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Action of Protein Kinase A and C Activators on Germinal Vesicle Breakdown and One-Cell Embryos in the Mouse

Daekee Lee, Kyungjin Kim and Wan Kyoo Cho

Department of Zoology, College of Natural Sciences, Seoul National University, Seoul 151-742, Korea

Experiments were performed to examine the role of cAMP-dependent protein kinase (PK-A) and diacylglycerol-dependent protein kinase (PK-C) during the meiotic resumption and the first mitotic cell cycle of mouse embryogenesis. Meiotic GV oocytes and one-cell embryos derived from *in vitro* fertilization were cultured *in vitro*, and morphological changes in response to activators of PK-A and PK-C were examined. Treatments with a membrane-permeable cAMP analog, dbcAMP (0.1 mg/ml), phosphodiesterase inhibitor, IBMX (0.1 mM), biologically active phorbol ester, TPA (10 ng/ml), or a synthetic diacylglycerol, sn-diC$_8$ inhibited resumption of meiosis. Combination of PK-A and PK-C activator brought about further inhibition. On the contrary, dbcAMP (0.1 mg/ml), IBMX (0.2 mM), TPA (10 ng/ml), and sn-diC$_8$ (0.5 mM) did not inhibit pronucleus membrane breakdown (PNBD) when added S or G2 phase of cell cycle. However, activators of PK-C inhibited cleavage of one-cell embryos. This result indicates that the action mechanism of PK-A and PK-C on dissolution of nuclear membrane in primary meiotic arrest oocytes may be different from that of mitotic one-cell embryos.

KEY WORDS: Germinal vesicle breakdown, Pronuclear memberane breakdown, Protein kinase A and C

In most animal species including mammal, amphibian, and starfish, fully grown oocytes enclosed in follicles are arrested at the prophase of the first meiotic division. Meiotic resumption of mammalian oocytes enclosed in follicles is induced under the stimulus of gonadotropin *in vivo* as well as *in vitro*. Meiotic resumption is followed by dissolution of nuclear membrane (germinal vesicle breakdown: GVBD), chromatin condensation, extrusion of the first polar body and then rearrest at the second metaphase; it designates as oocyte maturation (reviewed by Masui and Clarke, 1979).

When fully grown immature oocytes were removed from their follicular environment, spontaneous meiotic resumption occurred without stimulus of gonadotropin (Pincus and Enzmann, 1935; Edwards, 1962). *In vitro* spontaneous meiotic resumption is prevented by membrane-permeable cAMP analogs (Cho *et al.*, 1974), inhibitors of cyclic nucleotide phosphodiesterase (PDE) (Dekel and Beers, 1980), or activators of diacylglycerol-dependent protein kinase (PK-C) (Urner and Schorderet-Slatkine, 1984; Bornslaeger *et al.*, 1986b). Previous studies also indicate that the changes in protein phosphorylation by cAMP-dependent protein kinase (PK-A) or diacylglycerol-dependent protein kinase (PK-C) may play an important role in resumption of meiosis (Bornslaeger *et al.*, 1986a, 1986b).

Maturation promoting factor (MPF) has been known in relation to dissolution of nuclear membrane and condensation of the chromosome. For instance, active MPF present in mature mouse oocytes can induce GVBD of amphibian and starfish oocytes as well (Kishimoto *et al.*, 1984; Sorensen *et al.*, 1985). MPF extracted from mitotic cells of starfish and amphibian blastomeres

arrested in metaphase can induce GVBD of immature oocytes in the same species (Kishimoto *et al.*, 1982; Wasserman and Smith, 1978). In mammals, active MPF is present in mature oocytes and blastomeres arrested in metaphase (Balakier, 1978; Tarkowski and Balakier, 1980). Also, it has been known that MPF activity appears during metaphase and disappears during interphase of cell cycle (reviewed by Masui, 1985; Ford, 1985). The above studies suggest that MPF action may be not the species-specific and meiotic/mitotic-specific.

It is presumed that MPF activity may be present in pronuclear membrane breakdown (PNBD) stage of one-cell embryos. If such is the case, it is of interest to examine whether exogeneous treatment of PK-A and/or PK-C activators would affect PNBD in one-cell embryos, whether MPF activity would be linked to protein phosphorylation by PK-A and/or PK-C, and how their effect on PNBD is related to that on GVBD. The present study adresses the above questions.

Materials and Methods

Animals

Randomly bred ICR mice were maintained in the Animal Breeding Center of Seoul National University under controlled light (07:00-21:00) and dark cycle with food and water *ad libitum*.

