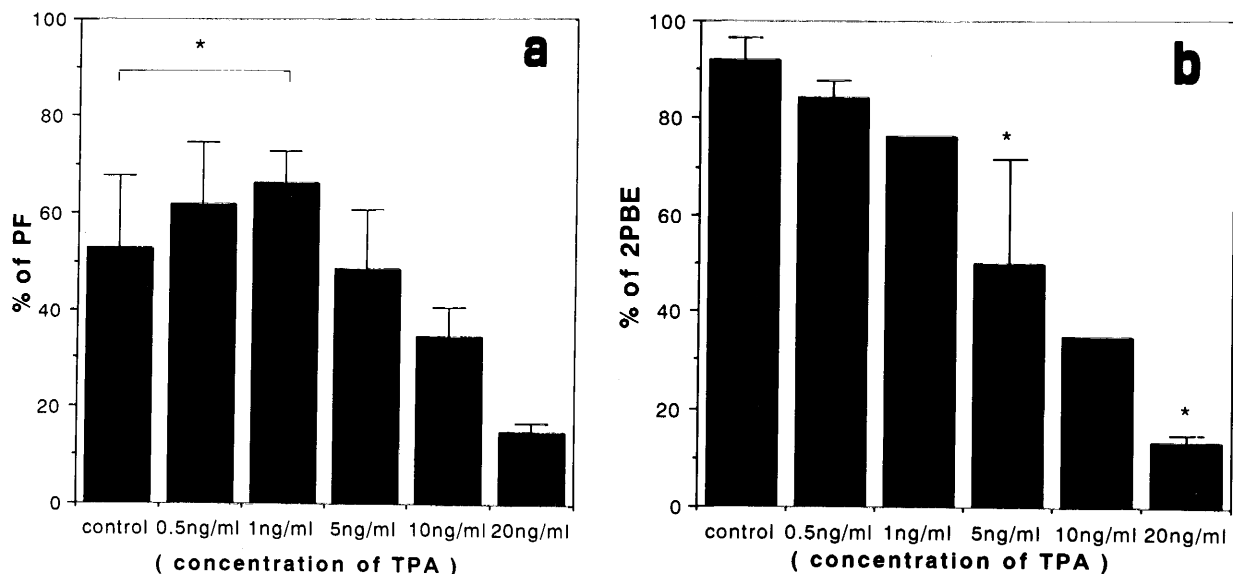

Fig. 1. Effect of TPA on PF and 2PBE during fertilization. Eggs began to be incubated in medium containing TPA 70 min after insemination. a) PF rate 6 h after insemination, (paired t-test: *P<0.05). Six hour after insemination was the best time to confirm the effect of facilitation of TPA, because most pronuclei were formed even in control oocytes 8 h after insemination. b) rate of 2PBE 6 h after insemination, (paired t-test: *P<0.05, compared with control group). Mean or mean ± S.D. The rates of 2PBE in the "1 ng/ml" and "10 ng/ml" TPA groups were checked only twice, but those of other groups were checked more than three times.

