A NOVEL PROTEIN KINASE C INHIBITOR IN BOVINE SPERMATOZOA AND ITS EFFECTS ON THE DEVELOPMENT OF FERTILIZED OVA

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ABSTRACT

We found a protein kinase C (PKC) inhibitor in bovine spermatozoa. The inhibitor was purified by Sephadex G-200 gel filtration chromatography, isoelectric focusing and Mono Q chromatography. Inhibition of the PKC activity observed with 2 μ g of the purified inhibitor was comparable with that with 5 μ mol of chlorpromazine, a known potent inhibitor of PKC. Molecular weight of this inhibitor was estimated to be about 63,000, and isoelectric point was pH 4.5. The inhibitor was inactivated when boiled, or digested with trypsin, whereas DNase and RNase were without effect, indicating that the inhibitor is a protein. The inhibitor is neither a protease nor a phosphatase. In fresh bovine spermatozoa, the PKC activity was almost undetectable probably because of the presence of the PKC inhibitor. When spermatozoa were incubated at pH 7.5 at 37°C for 12-16 h, more than half the activity of PKC inhibitor was lost, suggesting that the inhibitor was inactivated by proteolysis. When the purified inhibitor was microinjected into mouse fertilized ova, both the development of one-cell fertilized ova into two-cell stage ova, and two-cell late stage ova into four-cell stage ova were inhibited. In contrast, PKC microinjected into two-cell early stage ova promoted their development into the four-cell and eight-cell stages; two-cell early stage ova injected with saline remained in the same stage. The results suggest a regulatory role of PKC and the PKC inhibitor in the development of fertilized ova.

Protein kinase C (PKC) plays important roles in signal transduction and also in cell proliferation and carcinogenesis (21-24). Although calcium in cytoplasm has been known to function in the development of fertilized ova (19), no direct evidence has been presented that PKC is involved in this process. The assay of PKC has generally been carried out with histone as substrate in the presence of Ca^{2+} , phosphatidylserine, and diacylglycerol (15). In contrast, assay of PKC needs neither Ca^{2+} , phosphatidylserine, nor diacylglycerol, when protamine was used as substrate; Ca^{2+} rather inhibited the PKC activity (39).

Protamine is found only in mature spermatozoa. Does PKC exist in spermatozoa? We tried to extract PKC from bovine and human sperm and to measure its activity. However, PKC activity in spermatozoan extracts has been difficult to measure, in accordance with previous reports indicating the absence of PKC in ram spermatozoa (30) or its low level in human spermatozoa (31). However, we noticed that the activity of PKC purified from rat brain was inhibited by adding spermatozoan extract, suggesting the presence of a PKC inhibitor in spermatozoa (unpublished).

PKC inhibitors reported so far in literature include 1) synthetic compounds such as chlorpromazine (20), trifluoperazine (32), tamoxifen (26), aminoacridines (5), isoquinolinesulfonamides (6, 8, 27), and various synthetic peptides (7, 9, 10, 35); and 2) inhibitors of biological origin such as adria-

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Abbreviations: PKC, protein kinase C; PMSG, pregnant mare serum gonadotropin; HCG, human chorionic gonadotropin

mycin (37), sphingosine (6), sangivamycin (16), concanavalin A (28), defensins (3) and staurosporine (36, 38). Besides these, endogenous PKC inhibitors have been found in normal mammalian tissues. Schwantke and Le Peuce isolated a 20 kDa PKC inhibitor from rat brain (33), and McDonald and Walsh isolated from bovine brain a 17 kDa PKC inhibitor protein which has some resemblance to calmodulin (18). Hucho *et al.* (11) isolated from bovine brain cytosol a 40 kDa heat-stable protein with PKC-inhibiting activity. Balazovich *et al.* (1) suggested the presence of another type of PKC inhibitor protein which is associated with neutrophil-specific granule membrane.

We have isolated and characterized a novel PKC inhibitor from bovine spermatozoa, and demonstrated that the inhibitor exerts an inhibitory action on the development of fertilized ova.