Collection and culture of oocytes

Three-week-old female mice being sacrificed by cervical dislocation, four to six ovaries were obtained in each experiment. Ovaries were placed in an embryological watch glass and punctured with a fine needle to release oocytes from their follicles under a dissecting microscope (Wild). Intact GV-containing oocytes without cumulus cells were collected using a mouth-controlled micropipet. The puncture medium is a standard egg culture medium (SECM) (Biggers *et al.*, 1971) with a slight modification by decreasing the concentration of $NaHCO_3$ to 15 mM and by adding 10 mM HEPES, pH 7.4. The medium also contains PDE inhibitor, 3-isobutyl-1-methylxanthine (IBMX; 0.2 mM) in order to prevent the resumption of meiosis *in vitro*. Oocytes were cultured in multi-well plate (Lux products) containing 0.1 ml of medium at 37°C in humidified atmosphere of 5% CO_2 in air. Multi-well plate containing medium was preincubated for equilibration at least for 1 h prior to culture. All instruments and glasswares used in culture were sterilized with dry heat at 160°C for 1 h or autoclaved for 30 min at 15 lbs. Culture medium was filtered by Millipore membranes (pore size 0.45 μm; Millipore Co.) prior to use.

In vitro fertilization and culture of one-cell embryos

For *in vitro* fertilization, m-KRB solution (Toyoda and Chang, 1974) containing 30 mg/ml bovine serum albumin was used. The medium was sterilized through millipore filtration (0.45 μm, Millipore Co.). Embryological watch glasses containing 0.4 ml (for fertilization) or 0.5 ml (for sperm suspension) of medium overlaid with 2 ml of equilibrated mineral oil (Sigma) were prepared 12 h prior to use and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air (Hoppe and Pitts, 1973).

Six-to eight-week-old female ICR mice were superovulated by an intraperitoneal injection of 5 I.U. pregnant mare's serum gonadotropin (PMSG) and followed by 5 I.U. human chorionic gonadotropin (hCG) (Sigma) 48 h later. The mice were sacrificed at 13.5 h post hCG, the oviducts were excised and the ovulated eggs were obtained by tearing the oviducts with a fine needle. Two to three cumulus masses were placed in each embryological watch glass. After sixteen-to twenty-week-old male ICR mice were sacrificed, sperm suspension was made by squeezing out the sperm from the cauda epididymides. The sperm was incubated for 1.5-2 h at 37°C to allow for capacitation (Fraser, 1983). At 14 h post hCG, 20-30 μl of sperm suspension was pipetted into each watch glass containing the oocytes, and the watch glasses were subsequently incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Fertilized eggs possessing two pronuclei were separated from the sperm suspension at 10 h post insemination (hpi) (Howlett, 1986), washed in preincubated modified Whitten's medium containing 0.1 mM EDTA (m-WM + EDTA) (Abramczuk *et al.*, 1977) three times. One-cell embryos were incubated in petri dish (35 × 10 mm, Falcon Plas-

tic Co.) containing microdrops of m-WM + EDTA under mineral oil (Brinster, 1963) until either 13 hpi or 16 hpi, and further cultured in multi-well plate containing 0.1 ml of medium with testing drugs.

Chemical treatments

Dibutyryl cyclic AMP (dbcAMP) (10 mg/ml, Sigma) was prepared as stock solution in salt solution of SECM. Stock solution of IBMX (0.1 M, Sigma) was prepared in 0.3 N NaOH solution. 12-0-tetra-decanoyl phorbol-13-acetate (TPA) and $4\alpha-12$, 13 phorbol didecanoate ($4\alpha-$PDD) were prepared as 10 μg/ml stock solution in dimethylsulfoxide (DMSO). Sn-1, 2-dioctanoylglycerol (Sn-diC_8) (10 mM, Sigma) was prepared in chloroform and chloroform driven off under reduced pressure prior to use and then an equal volume of DMSO was added (Bornslaeger *et al.*, 1986b).