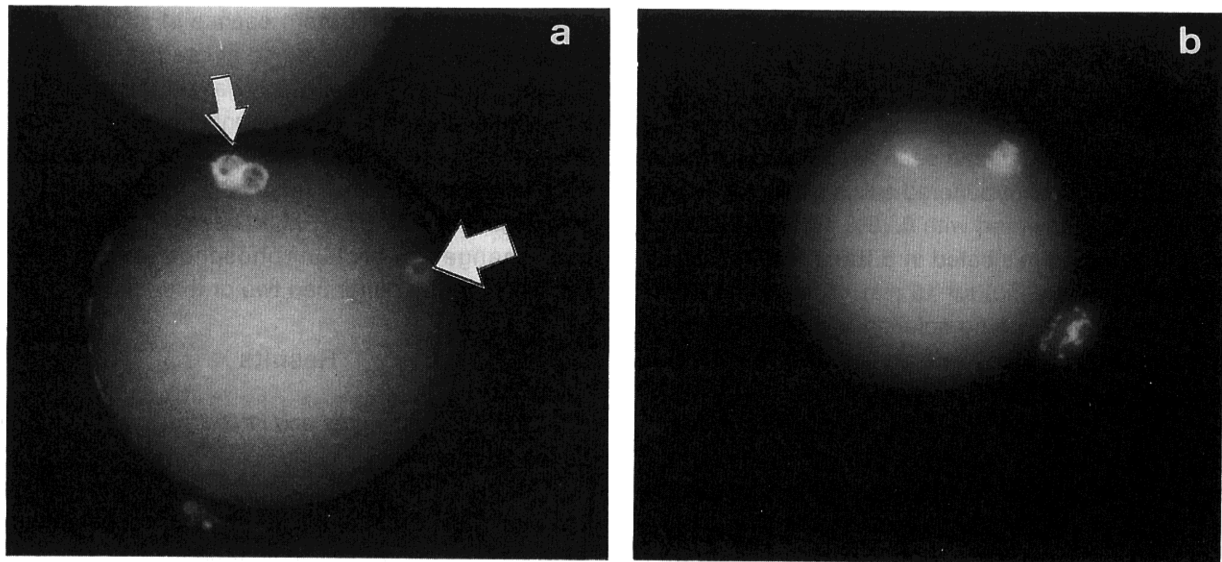

Fig. 2. Effect of TPA on PF and 2PBE during fertilization: Hoechst 33258 staining. a) male (large arrow) and female (small arrow) pronuclei of an egg formed 4 h after insemination: An egg was incubated in medium containing 5 ng/ml TPA. 2PBE was inhibited, but separation of female chromosomes occurred. Two female pronuclei were formed in the egg cytoplasm, as indicated by the arrow. b) Twenty ng/ml of TPA suppressed even decondensation of the sperm head and female chromosome separation.

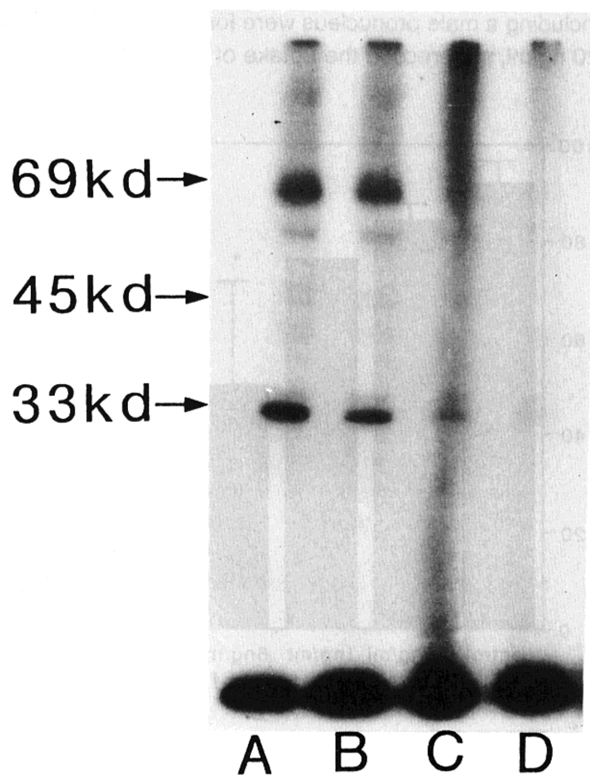

Fig. 3. Effect of TPA and staurosporine on pattern of protein phosphorylation in mouse eggs during fertilization. The pattern of protein phosphorylation examined 4–6 h after insemination is shown. 30 oocytes were used in each group. A) control, B) TPA (0.5 ng/ml), C) TPA (20 ng/ml), D) staurosporine (5 nM).

by 33-kd protein with a nonspecific increase in protein phosphorylation (Fig. 3). At a 20 ng/ml concentration of TPA, PF and 2PBE were apparently suppressed without decondensation of the sperm head and female chromosome separation (Fig. 2b). diC_8, in concentrations of 50 to 200 μM, had the same effect on PF as did TPA at 10–20 ng/ml, whereas the bioinactive phorbol ester, 4α-PDD, did not (data not shown). Staurosporine, a PKC inhibitor, 1–5 nM, suppressed the 2PBE significantly without suppressing PF (Fig. 4a, b). Staurosporine inhibited female chromosome separation and formed one female diploid pronucleus in each egg (Fig. 5a). Twenty nM of staurosporine, at a high concentration, even suppressed the PF (Fig. 5b).

Effect of PKC activators and inhibitors on PF and 2PBE after Ca ionophore activation

TPA, at lower concentrations, apparently accelerated PF after Ca ionophore activation as it did on

