Korean J Biol Sci 2: 87-91, 1998

Requirement of Protein Kinase C Pathway during Progesterone-induced Oocyte Maturation in Amphibian, *Rana dybowskii*

Jaya Bandyopadhyay, Arun Bandyopadhyay, Hae Mook Kang[1], Hyuk Bang Kwon, and Hueng Sik Choi*

Hormone Research Center and Department of Biology, Chonnam National University, Kwangju 500-757, Korea; [1]Department of Genetic Engineering, Chongju University, Chongju 360-764, Korea

Key Words:
Oocyte maturation
Protein kinase C
Staurosporine
U73122
Maturation-promoting factor
Rana dybowskii
Amphibian

The present study investigated the involvement of the phospholipase C (PLC) and protein kinase C (PKC) signaling pathways during progesterone-induced meiotic maturation in amphibian (*Rana dybowskii*) oocytes. Progesterone-induced germinal vesicle breakdown (GVBD) of oocytes was significantly inhibited by a PKC inhibitor, staurosporine and a PLC inhibitor, U73122, in a dose-dependent manner. In contrast, U73343, an inactive analogue of U73122, was ineffective in suppressing GVBD. PKC activity in oocytes reached a maximum level at 30 min after progesterone stimulation and this elevated PKC activity was effectively suppressed by U73122 or staurosporine, suggesting that the activation of PKC enzyme is closely linked to PLC signaling during oocyte maturation. In addition, these inhibitors blocked the maturation promoting factor (MPF) activity which appeared in oocytes in response to progesterone, suggesting that PKC activation is an important signal for MPF activity. Therefore, this study demonstrates that the activation of PKC via PLC signaling is directly linked to an intracellular protein kinase cascade related to the appearance of MPF activity during meiotic maturation in amphibian (*Rana dybowskii*) oocytes.

Fully grown amphibian oocytes, arrested in prophase of meiosis I, are induced to mature with progesterone (Schuetz, 1967), followed by the activation of maturation promoting factor (MPF) (Masui and Markert, 1971; Ford, 1985). Progesterone treatment of *Xenopus* oocytes rapidly induces the synthesis of c-*mos* proto-oncogene product, p39mos via a protein kinase cascade that precedes MPF activation which is essential for germinal vesicle breakdown (GVBD) (Sagata et al., 1989). Although it is well established that progesterone initially causes inhibition of adenylate cyclase (Sadler and Maller, 1981), the biochemical mechanisms underlying steroid action on the oocyte plasma membrane are still not fully characterized.

In addition to adenylate cyclase, many studies have also demonstrated that the phospholipid signaling pathway is involved in steroid-induced oocyte maturation. In various amphibian oocytes, it has been reported that progesterone causes a rapid increase in the level of inositol triphosphate (IP_3) (Stith et al., 1992; Chien et al., 1991) and diacylglycerol (DAG) (Wasserman et al., 1990; Chien et al., 1991; Han et al., 1992). In *Rana pipiens* oocytes, progesterone was found to activate plasma membrane-bound protein kinase C (PKC) (Kostellow et al., 1987), while the direct activation of PKC by 12-*O*-tetradecanoylphorbol 13-acetate (TPA) induced oocyte GVBD in the absence of any hormonal stimulation (Stith and Maller, 1987; Kleis-San Francisco and Schuetz, 1988; Kwon et al., 1992). Recently, Han and Lee (1995) have hypothesized that phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis as well as inositol 1,4,5-triphosphate [Ins (1,4,5)P_3]-induced Ca^{2+} release play a crucial role in regulating meiotic cell division in *Xenopus* oocytes. In contrast, other investigators have found that progesterone causes a marked decrease in DAG content within first 15 seconds and subsequent decrease in PKC activity in *Xenopus* oocytes, and these decrease in DAG and PKC activity may be necessary for the resumption of meiotic maturation (Smith, 1989; Varnold and Smith, 1990; Stith et al., 1991). Thus, although activation of PKC by TPA results in meiotic maturation in amphibian oocytes, it is still uncertain whether PKC activation is essential for progesterone-induced oocyte maturation. In this study, by utilizing staurosporine, an inhibitor of PKC and U73122, an inhibitor of PLC, we have demonstrated that the activation of PKC via PLC signaling plays an important role in inducing MPF activation and oocyte GVBD in amphibians, *Rana*

* To whom correspondence should be addressed.
Tel: 82-62-530-0503, Fax: 82-62-530-0500

dybowskii.