Observation of oocytes and embryos

Germinal vesicle (GV), germinal vesicle breakdown (GVBD) oocytes, pronuclear (PN), and pronuclear membrane breakdown (PNBD) embryos were scored by examination under stereomicroscope (Wild M5) at 100-fold magnification. For cytological examination, one-cell embryos were placed onto a glass slide, fixed in glacial acetic acid 1 vol. + ethanol 3 vol. for 10 min, stained with 0.5% aceto-lacmoid for 5 min, and then examined under phase microscope (Nikon).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Phosphoproteins were radiolabeled by incubating oocytes in 40 μl of phosphate-free SECM containing 0.8 mCi/ml ^{32}P-orthophosphate (Amersham) for 3 h; embryos in 40 μl of phosphate-free m-WM + EDTA containing 0.8 mCi/ml ^{32}P-orthophosphate for 2 h. Following radiolabeled, oocytes and embryos were washed thoroughly in 0.1% polyvinylpyrrolidone (PVP-40, Sigma, pH 7.0) solution and transferred to 1.5 ml Eppendorf tube. After three cycles of freezing and thawing, 1 μl of solution of RNase (50 μg/ml in 10 mM Tris, pH 7.5 and 5 mM $MgCl_2$) was added and the sample was incubated for 15 min at room temperature (Schultz *et al.*, 1983). Each sample was placed in 20 μl of SDS sample buffer (Laemmli, 1970). One-dimensional SDS-PAGE was performed as described previously (Laemmli, 1970). Proteins were stacked on 3% SDS polyacrylamide gels and separated on 10% SDS polyacrylamide gels with a current of 20 mA (for stacking); 30 mA (for separating). Gels were dried onto filter paper and exposed to Fuji X-ray film at -70°C for 6-12 days for autoradiography.

Statistical analysis

Students t-test and one-way analysis of variance (ANOVA) were used for analysis of data.

Results

Effect of IBMX and TPA on GVBD

Denuded GV oocytes were incubated in medium with various concentrations of IBMX which is an inhibitor of PDE. Meiotic resumption of GV oocytes was inhibited by increasing concentration of IBMX, while most GV oocytes showed GVBD in control medium (Fig. 1A). GV oocytes were still shown GV stage up to 20-h incubation period at 0.1 mM IBMX (data not shown).

The active phorbol ester, TPA inhibited the meiotic resumption of denuded oocytes in a dose-dependent manner (Fig. 1B). GV oocytes maintained their GV stage up to 20-h incubation period at 10 ng/ml of TPA, as being similar to IBMX-including condition (data not shown).

A more natural activator of PK-C, sn-diC_8 also inhibited the meiotic resumption, whereas inactive phorbol ester such as $4\alpha-$PDD failed to inhibit meiotic resumption (Fig.2A). When the oocytes were cultured in the presence of both IBMX and TPA, meiotic resumption was, interestingly, further inhibited (Fig. 2B).

Effects of IBMX and TPA on PNBD

In order to determine the period of cell cycle when drugs were added, morphological changes during the first cell cycle were initially examined (Fig. 3). PNBD occurred at 16-20 hpi, cleavage subsequently occurred at 18-22 hpi (Fig. 4).

One-cell embryos containing male and female pronucleus were incubated in various concentrations of IBMX or TPA in order to investigate the possible role of protein kinase A or C in PNBD.

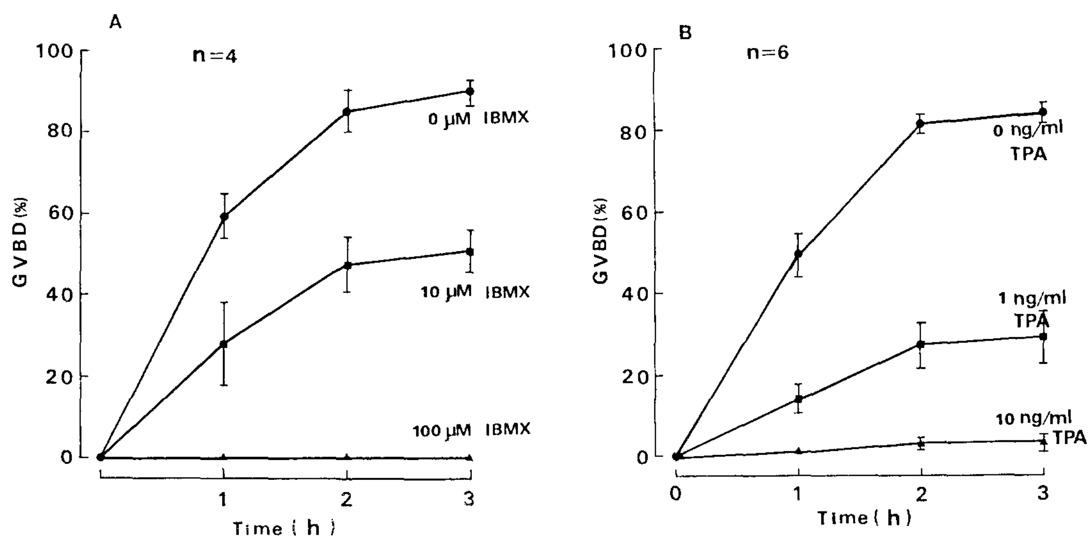

Fig. 1. Effect of IBMX and TPA on GVBD. Denuded oocytes were collected in SECM plus 0.2 mM IBMX and then transferred to media containing IBMX (A) and TPA (B) with various concentrations. Oocytes showing GVBD were scored at different incubation times. Each point represents the mean ± SE.