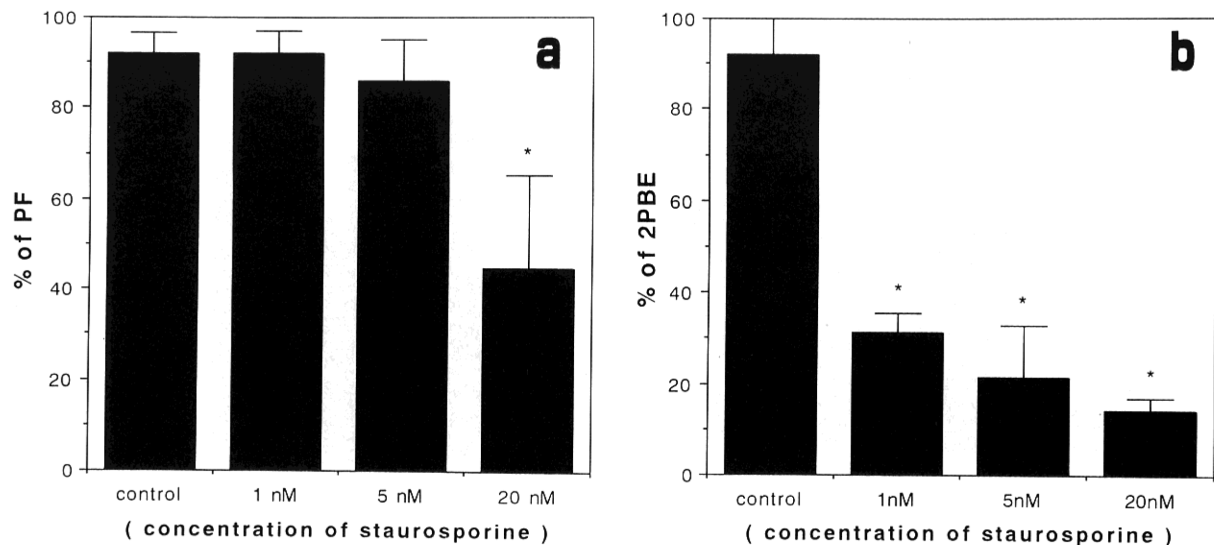

Fig. 4. Effect of staurosporine on PF and 2PBE during fertilization. Eggs began to be incubated in medium containing staurosporine 70 min after insemination. a) rates of PF 8 h after insemination. b) rate of 2PBE 8 h after insemination. To evaluate the inhibitory effect of staurosporine on 2PBE, rates of 2PBE 8 h insemination were investigated in this experiment. Mean ± S.D. (paired t-test, *P<0.05, compared with control group)

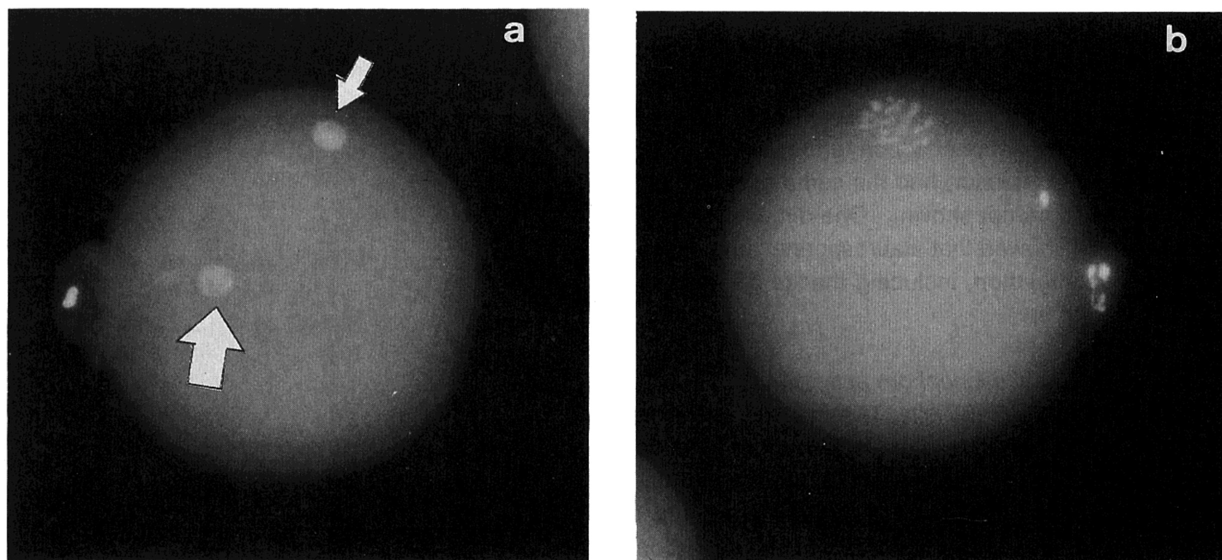

Fig. 5. Effect of staurosporine on PF and 2PBE during fertilization: Hoechst 33258 staining. a) Effect of 1 nM staurosporine during fertilization. One male (large arrow) and one female (small arrow) pronuclei were formed in an egg. The second polar body was not formed and the female chromosomes were not separated. b) Effect of 20 nM staurosporine during fertilization. Decondensation of a sperm head and female chromosomes in the egg cytoplasm was suppressed.

fertilization (Fig. 6). The rate of PF 4 h after Ca ionophore activation of oocytes that were then incubated in medium containing 1 ng/ml of TPA was significantly higher than that of oocytes incubated in TPA-free medium. Furthermore, staurosporine, even at a lower concentration of 1 nM, suppressed 2PBE and female chromosome separation (Fig. 7). The chromosome decondensation and PF, however, were not suppressed at the lower concentration of staurosporine, although 2PBE and female chromosome separation were sup-