Materials and Methods

Animals

Hibernating frogs (*Rana dybowskii*) were collected during November-December from streams in the Kangwon area of Korea and kept in a state of artificial hibernation in a room without heat or light. Animals were housed in a glass or plastic boxes containing added stones to provide a more natural habitat during hibernation. Water was allowed to flow continuously through the containers.

Hormones and reagents

Progesterone, PKC inhibitor, staurosporine, and histone type III-S were purchased from Sigma. PLC inhibitor, U73122 and its inactive analogue, U73343 were obtained from Research Biochemical International. The PKC assay kit and radiochemical [γ-^{32}P]-ATP (3,000 mCi/mmol) were purchased from Amersham International.

Isolation and culture of oocytes

Animals were sacrificed by decapitation, ovaries were removed and immediately placed in amphibian Ringer medium (AR; Kwon and Schuetz, 1985). Fully grown oocytes were manually stripped of their outer follicular envelopes (epithelium, theca) under magnification. Defolliculated oocytes were then treated with calcium-free AR to obtain "denuded oocytes" by removing the single layer of follicular cells that remain attached to the oocyte surface as described earlier (Kwon and Lee, 1991). Samples of 10 denuded oocytes were typically incubated in each well containing 1 ml of AR solution at 22-24℃ for 24 h in a shaking incubator (80 oscillations per minute). Oocytes were preincubated for 1 h in the absence or presence of different concentrations of staurosporine, U73122 or U73343 and further incubated for an additional 24 h with 3 μM of progesterone. Following incubations, oocytes were fixed in 5% trichloroacetic acid (TCA) and examined for GVBD under a dissecting microscope.

Measurement of PKC activity

PKC activity in oocytes was measured as the rate of phosphorylation of peptide substrate using the PKC assay kit. Denuded oocytes were incubated in the absence (control) or presence of progesterone (3 μM) alone or in combination with staurosporine (10 μM) or U73122 (50 μM), respectively. Incubations were terminated at different times by washing oocytes with ice-cold AR solution. Oocytes were homogenized in ice-cold homogenizing buffer containing 50 mM of Tris/HCl, pH 7.5, 0.3% of 2-mercaptoethanol, 5 mM of EDTA, 10 mM of EGTA, 50 μg of PMSF, 10 mM of benzamidine, 2 μg/ml of leupeptin, and 0.1 mM of okadaic acid. Twenty five microliters of each homogenized sample (in duplicate) were subjected to PKC assay according to the manufacturer's instruction using 0.2 μCi [^{32}P]-ATP per 25 μl sample. Radioactivity was measured in a liquid scintillation counter (Packard 2300, USA) and expressed as cpm [^{32}P]-ATP incorporated.

H1 kinase assay

H1 kinase activity was assayed for MPF activity as described previously by Haccard et al. (1995). Briefly, 10 denuded oocytes were incubated in 1 ml of AR solution containing of 10 μM of staurosporine or 50 μM of U73122 in the presence of 3 μM progesterone at 22-24℃. At designated time points oocytes were washed with ice-cold AR and then homogenized in 400 μl of oocyte extraction buffer containing 80 mM of β-glycerophosphate (pH 7.4), 20 mM of EGTA, 15 mM of $MgCl_2$, 1 mM of DTT, 100 μM of sodium orthovanadate, 10 mM of sodium fluoride, 10 μg/ml of leupeptin, 5 μg/ml of aprotonin, and 100 mM of PMSF. The homogenates were centrifuged at 10,000 x g for 10 min. Twenty microliters of each extract and 1 mg/ml of histone type III-S were incubated in a final 50 μl of buffer containing 20 mM of HEPES (pH 7.0), 5 mM of 2-mercaptoethanol, 10 mM of $MgCl_2$, 1 μCi of [^{32}P]-ATP, and 7 μM of protein kinase inhibitor as described by Carnero et al. (1995). Reactions were stopped by addition of 5 x sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and boiled for 5 min. Samples were separated on a 12% SDS-polyacrylamide gel, dried after staining with Coomassie blue and then exposed to imaging plate of the image analyzer (Fuji, BAS-1500) or autoradiographed with X-ray film (Fuji).

Data analysis

Data were analyzed by analysis of variance (ANOVA) or Student's t-test. All treatments were done in duplicate from three individual frogs. All values are expressed as means ± SEM (n=6).