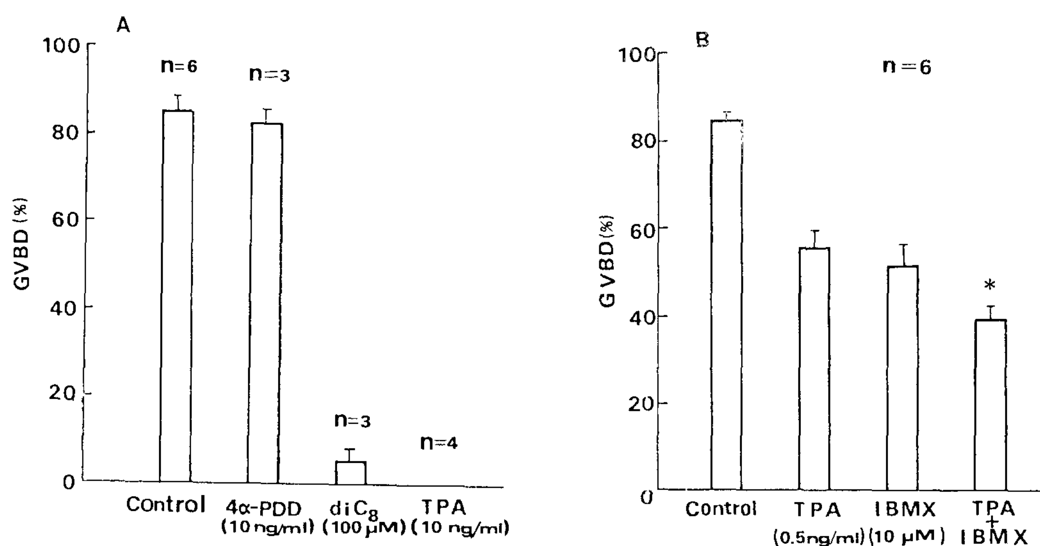

Fig. 2. (A) Effect of 4α-PDD or sn-diC_8 on GVBD. Experimental protocol is the same as that described in Fig. 1. Inactive PK-C activator, 4α-PDD did not inhibit GVBD. Whereas natural activator of PK-C, sn-diC_8 inhibited GVBD. (B) Combined effect of IBMX and TPA on GVBD. Asterisk indicates that more inhibition caused by combining IBMX with TPA ($p<0.01$). Each bar represents mean ±SE.