MATERIALS AND METHODS

Materials

PKC was purified from rat brain soluble fraction as described by Inoue *et al.* (12). Diacylglycerol, III-S histone, Ampholine and chemicals used in Whitten culture medium (glucose, crystalline BSA, polyvinylpyrrolidone, and EDTA) were purchased from Sigma (St. Louis, MO, U.S.A.). Animals (6-week-old female and 8-week-old male Kunming mice) were supplied from the Department of Laboratory Animals, China Medical University. Fresh bovine spermatozoa were obtained at a local slaughterhouse and transported to our laboratory packed in ice. Pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (HCG) were obtained from the Changchun Biological Products Research Institute (Changchun, Jilin, China) and the Shanghai Biological Products Research Institute (Shanghai, China), respectively.

Determination of the PKC Activity

PKC activity was determined by the method of Kikkawa *et al.* (15) by measuring the incorporation of ^{32}P from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into III-S histone. Reaction mixture (0.14 ml) contained 5 μmol of Tris/HCl (pH 7.5), 1.25 μmol of Mg acetate, 50 μg of III-S histone, 2.5 nmol of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ ($15\text{--}20 \times 10^6$ cpm/nmol), 125 nmol of CaCl_2 , 10 μg of phosphatidylserine, and 0.4 μg of diacylglycerol. The reaction was started by the addition of reaction mixture to tubes containing 90 μl distilled water and 20 μl PKC to

be assayed, and terminated after incubation for 3 min at 30°C by adding 3 ml of 25% trichloroacetic acid. Acid-precipitated materials were collected on a Toyo Roshi membrane filter (pore size, $0.45 \mu\text{m}$), and radioactivity was counted in a scintillation counter.

Determination of the Inhibitor Activity

Activity of purified rat brain PKC (0.4 μg) was measured in the presence and absence of appropriate amount of PKC inhibitor. Inhibitor activity was expressed as a percentage decrease, taking the PKC activity in the absence of inhibitor as 100%. One unit of PKC inhibitor was defined as the activity which causes 50% inhibition of the PKC activity.

Microinjection of PKC and PKC Inhibitor into Fertilized Ova

Micropipets were prepared from glass capillaries (outer diameter 2 mm) using a micropipet puller (W-P Instruments, New Haven, CT, U.S.A.). Diameter of the micropipet tip was 0.5–1.0 μm . Solutions were delivered into the micropipet through its rear open end by means of a 20 gauge Angiocath (Deseret, Sandy, UT, U.S.A.), and microinjected into fertilized ova by using a micromanipulator (MP-1, Narishige, Tokyo, Japan) under an inverted microscope (TMS, Nikon, Tokyo, Japan). Temperature was maintained at 37°C using a plastic housing equipped with an incubator. The volume of injected solution was approximately 2 pl. When the microinjection caused cell damages such as bleb formation, these ova were excluded.

Culture of Fertilized Ova

Ova were transferred to the Whitten culture medium, pre-equilibrated at 37°C and in 5% $\text{CO}_2/95\%$ O_2 . The Whitten culture medium contained 88 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4 , 1.2 mM $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 23 mM NaHCO_3 , 5.5 mM glucose, 1.7 mM Ca lactate, 50 mM Na lactate, 0.23 mM Na pyruvate, 0.1 mM EDTA, 1 mg/ml polyvinylpyrrolidone and 3 mg/ml BSA. The ova were incubated in a Queue cell culture incubator (Box 1901, Parkersburg, WV, U.S.A.).

Other Methods

Protein was determined by the method of Lowry *et al.* (17). Polyacrylamide gel electrophoresis was car-

Fig. 1 Sephadex G-200 column chromatography of a PKC inhibitor protein. The PKC inhibitor activity was determined as described under 'Materials and Methods' with 10 μ g of purified rat brain PKC and 40 μ l each of eluate fractions from the Sephadex column. The inhibitor activity was represented as percentage decrease in the PKC activity. ●—●, inhibitor activity; ·····, absorbance at 280 nm

ried out at 400 V for 4 h. Samples were dissolved in 0.5 M Tris/HCl (pH 6.8) containing 2 M glycerol and 0.7 M 2-mercaptoethanol and subjected to electrophoresis. The separating gel consisted of an increasing gradient of polyacrylamide from 5 to 18%. Proteins were stained with Coomassie Brilliant Blue. BSA (68 kDa), ovalbumin (45 kDa), pepsin (34.7 kDa) and trypsin (23.8 kDa) were used as molecular weight markers.