Results

Inhibition of progesterone-induced oocyte maturation by PLC and PKC inhibitors

To ascertain whether PLC and PKC are essential for the progesterone-induced meiotic maturation in amphibian (*Rana dybowskii*), oocytes were isolated and incubated for 24 h in the presence of progesterone (3 μM) with increasing doses of PKC inhibitor, staurosporine (0.1~10 μM), PLC inhibitor, U73122 (10~100 μM), or inactive analogue of U73122, U73343 (10~100 μM) and examined for GVBD, as an indicator of oocyte maturation. As shown in Fig. 1, progesterone significantly induced oocyte GVBD (94%) and this induction was suppressed in the presence of staurosporine (10 μM,

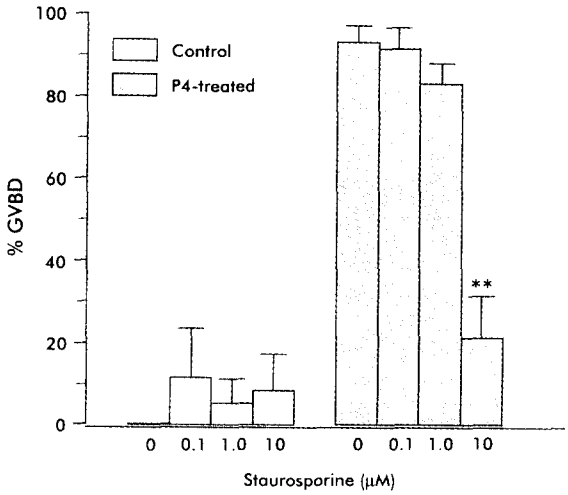

Fig. 1. Effect of a PKC inhibitor (staurosporine) on progesterone-induced oocyte maturation in *R. dybowskii*. Denuded oocytes were pre-incubated in the presence of increasing concentrations of staurosporine (0.1, 1 and 10 μM) for 1 h followed by the addition of progesterone (P4; 3 μM) and incubated further for 24 h. Cultures were terminated by fixing the cells in 5% TCA and examined for GVBD. Observations are expressed as mean±SEM (n=6) for three independent animals with duplicates. ** P<0.01, when compared to P_4 group.

p<0.01 when compared to control) indicating that PKC activation is necessary for oocyte maturation. To test the possible involvement of PLC in oocyte maturation, we examined the effect of PLC inhibitor, U73122 on GVBD of oocyte in vitro (Fig. 2). The treatment of U73122 to oocytes effectively blocked progesterone-induced oocyte GVBD in a dose-dependent manner (p<0.01), whereas its inactive analogue, U73343, was ineffective in the inhibition. Staurosporine or U73122 alone had little or no effect on the oocyte maturation. Hence, these results suggest that PKC and PLC play an important role in the progesterone-induced oocyte maturation in amphibian.

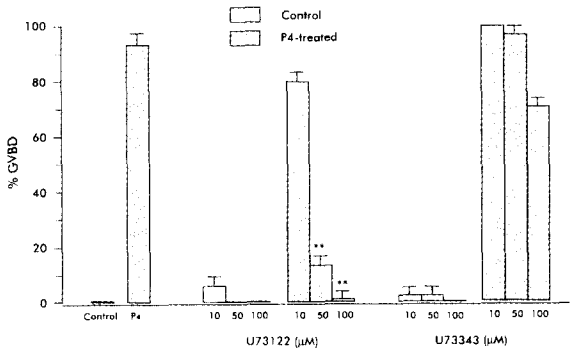

Fig. 2. Effect of PLC inhibitor (U73122) on progesterone-induced oocyte maturation in *R. dybowskii*. Denuded oocytes were pre-incubated in the presence of increasing concentrations (10, 50 and 100 μM) of U73122 or U73343, an inactive analogue of U73122, for 1 h and then incubated further for 24 h with or without 3 μM of progesterone (P4). After incubation, the oocytes were fixed with 5% TCA and examined for GVBD. Observations are expressed as mean±SEM (n=6) for three independent animals with duplicates. ** P<0.01, when compared to P_4 group.