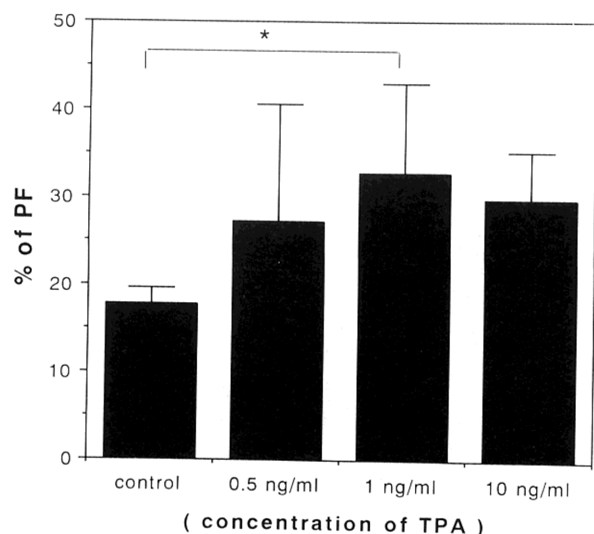

Fig. 6. Effect of TPA on PF after Ca ionophore activation. Oocytes began to be incubated in medium containing TPA 5 min after Ca ionophore activation. Rate of PF was investigated 4 h after the activation. Mean ± S.D.

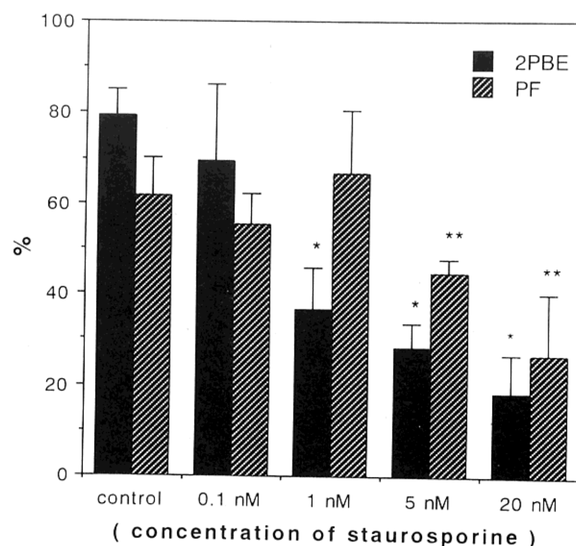

Fig. 7. Effect of staurosporine on PF and 2PBE after Ca ionophore activation. Oocytes began to be incubated in medium containing staurosporine 5 min after Ca ionophore activation. PF and 2PBE were examined 6 h after activation. Mean ± S.D. (paired t-test, *P, **P<0.05, compared with control group)

pressed. One diploid female pronucleus was formed without a second polar body (Fig. 8). At higher concentrations, staurosporine suppressed both PF and 2PBE as it had the same effect on fertilization (Fig. 7). H7, another PKC inhibitor, had the same effect as did staurosporine (data not shown). One-dimensional gel electrophoresis showed that staurosporine suppressed protein phosphorylation, including that of 33- and 45 kd proteins (Fig. 9).

TPA activation and Ca ionophore activation

The percentage of 2PBE after TPA-induced activation was much lower than that after Ca ionophore-induced activation (Fig. 10), but the accumulation of actin filament was observed (Fig. 11a). Two female pronuclei were formed in an oocyte (Fig. 11b). When activation with both Ca ionophore (20 μM) and TPA (200 ng/ml) was simultaneously induced, the rate of PF increased significantly in comparison with that when induction was by either Ca ionophore or TPA alone (Fig. 12). The rate of 2PBE after activation induced by both Ca ionophore and TPA was much lower than that after Ca ionophore activation only.

Effect of BAPTA-AM on 2PBE and PF in TPA activation

When oocytes were activated by TPA in calcium free medium, 2PBE and PF were effectively suppressed (Table 1). The formation of the polar body-like structure

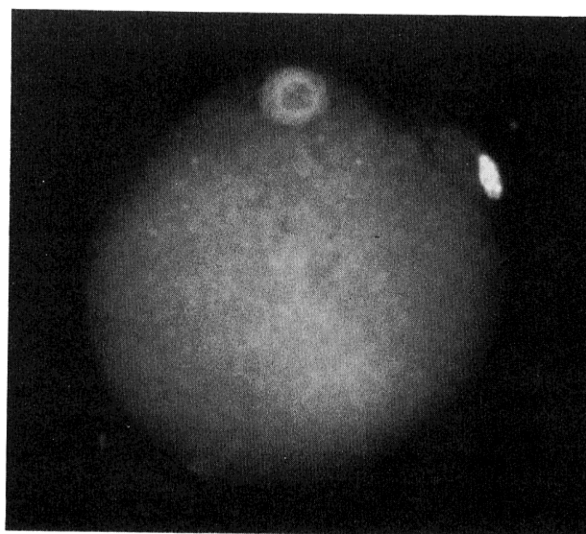

Fig. 8. Effect of staurosporine on PF and 2PBE after Ca ionophore activation: Hoechst 33258 staining. Oocytes began to be incubated in medium containing 1 nM staurosporine 5 min after Ca ionophore activation. Only one female pronucleus was formed in an oocyte. The second polar body was not formed.