RESULTS

Purification of a PKC Inhibitor from Bovine Spermatozoa

All procedures were carried out at 0–4°C. Fresh bovine spermatozoa (2 ml) were mixed with 2 ml of 20 mM Tris/HCl (pH 7.5) containing 0.25 M sucrose, 10 mM EGTA and 2 mM EDTA, and centrifuged at 2,000 rpm for 10 min. The supernatant was discarded. The same operation was repeated to remove impurities. The pellet was suspended in the same buffer (2 ml), homogenized in a sonic dismembrator (Fisher Scientific, F50, Pittsburgh, PA, U.S.A.) for 1 min. After centrifugation at 100,000 g for 1 h, PKC inhibitor activity was largely recovered in the soluble fraction. The activity detected in the pellet was negligible, if at all. The soluble fraction (2 ml, 17.8 mg protein) was applied to a Sephadex G-200 column (1.5 $\times$ 90 cm) which had been equilibrated with 20 mM Tris/HCl (pH 7.5) containing 10 mM 2-mercaptoethanol, 0.5 mM

Fig. 2 Isoelectric focusing of the PKC inhibitor protein. Isoelectric focusing and determination of the PKC inhibitor activity were carried out as described in the text and in the legend to Fig. 1. ■—■, inhibitor activity; ·····, pH

EDTA and 0.5 mM EGTA. Elution was carried out with the same buffer, and fractions of 2 ml each were collected. The PKC inhibitor activity was detected in fractions 22–27 (Fig. 1). Active fractions (12 ml, 4.77 mg protein) were combined and concentrated to 2 ml using an Amicon ultrafiltration apparatus (M-52, Lexington, MA, U.S.A.) with a PM-10 filter. The concentrate was subjected to isoelectric focusing using a 110 ml column (LKB) of carrier ampholyte (pH 4–6.5) at a final concentration of 1.5%. After electrophoresis at 600 V for 38 h, fractions of 2 ml each were collected. The PKC inhibitor activity was recovered in fractions 23–27 (Fig. 2). The pH of the peak fraction determined at 4°C was about 4.5. Combined active fractions (10 ml, 0.44 mg protein) were concentrated to 1 ml by ultrafiltration and applied to a Mono Q column (HR 5/5, Pharmacia) which had been equilibrated with 20 mM Tris/HCl (pH 7.5) containing 0.25 M sucrose, 1.2 mM EGTA, 20 mM 2-mercaptoethanol and 0.1 mM phenylmethylsulfonyl fluoride. Elution was carried out with a linear gradient of NaCl (0.2–0.7 M) at a flow rate of 1 ml/min, and 1 ml-fractions were collected. The PKC inhibitor activity was recovered in 0.45–0.55 M fractions of the NaCl gradient (Fig. 3). Active fractions (3 ml, 0.065 mg protein) were combined and dialyzed first against a large excess of 20 mM Tris/HCl (pH 7.5) containing 10 mM 2-mercaptoethanol, 0.5 mM EGTA, 0.5 mM EDTA and 20% sucrose, and then against 0.9% NaCl. The purified PKC inhibitor was concentrated to 2 ml by ultrafiltration and used for subsequent studies. A typical

Table 1 *Purification of a PKC Inhibitor Protein from Bovine Spermatozoa*

Steps	Total protein (mg)	Total inhibitor activity (units)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
100,000 g supernatant	17.80	540.91	30.4	1	100
Sephadex G-200 chromatography	4.77	366.45	76.8	2.5	67.7
Isoelectric focusing	0.44	280.94	638.5	21.0	51.9
Mono Q column chromatography	0.065	145.26	2,234.8	73.5	26.9

PKC inhibitor protein was purified from 2 ml of bovine spermatozoa. Details of the purification and assay of the PKC inhibitor activity are described in the text and legend to Fig. 1.