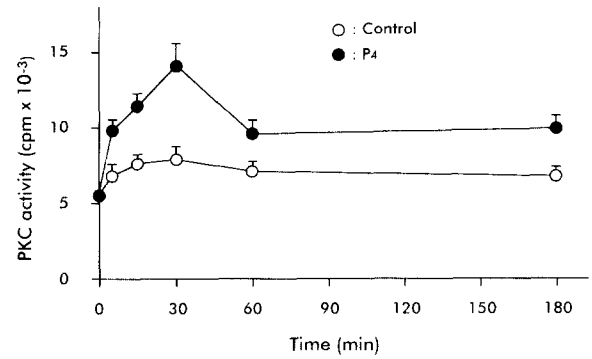

Fig. 3. Time course of PKC activity during progesterone-induced oocyte maturation in *R. dybowskii*. Denuded oocytes were incubated in AR solution with (●) or without (○) 3 μM of progesterone (P_4) and cultured for 3 h. The oocytes were collected at designated time points and PKC activity in oocytes was measured. Data are represented as mean±SEM (n=6) of three independent experiments in duplicates.

Progesterone-induced PKC activation and its inhibition by PLC and PKC inhibitors

To ascertain whether progesterone induced PKC activation during oocyte maturation, the activity of PKC in oocytes was measured through the culture period in the presence or absence of progesterone. As shown in Fig. 3, PKC activity markedly increased and reached a maximum level (2-fold increase vs control) at 30 min in response to progesterone and gradually declined thereafter, whereas PKC activity in control oocytes changed insignificantly during the culture period, indicating that PKC activation occurs within 30 min after progesterone stimulation.

The effect of staurosporine or U73122 on PKC activation was examined at 30 min after progesterone treatments. As shown in Fig. 4, elevated PKC activity with progesterone treatment was significantly inhibited in the presence of 10 μM staurosporine or 50 μM U73122 (p<0.01) which indicates that progesterone stimulates the activation of PKC via the involvement of PLC pathway during oocyte maturation of *Rana dybowskii*.

Effect of PLC and PKC inhibitors on progesterone-induced MPF activation

Since progesterone-induced oocyte GVBD was inhibited by PLC and PKC inhibitors, experiments were carried out to ascertain whether these inhibitors affect the progesterone-induced in vivo activation of H1 kinase which is used as a general marker for MPF activation. For this purpose the phosphorylation pattern of histone H1 protein, by oocyte extracts treated with progesterone, was studied. A marked increase in H1 kinase activity was observed in extracts at 6-9 h of progesterone stimulation and a second increase at 24 h of culture (Fig. 5A). The first rise of H1 kinase activity between 6-9 h preceded GVBD, which occurred by 18 h of culture and this observation is consistent with the time course pattern of GVBD in *Rana dybowskii* oocyte as

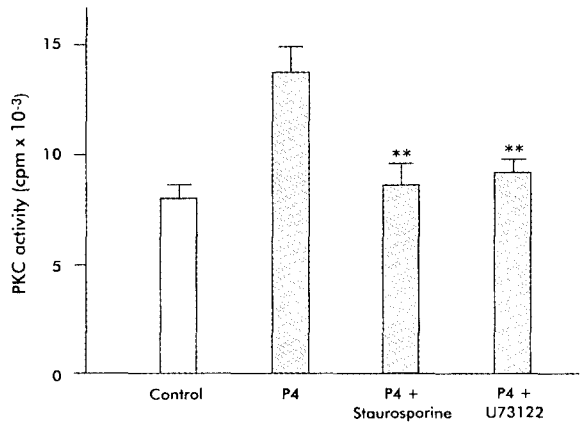

Fig. 4. Inhibition of progesterone-induced PKC activity by staurosporine and U73122 in *R. dybowskii* oocytes. Denuded oocytes were incubated with 10 μM of staurosporine or 50 μM of U73122 in the presence of 3 μM of progesterone for 30 min. The oocytes were homogenized in ice-cold homogenizing buffer and PKC activity was then measured. Data are represented as mean ± SEM (n=6) of three independent experiments in duplicates. ** P<0.01, when compared to P_4 group.