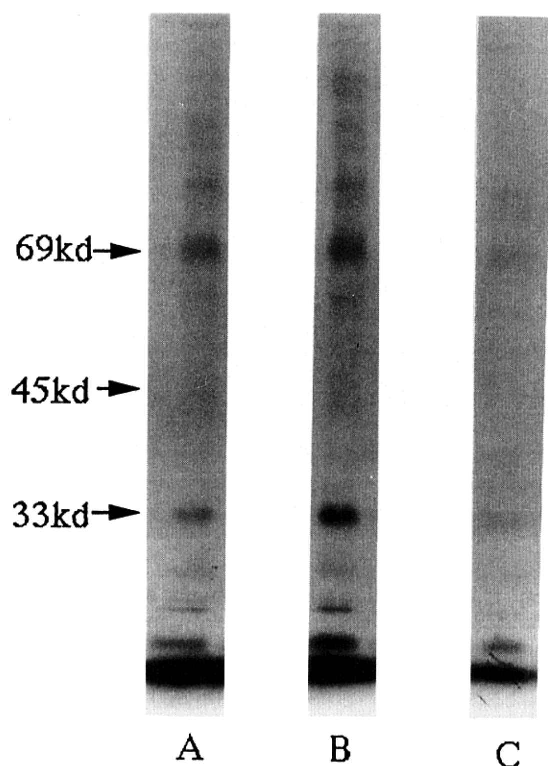

Fig. 9. Effect of staurosporine on pattern of protein phosphorylation of mouse oocytes after Ca ionophore activation. Before activation, oocytes were preincubated for 2 h in medium containing 1 mCi/ml [^{32}P]orthophosphate. 30 oocytes were used in each band. A) control: inactivated oocytes incubated for 2 h only in medium containing 1 mCi/ml [^{32}P]orthophosphate; B) oocytes activated by Ca ionophore for 5 min after 2 h preincubation; C) oocytes activated by Ca ionophore for 5 min in medium containing 1 nM staurosporine after 2 h preincubation.

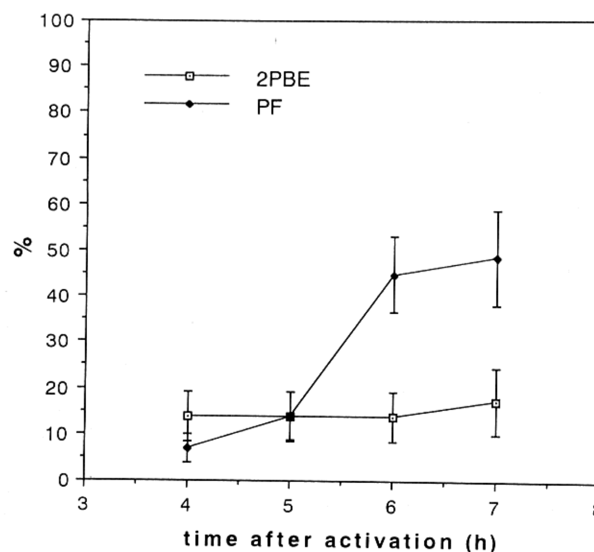

Fig. 10. Rates of PF and 2PBE after TPA (200 ng/ml) activation. Oocytes were activated by 200 ng/ml of TPA for 5 min and incubated in mKRB after they were washed. Mean ± S.D.

[20] was suppressed in proportion to the decrease in the accumulation of microfilaments (Fig. 13). Chromosome separation, however, was not inhibited, even at 20 μM of BAPTA-AM (Fig. 14). One-dimensional gel electrophoresis revealed that BAPTA-AM, even at 20 μM, did not inhibit protein phosphorylation, including that of 33- and 45 kd proteins (Fig. 15).

Discussion

PKC, which is activated by diacylglycerol, is an important messenger of signal transduction in many kinds of cells including oocytes and eggs, but the real function of PKC in mammalian M-II oocytes remains unclear because discrimination between specific and nonspecific functions of PKC is very difficult in *in vivo* experiments. Only a few studies have demonstrated several functions of PKC in mammalian M-II oocytes. Several studies reported the induction by PKC of an increase in intracellular Ca^{2+}, including Ca^{2+} oscillation associated with calcium influx [2, 14]. And other studies demonstrated the association of PKC with CGE although it remains unclear whether CGE is dependent on or independent of the intracellular Ca^{2+} increase induced by PKC activation [11, 17, 32, 33]. In the present study, therefore,we investigated another specific effect of PKC.

Effects of PKC activators and inhibitors on PF and the 2PBE on fertilization

TPA, a PKC activator, facilitated PF at a low concentration. Staurosporine, a PKC inhibitor, suppressed PF at 20 nM though this concentration of staurosporine was higher than the physiological level. This indicates that PKC may have a facilitating effect on PF. TPA inhibited PF at higher concentrations, implying that this inhibition is derived from a down-regulation of PKC. In mouse epidermal cells, 2 nmol of TPA resulted in a rapid decrease in cytosolic, particulate, and total epidermal PKC activity even at 6 h which remained decreased by 70% at 96 h [34]. It has been demonstrated that the reduction in PKC induces a decrease in protein phosphorylation caused by PKC itself in some kinds of cells [35, 36]. In the present study, the phosphorylation