Fig. 3 Mono Q column chromatography of the PKC inhibitor protein. Chromatographic procedures and assay of the PKC inhibitor are as described in the text and legend to Fig. 1. ●—●, inhibitor activity; ·····, NaCl gradient

Fig. 4 Electrophoresis of the purified inhibitor. Polyacrylamide gel electrophoresis was carried out under conditions described in the text; 80 μ g of the protein were loaded (A). BSA (68 kDa), ovalbumin (45 kDa), pepsin (34.7 kDa) and trypsin (23.8 kDa) served as molecular weight markers (B)

Fig. 5 Dose-dependent inhibition of PKC by the PKC inhibitor protein. The PKC activity in the presence of the inhibitor was expressed as logarithm of percent activity taking the activity in the absence of the inhibitor as 100%. The PKC reaction with 0.4 μ g of purified rat brain PKC was carried out as described under 'Materials and Methods' in the presence of 0–40 μ l of the PKC inhibitor (0.2 μ g/ μ l).

example of the purification procedure is presented in Table 1.

Properties of the Purified PKC Inhibitor

The purified PKC inhibitor gave apparently a single protein band, when 80 μ g protein was subjected to polyacrylamide gel electrophoresis (Fig. 4). The molecular weight was estimated to be about 63,000.

Fig. 5 shows that the inhibition of PKC by the inhibitor was dose-dependent when PKC was assayed with III-S histone as substrate in the presence of Ca^{2+} , phospholipid and diacylglycerol. The

Table 2 Comparison of Inhibitory Effects of the PKC Inhibitor and Chlorpromazine

	Activity (cpm)	Inhibition (%)
PKC	15,000	0
PKC + inhibitor	7,000	46
PKC + chlorpromazine	6,000	60

PKC reaction was carried out with 0.4 μ g of purified rat brain PKC as described under 'Materials and Methods'. Where indicated, 2 μ g of the purified bovine spermatozoa PKC inhibitor or 5 μ mol of chlorpromazine was included in the reaction mixture.

Table 3 Effects of Various Treatments of the PKC Inhibitor

Additions	Inhibition (%)
None	0
PKCI	48
PKCI (boiled) ¹	8
PKCI pretreated with trypsin ²	11
PKCI pretreated with DNase ³	46
PKCI pretreated with RNase ⁴	47

PKC reaction was carried out with 0.4 μ g purified rat brain PKC, except that 10 μ l of the PKC inhibitor (PKCI) (0.2 μ g/ μ l) were added after various treatments as indicated. Mean values of 3 to 5 experiments are shown. ¹The PKC inhibitor was boiled for 3 min prior to the addition to the assay mixture. ²The PKC inhibitor had been treated with 0.2 mg/ml trypsin for 24 h at 30°C. ^{3,4}The PKC inhibitor had been treated with 300 μ g/ml DNase or 100 μ g/ml RNase for 6 h at 30°C.

inhibition by 2 μ g of the inhibitor was comparable with that by 5 μ mol of chlorpromazine, a potent inhibitor of PKC (20), indicating that inhibitory effect of the bovine spermatozoa PKC inhibitor is very potent (Table 2). Table 3 shows effects of various treatments on the inhibitory activity of the PKC inhibitor. Trypsin digestion at 30°C for 24 h, and boiling for 3 min caused a marked reduction in the inhibitory activity, whereas treatment with DNase or RNase was without effect. Activity of PKC could be altered by proteolysis of the enzyme, or by dephosphorylation of substrate protein of PKC, but inhibitory activity of the spermatozoan PKC inhibitor was not affected by protease inhibitors (phenylmethylsulfonyl fluoride and leupeptin) or by phosphatase inhibitors (NaF and L-serine).

Table 4 Effects of Protease and Phosphatase Inhibitors

Additions	PKC inhibiting activity (%)
None	0
PKCI	44
PKCI + NaF	45
PKCI + L-serine	42
PKCI + PMSF	44
PKCI + leupeptin	46

PKC reaction with 0.4 μ g of purified rat brain PKC was carried out under the standard assay conditions in the presence of 10 μ l of the purified PKC inhibitor (PKCI) (0.2 μ g/ μ l). Where indicated, 40 mM NaF, 0.1 mM L-serine, 1 mM phenylmethylsulfonyl fluoride (PMSF) or 10 μ g/ml leupeptin was included in the reaction mixture. NaF or L-serine was added as a phosphatase inhibitor, and PMSF or leupeptin as a protease inhibitor. Mean values of 3 to 5 experiments are shown.