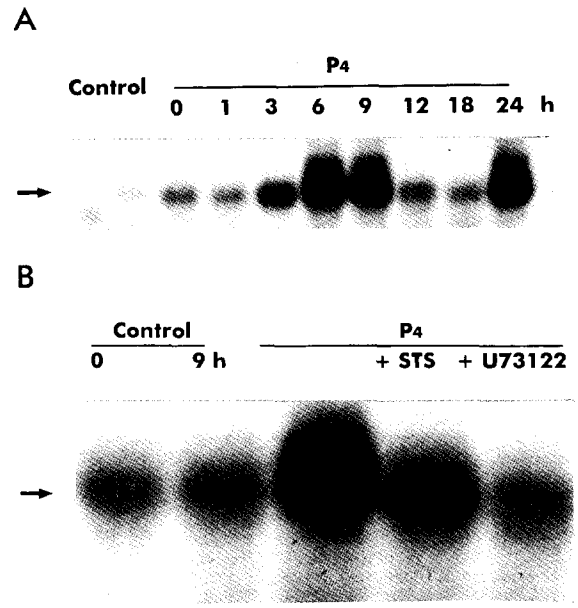

Fig. 5. Effect of PKC and PLC inhibitors on progesterone-induced MPF activity in *R. dybowskii* oocytes. A, H1 kinase activity was assayed for the measurement of MPF activity in progesterone-stimulated oocytes. Denuded oocytes were incubated in the presence of progesterone (3 μM) for 24 h. At designated time intervals, oocytes were harvested and assayed for MPF activity as described in Materials and Methods. Arrow indicates the position of the phosphorylated histone H1 protein. The bands (Control) shown in the first and second lanes from left represent the autophosphorylation of histone H1 protein in the absence of oocyte extracts and the endogenous phosphorylation of oocyte extracts in the absence of histone H1 protein, respectively. Numbers at the top of the gel indicate the time of incubation in hours (hr). B, Inhibition of progesterone-induced MPF activity by staurosporine or U73122. Oocytes were incubated for 9 h with 3 μM progesterone alone (P_4), progesterone plus 10 μM staurosporine (+STS), or progesterone plus 50 μM U73122 (+U73122). After culture, MPF activity in oocytes was assayed. Oocytes cultured for 0 h or 9 h without progesterone stimulation were used as controls. Arrow indicates the position of the phosphorylated histone H1 protein.

described earlier (Kwon et al., 1992).

In order to examine the effects of staurosporine and U73122 on progesterone-induced H1 kinase activation, oocytes were cultured for 9 h in the presence or absence of staurosporine or U73122 with or without progesterone and H1 kinase activities in oocytes were measured. As shown in Fig. 5B, progesterone-induced H1 kinase activation was suppressed by both the inhibitors, indicating that PKC activation is closely linked to MPF activation which is necessary for GVBD in *Rana* oocytes.

Discussion

The present study demonstrates that the signaling pathway of PLC and PKC plays an important role in progesterone-induced meiotic maturation in *Rana dybowskii* oocytes. This conclusion is based on our observations that the potent inhibitors of PKC and PLC effectively suppressed the progesterone-induced PKC activation, MPF activation, and oocyte maturation.

Although the biochemical mechanisms of steroid action on membrane are not fully understood, considerable evidence indicate that steroids trigger production of second messengers in the oocyte membrane (Kostellow et al., 1987; Chien et al., 1991). In *Rana dybowskii* oocytes, we observed that progesterone induces an immediate and transient increase in PKC activity within first 30 min of stimulation (Fig. 3). Since progesterone-induced oocyte GVBD was blocked by a PKC inhibitor, staurosporine (Fig. 1), it is evident that this PKC activation is essential for oocyte maturation. Microinjection of a PKC isoform was found to induce oocyte GVBD without hormonal stimulation in *Xenopus* oocytes (Carnero et al., 1995) and this data also supports the idea that PKC in oocytes play a positive role during oocyte maturation.

In general, interaction of hormone and receptor in the plasma membrane was known to generate two second messengers from phospholipids, such as inositol triphosphate (IP_3) and diacylglycerol (DAG) in amphibians (Morrill et al., 1994). In *Rana pipiens*, it has been shown that labeled phospholipids disappear sequentially 5-90 min after hormone stimulation, suggesting that phospholipid activation occurs as part of a cascade of membrane events (Chien et al., 1986). Moreover, progesterone was found to induce rapid and successive changes in major phospholipid classes in membranes such as ethanolamine, choline, and inositol-containing phospholipids in amphibian oocytes. Several investigators also suggested that generation of DAG and subsequent activation of PKC is due to the PLC mediated hydrolysis of phosphatidyl choline (PC) (Chien et al., 1991) and these observations are consistent with our results, since progesterone-induced activation of PKC, and oocyte GVBD were inhibited by a PLC inhibitor, U73122, in *Rana dybowskii* oocytes (Figs. 2

and 4). Thus, it is likely that PLC-mediated second messengers may play an important role in oocyte maturation in *Rana dybowskii*. The data presented here, together with previous results, demonstrate that progesterone action on oocytes is mediated by membrane-associated second messenger systems such as PLC and PKC pathways in amphibian oocytes.