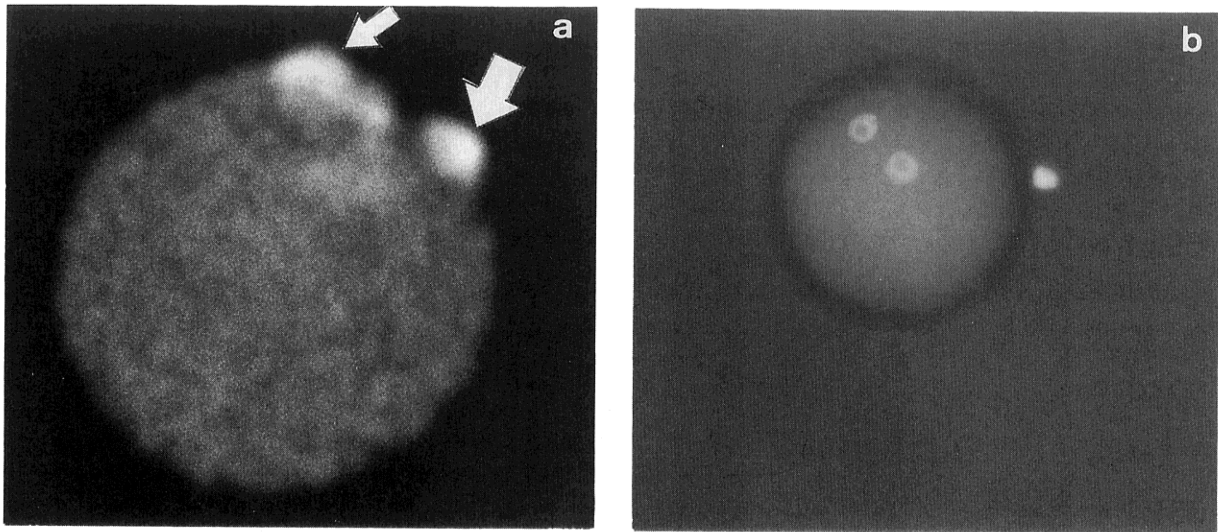

Fig. 11. Accumulation of actin filaments and PF after TPA activation. a) accumulation of actin filaments stained by FITC-phalloidin. The staining procedure is described in Materials and Methods. The small arrow indicates the first polar body. The large arrow indicates accumulation of actin filaments. b) PF after TPA activation stained by Hoechst 33258. Two pronuclei were formed in an oocyte without 2PBE.

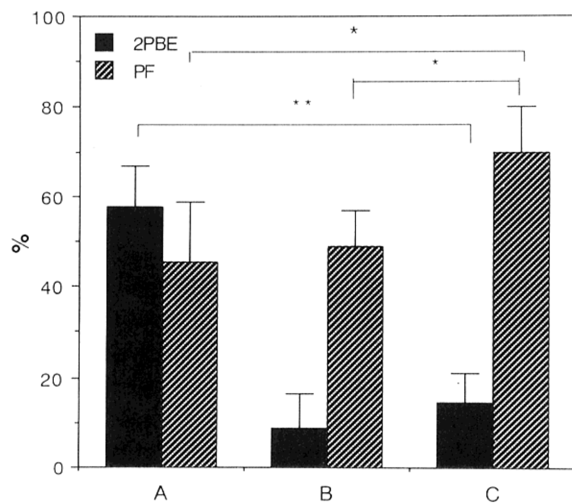

Fig. 12. Effect of both Ca ionophore and TPA activation on PF and 2PBE. A, Ca ionophore (20 μM) activation. B, TPA (200 ng/ml) activation. C, Ca ionophore (20 μM) plus TPA (200 ng/ml) activation. PF and 2PBE were examined 6 h after activation (paired t-test: *P, **P<0.05).

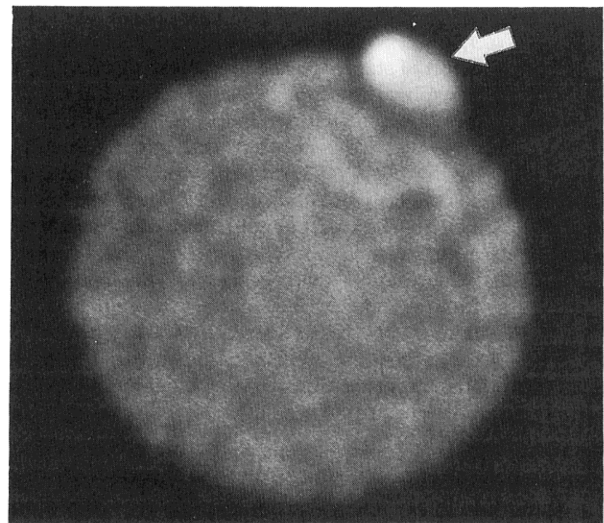

Fig. 13. Decrease in accumulation of actin filaments stained by FITC-phalloidin. Oocytes were incubated in Ca^{2+}-free medium after TPA activation. No accumulation of actin filaments was visible. The small arrow indicates the first polar body.

of 33 kd protein, which was facilitated by TPA as shown by Endo *et al.* [21], was decreased.