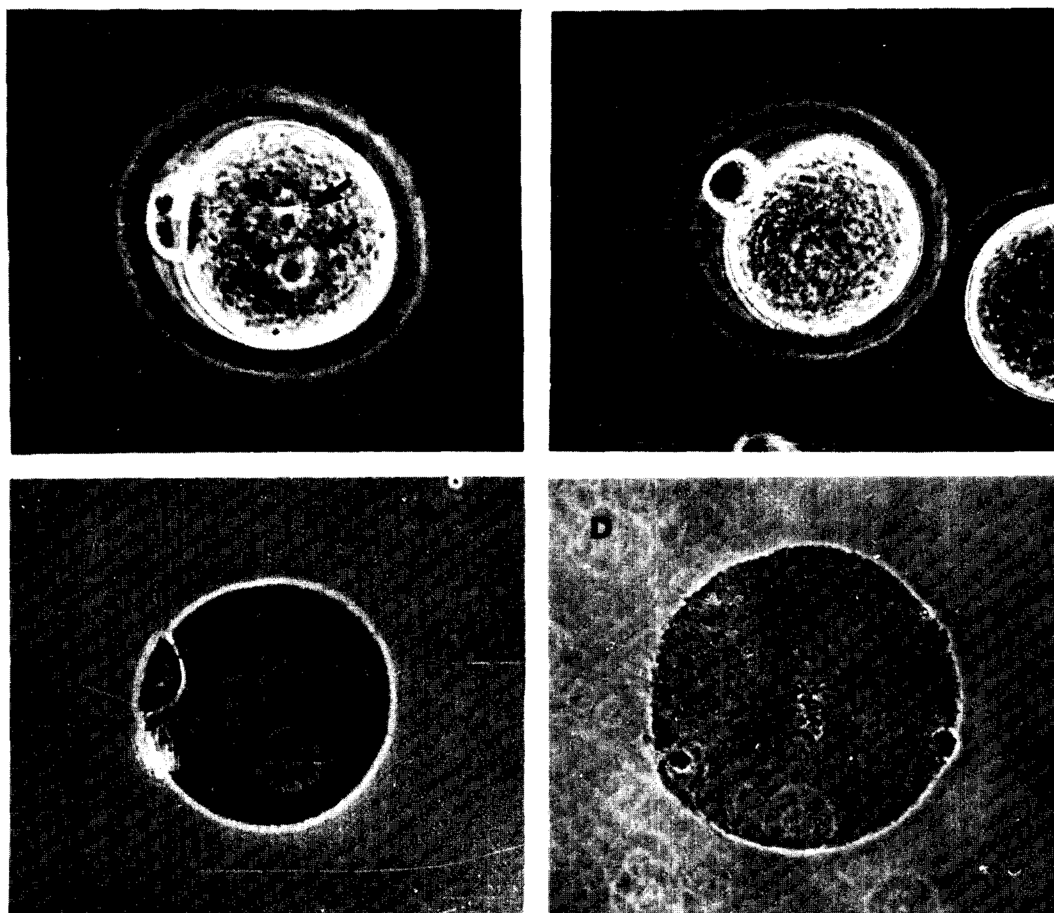

Fig. 3. Morphological changes of one-cell embryos. Phase photographs of (a) one-cell embryo containing male and female pronucleus (arrows), (b) one-cell embryo shown PNBD. Cytological observation of (c) one-cell embryo containing male and female pronucleus (arrows), (d) one-cell embryo shown PNBD (arrow indicates mitotic chromosomes). Photographs were taken by a Nikon inverted microscope (X200).

Treatment pronuclear one-cell embryos with IBMX from 16 hpi of the first cell cycle did not inhibited PNBD contrary to the action of IBMX on GV oocytes (Fig. 5A and see Fig. 1A). TPA failed to inhibit PNBD either (Fig. 5B). However, when TPA was added from 16 hpi, cleavage of one-cell embryos was clearly inhibited and this inhibition was evident even at 36 hpi (Fig. 6).

Changes in protein phosphorylation pattern during GVBD and PNBD

The previous data (Figs. 1,5) indicate that action mechanism of PK-A and PK-C on GVBD and PNBD may differ from each other. Then, the question is whether pattern of protein phosphorylation in response to PK-A and PK-C might be different between GVBD and PNBD. Fig. 7 depicted electrophoretic data showing three major classes of protein phosphorylation patterns: 1) protein phosphorylation patterns of molecular weight 24, 36, 76, 98, and 117 KD were common in GVBD and PNBD, 2) protein phosphorylation increased during GVBD (53 and 66 KD) and PNBD (50 KD), respectively, and 3) protein phos-

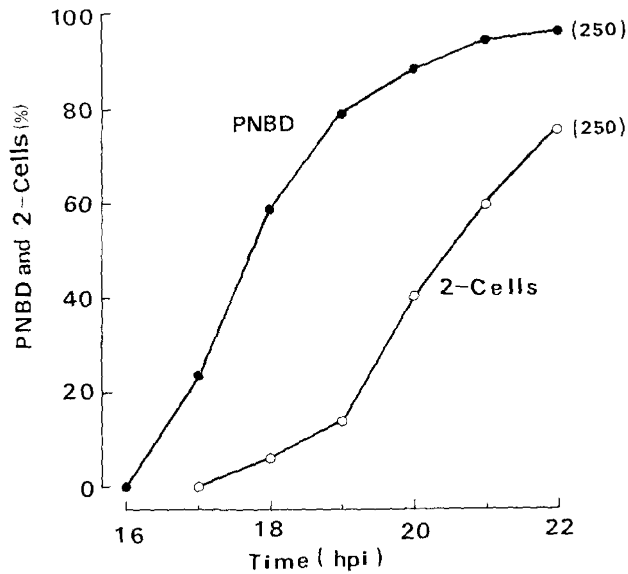

Fig. 4. The time course of first mitosis. One-cell embryos derived from *in vitro* fertilization were cultured in m-WM + 0.1 mM EDTA. The cumulative percentage of eggs with PNBD and two-cell embryos was calculated at different incubation periods expressed hours post insemination (hpi). Number of eggs examined is shown in parenthesis.