These results suggest that the spermatozoan inhibitor does not act on PKC via proteolysis or phosphorylation processes.

These results altogether indicate that bovine spermatozoa contain a fairly potent PKC inhibitor which is a protein of approximately 63 kDa.

Effect of the PKC Inhibitor and PKC on the Development of Fertilized Ova

In order to demonstrate a possible function of the PKC inhibitor, its effect on the development of fertilized ova has been investigated. In this experiment, superovulation was induced by intraperitoneal injection of 5 units of PMSG, which was followed, 48 h later, by intraperitoneal injection of 5 units of HCG. Female mouse injected with PMSG and HCG was immediately housed together with a male mouse. Insemination has been known to occur 10 h after the HCG injection, and vaginal emboli resulted from the copulation has been taken as a possible sign of insemination. The two-cell stage in the development of fertilized ova can be divided into two phases, an early one (32–36 h post-insemination) and a late one (38–42 h post-insemination). Fertilized ova in the two-cell stage *in vivo* can pass the early and late phases smoothly and further proceed into the four-cell stage. *In vitro*, on the other hand, only two-cell late stage ova and not two-cell early stage ova can develop into the four-cell stage, because of the two-cell stage block (4). Fertilized ova were therefore taken out 10 h after

Fig. 6 Effect of the microinjected PKC inhibitor on the development of fertilized ova. Experimental procedures are as described in the text and under 'Materials and Methods'. A: Fertilized ova microinjected with 2 pl each of 0.9% NaCl as a control developed into two-cell stage ova 36 h after insemination. $\times 400$. B: Fertilized ova microinjected with 2 pl each of the PKC inhibitor ($0.2 \mu\text{g}/\mu\text{l}$) failed to proceed to the two-cell stage. $\times 400$. C: Two-cell late stage ova microinjected with 2 pl of 0.9% NaCl as a control developed into four-cell stage ova 42 h after insemination. $\times 200$. D: Two-cell late stage ova microinjected with 2 pl each of 0.2 mg/ml PKC inhibitor remained in the two-cell stage. $\times 200$

insemination from the ovary of mice which had vaginal emboli. Two-cell stage ova of early and late phases were taken out from the oviduct 36 h and 42 h after insemination, respectively. Fertilized ova and two-cell late stage ova were microinjected with 2 pl of the purified PKC inhibitor protein ($0.2 \mu\text{g}/\mu\text{l}$). Two-cell early stage ova were microinjected with 2 pl of purified rat brain PKC ($0.4 \mu\text{g}/\mu\text{l}$). As a control, fertilized ova and two-cell early and late stage ova were microinjected with 2 pl of 0.9% NaCl. All these ova were then subjected to culture in the Whitten culture medium at 37°C for 26–32 h.

When the PKC inhibitor protein was microinjected, fertilized ova failed to develop into the two-cell stage, and two-cell late stage ova failed to develop into the four-cell stage. In the control group, fertilized ova developed into two-cell stage ova 36 h

after insemination, and two-cell late stage ova proceeded into the four-cell stage 42 h after insemination (Fig. 6). Fertilized ova microinjected with 2 pl of boiled PKC inhibitor also developed into two-cell stage ova.

As shown in Fig. 7, two-cell early stage ova microinjected with PKC developed into the four-cell stage and further into the eight-cell stage, whereas the control ova microinjected with 0.9% NaCl stayed in the two-cell stage because of the two-cell stage block.