Some of our results suggest that PKC regulates 2PBE. The finding that TPA induced the formation of two female pronuclei in an egg without 2PBE supports the concept of an inhibitory effect of PKC on 2PBE. It is known that PKC induces phosphorylation of heavy meromyosin (HMM) in smooth muscle cells [37]. Phosphorylation of HMM by PKC reduces phosphorylation of HMM by myosin light chain (MLC) kinase that is necessary for the activation of actin-myosin movement, and the phosphorylation of HMM by PKC reduces actin-

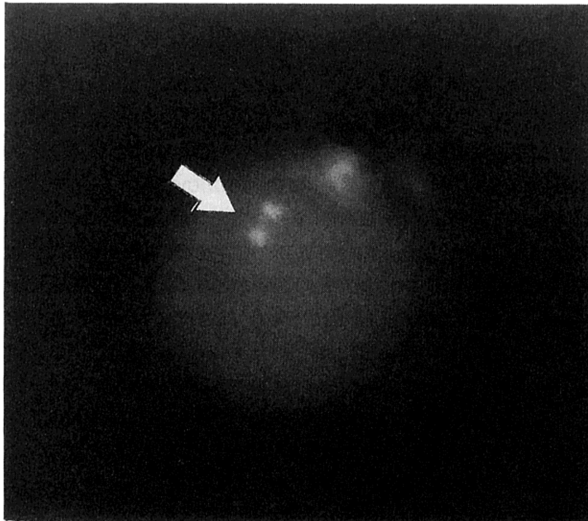

Fig. 14. Effect of BAPTA-AM on chromosomes in an oocyte after TPA activation. Oocytes were activated and incubated in 20 μM BAPTA-AM. An arrow indicates the separation of the female chromosome.

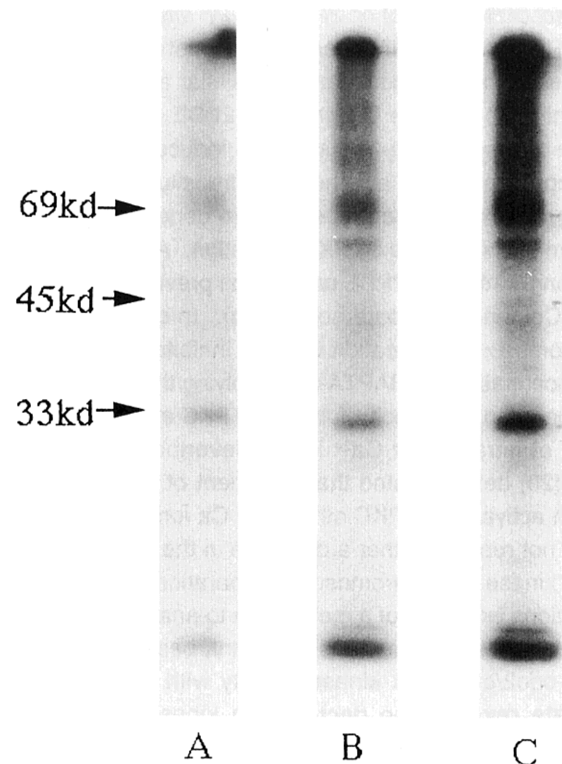

Fig. 15. Effect of BAPTA-AM on protein phosphorylation after TPA activation. Before activation, oocytes were preincubated for 2 h in medium containing [^{32}P]orthophosphate, 1 mCi/ml. 30 oocytes were used in each group. A) control: inactivated oocytes incubated for 2 h only in medium containing 1 mCi/ml [^{32}P]orthophosphate; B) oocytes activated by TPA, 200 ng/ml, for 5 min after 2 h preincubation; C) oocytes activated in medium containing 20 μM BAPTA-AM for 5 min after 2 h preincubation.

activated MgATPase activity [37]. And phosphorylation of MLC kinase by PKC reduces the affinity of MLC kinase for calmodulin [38]. These sutudies show that PKC suppresses the actin-myosin movement. A similar system may exist in mouse oocytes. A PKC inhibitor, staurosporine, also inhibited 2PBE. But because staurosporine inhibited female chromosome separation and induced the formation of one diploid female pronucleus in an egg, it is unclear whether the main inhibitory effect of staurosporine on 2PBE is due to the inhibition of chromosome separation or the inhibition of microfilament accumulation on the egg surface.

Effect of PKC activators and inhibitors on PF and 2PBE after Ca ionophore activation

Ca ionohore activates oocytes by inducing intracellular Ca^{2+}. Ca ionophore activates oocytes immediately. We compared the results of Ca ionophore activation with those of *in vitro* fertilization. TPA, 1 ng/ml, facilitated PF induced by Ca ionophore as it did on fertilization, and staurosporine inhibited PF at higher concentration, implying that activation of PKC has a supportive effect on calcium ion for PF. Staurosporine inhibited PF at higher concentrations, but it did not inhibit PF at low concentrations, including chromosome decondensation and nuclear envelope formation. Staurosporine, however, inhibited female chromosome separation, even at a low concentration, and one diploid female pronucleus was formed in an oocyte as staurosporine had the same effect on fertilization. This finding shows that female chromosome separation is profoundly dependent on the activation of PKC.