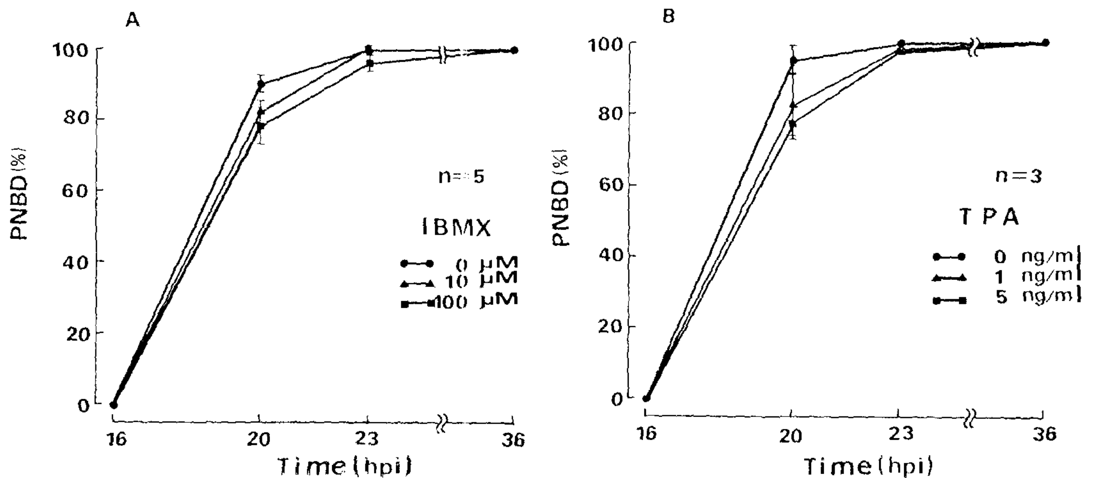

Fig. 5. Effect of IBMX and TPA on PNBD. One-cell embryos collected at 16 hpi were cultured with various concentrations of IBMX (A) and TPA (B). The percentage of one-cell embryos showing PNBD was calculated at the indicated times. Each point represents the mean ± SE.

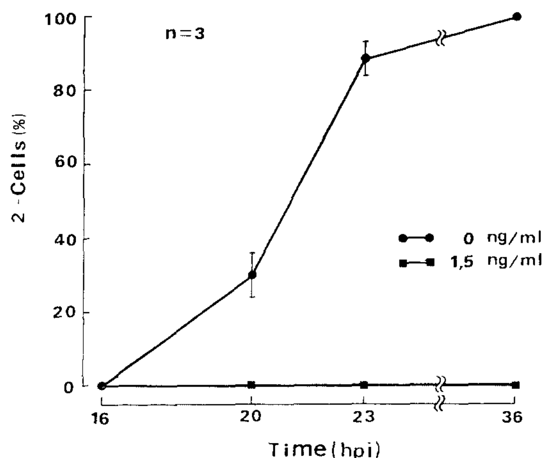

Fig. 6. Effect of TPA on the first cleavage. One-cell embryos collected at 16 hpi were cultured with various concentrations of TPA. The percentage of two-cell embryos was calculated at the indicated times. Each point represents the mean ± SE.

phorylation of molecular weight 81 KD decreased only during GVBD.

Discussion

There is a substantial number of reports supporting the notion that cAMP plays an important role in the physiological regulation of meiotic resumption in mammalian oocytes (reviewed by Sato and Koide, 1987). The effect of IBMX on GVBD of denuded oocytes as shown in Fig. 1A coincides with the previous reports (Dekel and Beers, 1980; Bornslaeger *et al.*, 1984). When the catalytic subunit of PK-A was microinjected into GV oocytes, spontaneous meiotic resumption was inhibited; whereas inhibitor of PK-A was microinjected into GV oocytes, GVBD was found to occur in the presence of IBMX (Bornslaeger *et al.*, 1986a). It appears, then, that inhibition of meiotic resumption by IBMX is mediated by activation of PK-A.

It has been known that PK-C plays a crucial role in signal tansduction for activation of cellular function and proliferation in a variety of biological system (reviewed by Nishizuka, 1984). The present finding that PK-C activator, TPA inhibited GVBD in denuded oocytes is well consistent with the previous reports (Urner and Schorderet-Slatkine, 1984; Bornslaeger *et al.*, 1986b). It may suggest that protein phosphorylation by PK-C is involved in inhibition of meiotic resumption.

Although it remains to be elucidated whether PK-A and PK-C would work together in inhibition of meiotic resumption, the present study revealed, however, that combination of IBMX and TPA resulted in a strong inhibition of meiotic resumption (Fig. 2B). Considering the above finding, together with the previous report that PK-C activator did not affect either the level of cAMP or decrease of PK-A activity in the mouse oocytes (Bornslaeger *et al.*, 1986b), it suggests that specific protein(s) phosphorylated by PK-A and/or PK-C involved in meiotic resumption is common.