DISCUSSION

It is well known that Ca^{2+} plays an important role in cell proliferation. A transient increase in intracellular Ca^{2+} concentration is a prerequisite for the development of fertilized ova (29). Outflow of H^+

Fig.7 Effect of microinjected PKC on the development of two-cell early stage ova. Experimental procedures are as described in the text and under 'Materials and Methods'. Two-cell early stage ovum microinjected with 2 pl of purified rat brain PKC ($0.4 \mu\text{g}/\mu\text{l}$) developed into four-cell stage ovum 42 h after insemination (A; $\times 400$), and further into the eight-cell stage on longer culture in the Whitten culture medium (B; $\times 200$). Two-cell early stage ova microinjected with 2 pl each of 0.9% NaCl (C; $\times 100$) remained in the two-cell stage under the same culture conditions.

from cells has been observed in developing fertilized ova, resulting in an increase in intracellular pH from 6.6 to 7.2, which is also a prerequisite for the development (13). The mechanism of the increment in intracellular Ca^{2+} concentration has been a target of current studies. Chapron *et al.* (2) suggested that epidermal growth factor opens Ca^{2+} channel and induces Ca^{2+} entry into cells. It has been

also observed that microinjection of Ca^{2+} or inositol-1,4,5-trisphosphate (IP_3) promotes the development of fertilized ova, suggesting that the IP_3 -induced Ca^{2+} release from endoplasmic reticulum is involved in the increase in intracellular Ca^{2+} concentration. Then how does Ca^{2+} function in ova? It is possible that Ca^{2+} acts through binding with calmodulin, a Ca^{2+} -binding regulatory protein known to be abundant in ova. Another possibility is that Ca^{2+} acts through activation of PKC; both a certain concentration of Ca^{2+} and diacylglycerol are required in the activation of classical PKCs (α , βI , βII and γ) (24, 25). cDNA cloning analysis of different PKC isozymes showed that the classical PKCs have four conserved (C_1 to C_4) and five variable (V_1 to V_5) regions. The C_1 region is a putative membrane-binding domain. The C_2 region appears to be related to the Ca^{2+} requirement of the enzyme (25). When activated by an increase in intracellular Ca^{2+} concentration, PKC is known to associate with cell membrane. One of the known functions of PKC is the activation by phosphorylation of a Na^+/H^+ exchange protein. As a result of this activation, H^+ is released from cells resulting in an increase in intracellular pH which is advantageous to cell division (34). Available evidence has also indicated that activated PKC regulates gene expression through acting on some transcriptional factors, such as CREB, AP-1 and I- κB , directly or indirectly (14).

PKC does exist in spermatozoa, but it has been difficult to measure its activity (30, 31). This may have been due, at least in part, to the presence of an inhibitor of PKC in spermatozoa. We have shown for the first time the presence of a 63 kDa PKC inhibitor in bovine spermatozoa and purified it through Sephadex G-200 gel filtration chromatography, isoelectric focusing and Mono Q column chromatography. Its inhibitory activity is fairly potent; inhibition observed with $2 \mu\text{g}$ of the purified inhibitor is comparable with that with $5 \mu\text{mol}$ of chlorpromazine. The inhibitor is neither phosphatase nor protease. We found recently that when bovine spermatozoa homogenate was incubated at pH 7.5 and 37°C , approximately 50% and 60% of the PKC inhibitor activity were lost after the incubation for 12–16 h and 20 h respectively, suggesting that the inhibitor was inactivated by some protease(s) in spermatozoa. The nature of this proteolytic inactivation remains to be elucidated.

In the present study, the microinjected spermatozoa PKC inhibitor clearly inhibited the development of fertilized ova. This inhibition was observed in the development not only from one-cell stage

ova to two-cell stage ova, but also from two-cell late stage ova to four-cell stage ova. *In vitro*, two-cell early stage ova can not proceed into the four-cell stage because of the two-cell stage block (4). However, microinjection of 2 pl of purified rat brain PKC (0.4 $\mu\text{g}/\mu\text{l}$) allowed the two-cell early stage ova to develop into the four-cell and eight-cell stages, suggesting a promoting role of PKC in the development of fertilized ova. Recently, we detected immunohistochemically α , β and ϵ isozymes of PKC in the bovine sperm tail and β isozyme in the sperm head. The PKC inhibitor was also detected in the head of fresh bovine spermatozoa by an immunofluorescence method (Haihui Xue, Yu Yang, Zhihong Zong, Shengwu Wang and Bingzhi Yu; to be published). A major interest related to these observations is whether or not the PKC in spermatozoa actually enters ova and promotes its development. Further studies along this line are in progress.

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