TPA activation and Ca ionophore activation

In the present experiments, the results show a correlation between calcium ion and PKC. TPA and Ca ionophore facilitated PF cooperatively, whereas TPA suppressed 2PBE. The rate of 2PBE induced by both Ca ionophore and TPA did not correspond to that induced by Ca ionophore alone, showing that the activation of PKC has a supportive effect on calcium ion for PF and an inhibitory effect on calcium ion for 2PBE.

Effect of BAPTA-AM on 2PBE and PF after TPA activation

PF and 2PBE induced by TPA were severely suppressed in the absence of intracellular and extracellular calcium ions. The fact that PF, 2PBE, and the formation of a polar body-like structure induced by TPA were suppressed in the absence of extracellular calcium ion, suggests that these processes are dependent on a calcium influx induced by PKC activation. And, BAPTA-AM strongly inhibited 2PBE and PF, as previously described by Colonna [14] (data not shown). In contrast, female chromosome separation was not inhibited even by high concentrations of BAPTA-AM, implying that chromosome separation is a specific function of PKC almost independent of intracellular Ca^{2+} ions. Nevertheless, Moore *et al.* [20] demonstrated that treatment of mouse oocytes with activators of PKC other than Ca ionophore A23187 did not result in either a decrease in the level of histone H1 kinase or in chromosome separation in opposite directions indicative of a metaphase-to-anaphase transition during the first hour after treatment, because the assay for cdc2/cyclin B1 kinase activity with a peptide substrate revealed no decrease in kinase activity after treatment with PMA or diC_8. But the chromosome separation demonstrated by Moore *et al.* is dynamic separation with a second polar body-like structure, which is different from the chromosome separation to which we refer. They also observed that the activation of PKC induced a dissembling of the spindle in both hamster and mouse eggs. Their findings are, therefore, consistent with the present data showing that chromosome separation is a specific event of PKC. They demonstrated that more than 80% of hamster and mouse oocytes did not display a visible spindle within 20 min after their treatment with PMA, a PKC activator, and diC8. We confirmed a similar phenomenon in TPA-activated mouse oocytes in Ca^{2+}-free medium and in a medium that included BAPTA-AM. These results suggest that chromosome separation and dissembling of the spindle microtubules are not dependent on the increase in intracellular Ca^{2+} ions if PKC has been significantly activated. In some kinds of cells, topoisomerase II is known to be nessesary for chromosome separation in mitotic division. Some studies demonstrated that topoisomerase II was phosphorylated and activated by PKC [39, 40]. No study shows that topoisomerase exists in mouse oocytes. One paper, however, showed that female chromosome separation was blocked by etoposide, a inhibitor of topoisomerase II [41]. Topoisomerase II may act on chromosome separation in a mouse oocyte.

Protein phosphorylation

In the present study, phosphorylation of 33- and 45-kd protein was observed because the change in their phosphorylation during fertilization or oocyte activation has been confirmed [21] and been detected easily by using a relatively small number of oocytes.

We could not find a real association of 33- and 45 kd protein phosphorylation with chromosome separation in this experiment, but phosphorylation of these proteins occurred in a mouse M-II oocyte even when both extracellular and intracellular Ca^{2+} ions were chelated. Protein kinase C inhibitors suppressed chromosome separation, while also suppressing phosphorylation of these proteins. These findings suggest that phosphorylation of 33- and 45 kd proteins is caused by activation of PKC independent of the calcium pathway and that chromosome separation may be dependent on their 33- and 45 kd proteins phosphorylation. Although Endo *et al.* [12] showed that exogenous PKC induced the *in vitro* phosphorylation of 70-, 55-, and 20 kd proteins in mouse oocyte extract, they did not observe 33- and 45 kd protein phosphorylation. PKC may phosphorylate 33- and 45 kd proteins through another kinetic substance indirectly. In this study, we could not clearly show phosphorylation of 70-, 55-, and 20 kd proteins. The reason may be that the number of oocytes used for one-dimensional gel electrophoresis was not enough to detect it. Further experiments are need to clarify the change in protein phosphorylation.

Conclusion

PKC has a significant effect on mouse fertilization. Separation of the female chromosomes in the fertilized mouse egg was specifically induced by the activation of PKC, independent of the intracellular increase in Ca^{2+} caused by the release of Ca^{2+} from intracellular calcium stores or the influx of extracellular Ca^{2+}. Although we did not mention that a calcium-independent type of PKC exists in mouse oocytes, the present data demonstrates that PKC itself causes a separation of the female chromosomes independent of the calcium pathway. Calcium ion may facilitate such PKC activity. Future analysis of the types of PKC in mouse oocytes will be required to evaluate the association of calcium ion with PKC.

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