During oocyte maturation, active MPF appeared at GVBD and reached a peak level at each meiotic metaphase (Hashimoto and Kishimoto, 1988). MPF activity has been found in mitotically dividing cells such as cleaving blastomeres of starfish (Kishimoto *et al.*, 1982), amphibian (Wasserman and Smith, 1978), mammal (Balakier, 1978), and mammalian cultured cells synchronized at M-phase (Sunkara *et al.*, 1979; Nelkin *et al.*, 1980). Moreover, MPF activity appeared to oscillate during each cell cycle (Gerhart *et al.*, 1984; Ford, 1985; Masui, 1985). Although a direct demonstration whether MPF activity is present in PNBD embryos is not yet available, it can be assumed that MPF activity may appear in PNBD, at least, when the first mitosis begins.

It remains unknown whether PK-A and/or PK-C may affect MPF activity. However, it is thought that the appearance of active MPF at GVBD stage of oocytes may be controlled by the phosphorylation of specific oocyte proteins by PK-A and/or PK-C. Interestingly, in the present study, exogenous treatment with PK-A and PK-C activators did not inhibit PNBD of mitotic one-cell embryos, in contrast to inhibition of meiotic resumption in GV oocytes. There are several possibilities to explain the differential action of PK-A and/or PK-C activators in mitotic one-cell embryos and GV oocytes. One possibility is that treatments mitotic one-cell embryos with PK-A or PK-C activator from 16 hpi (G2 phase) in the present study may be passed a certain critical

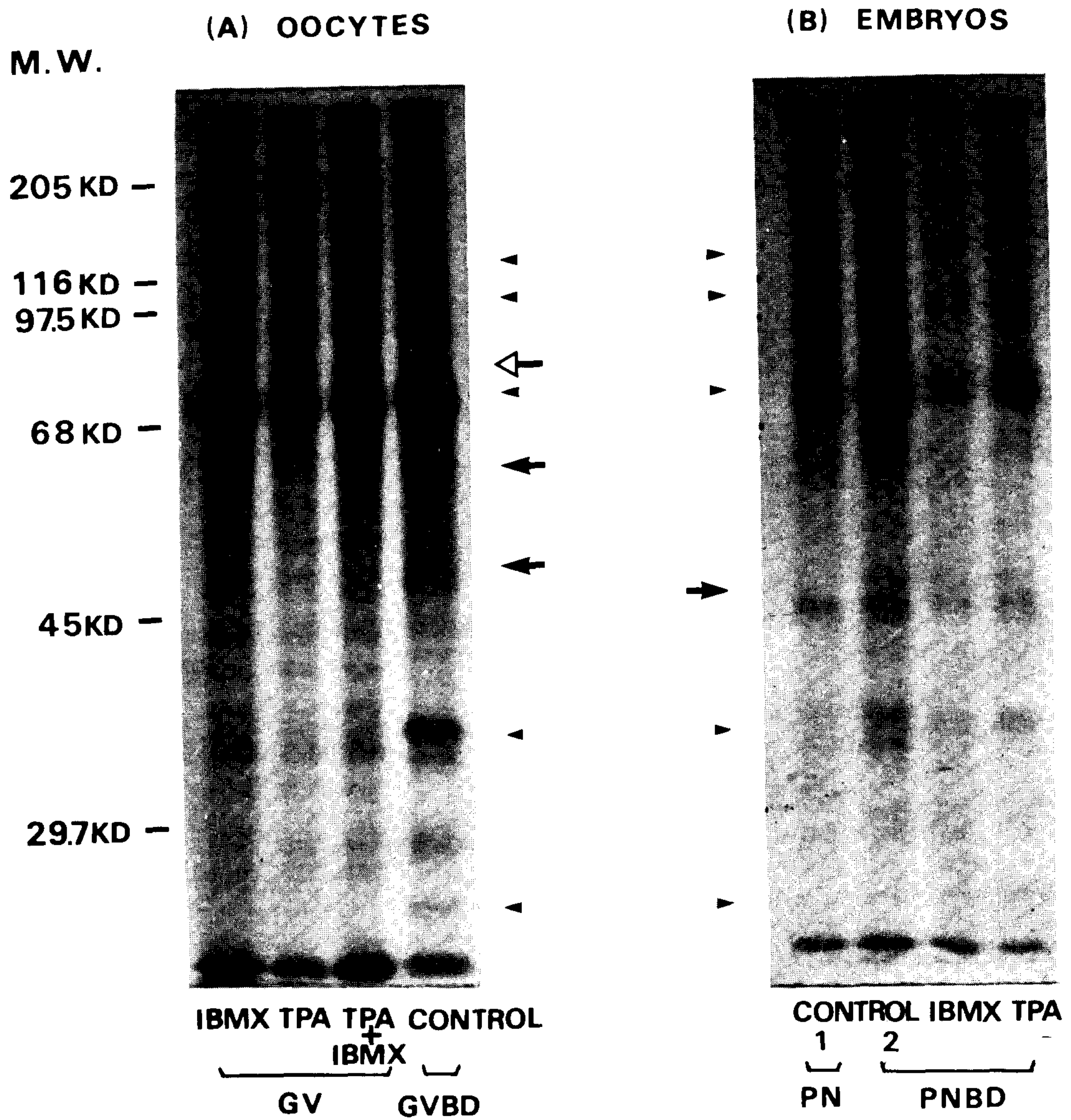

Fig. 7. The patterns of protein phosphorylation during meiotic resumption (A) and pronuclear membrane breakdown (B). Experimental protocol was described in materials and methods. ◀: common protein phosphorylation patterns in GVBD and PNBD; ⬅: protein phosphorylation increased during GVBD and PNBD; ⇦: protein phosphorylation decreased only during GVBD.

period to be effective in the control of PNBD. Several reports showed that the treatment GV oocytes with PK-A activators was passed a certain critical period where intraoocyte cAMP level was already dropped, meiotic resumption no longer affected (Cho *et al.*, 1974; Schultz *et al.*, 1983). Even pronuclear one-cell embryos were treated with PK-A or PK-C activators from 13 hpi (S phase), PNBD was not inhibited, though (data not shown). Another possibility is that the concentration of 0.1 mM IBMX used in this study may not be effective in inhibiting PDE activity thoroughly in one-cell embryos. In fact, the previous report showed that inhibition of GVBD was transient unless high concentration of inhibitor was employed in meiotic resumption (Bornslaeger *et al.*, 1984). However, this possibility seems to be unlikely since higher concentration of IBMX (1 mM) still failed to inhibit PNBD (data not shown).

Although a direct demonstration is not yet available, Poueymirou *et al.*, (1987) recently proposed that PK-A and/or PK-C is present in one-cell embryos. If such is the case, a third possibility that the protein(s) phosphorylated by PK-A and/or PK-C may be not related to appearance of MPF activity in one-cell embryos can be considered. Then, the present study suggests that MPF activity may be no longer regulated by PK-A or PK-C in mitotic one-cell embryo, and the protein(s) specifically phosphorylated by PK-A and/or PK-C at GV stage and subsequently dephosphorylated at GVBD stage may play an important role in regulation of MPF activity. Then 81 KD protein found in the present study may be important for controlling meiotic resumption.

Overall, it is tempting to postulate that the activation mechanism of MPF in meiotic arrest oocyte may be different from that of mitotic one-cell embryo. The detail molecular mechanism by PK-A and/or PK-C which is involved in the differential control over GVBD and PNBD remains to be elucidated.

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생쥐 GV난자와 1-세포기 배아의 핵막붕괴에 미치는 Protein Kinase A와 C의 작용

이대기 · 김경진 · 조완규(서울대학교 자연과학대학 동물학과)

난자성숙 재개와 1-세포기 배아의 세포주기에서, cAMP-의존성 protein kinase A와 diacylglycerol-의존성 protein kinase C가 핵막붕괴에 미치는 영향을 조사하였다. 난자성숙 재개는 dbcAMP (0.1 mg/ml), IBMX (0.1 mM), TPA(10 ng/ml), 또는 diacylglycerol (0.1 mM)에 의해 억제되었다. 또한 protein kinase A와 protein kinase C 활성제를 같이 처리하면 난자성숙이 더욱 억제되었다. 그러나 1-세포기 배아의 전핵막붕괴에는 아무런 영향도 미치지 못하였으며, 단지 protein kinase C 활성제만이 세포질 분열을 억제하였다. 이상의 결과로부터, protein kinase A와 protein kinase C에 의한 단백질 인산화 양상이 GV난자의 핵막붕괴와 1-세포기 배아의 전핵막붕괴에 미치는 세포내 작용기작은 상이함을 알 수 있었으며, 전기영동 결과, 81 KD 단백질이 난자성숙 재개에 중요한 역할을 하리라 사료되었다.