Although it is well known that progesterone triggers a kinase cascade reaction to induce the synthesis of c-*mos* proto-oncogene protein which is an important component leading to MPF activation prior to GVBD in *Xenopus* oocytes (Sagata et al., 1988, 1989; Maller, 1990; Nebreda et al., 1995), it is still uncertain whether PLC or PKC is involved in a progesterone-induced cascade of events such as synthesis of c-*mos* protein in amphibian oocytes. In this study, it has been shown that progesterone-induced oocyte GVBD and MPF activation were blocked by staurosporine or U73122 respectively (Fig. 5B) which implies that PKC and PLC are directly involved in the progesterone-induced signal transduction pathway for MPF activation and oocyte maturation in *Rana* oocytes. Further studies are required to investigate whether PLC and PKC are involved in the regulatory mechanism of c-*mos* protein synthesis in *Rana* oocyte.

In summary, the present study has demonstrated that PKC and PLC are closely associated with progesterone-induced oocyte maturation in *Rana dybowskii*. These results support the idea that a cascade of plasma membrane signal transduction plays an important role during meiotic maturation in amphibian oocyte.

Acknowledgements

We thank Dr. Sang Young Chun for critical reading of the manuscript. This study was supported in part by research fund from Chonnam National University (H. S. Choi, 1996), Genetic Engineering Research Program of the Ministry of Education (H. M. Kang, 1994), and by the Ministry of Education (BSRI-96-4425).

References

Carnero A, Liyanage M, Stabel S, and Lacal JC (1995) Evidence for different signalling pathways of PKC zeta and ras-p21 in *Xenopus* oocytes. *Oncogene* 11: 1541-1547.

Chien EJ, Kostellow AB, and Morrill GA (1986) Progesterone induction of phospholipid methylation and arachidonic acid turnover during the first meiotic division in amphibian oocytes. *Life Sci* 39 : 1501-1508.

Chien EJ, Morrill GA, and Kostellow AB (1991) Progesterone-induced second messengers at the onset of meiotic maturation in the amphibian oocyte: interrelationships between phospholipid N-methylation, calcium and diacylglycerol release, and inositol phospholipid turnover. *Mol Cell Endocrinol* 81: 53-67.

Ford CC (1985) Maturation promoting factor and cell cycle regulation. *J Embryol Exp Morphol* 89 (Suppl): 271-284.

Haccard O, Lewellyn A, Hartley RS, Erikson E, and Maller JL (1995) Induction of *Xenopus* oocyte maturation by MAP kinase. *Dev Biol* 168: 677-682.

Han JK, Fukami K, and Nuccitelli R (1992) Reducing inositol lipid hydrolysis, Ins (1,4,5)P_3 receptor availability, or Ca^{2+} gradients lengthens the duration of the cell cycle in *Xenopus laevis* blastomeres. *J Cell Biol* 116: 147-156.

Han JK and Lee SK (1995) Reducing PIP2 hydrolysis, Ins (1,4,5)P_3 receptor availability, or calcium gradients inhibits progesterone-stimulated *Xenopus* oocyte maturation. *Biochem Biophys Res Commun* 217: 931-939.

Kleis-San Francisco S and Schuetz AW (1988) Role of protein kinase C activation in oocyte maturation and steroidogenesis in ovarian follicles of *Rana pipiens*: studies with phorbol 12-myristate 13-acetate. *Gamete Res* 21: 323-334.

Kostellow AB, Chien EJ, and Morrill GA (1987) Calcium-dependent phosphorylation of the amphibian oocyte plasma membrane: an early event in initiating the meiotic divisions. *Biochem Biophys Res Commun* 147: 863-869.

Kwon HB, Chang KJ, Yoo YR, Lee CC, and Schuetz AW (1992) Induction of ovulation and oocyte maturation of amphibian (*Rana dybowskii*) ovarian follicles by protein kinase C activation *in vitro*. *Biol Reprod* 47: 169-176.

Kwon HB and Lee WK (1991) Involvement of protein kinase C in the regulation of oocyte maturation in amphibians (*Rana dybowskii*). *J Exp Zool* 257: 115-123.

Kwon HB and Schuetz AW (1985) Dichotomous effects of forskolin on somatic and germ cell components of the ovarian follicles: evidence of cAMP involvement in steroid production and action. *J Exp Zool* 236: 219-228.

Maller JL (1990) *Xenopus* oocytes and biochemistry of cell division. *Biochemistry* 29: 3157-3166.

Masui Y and Markert CL (1971) Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. *J Exp Zool* 177: 129-146.

Morrill GA, Ma GY, and Kostellow AB (1994) Progesterone-induced phospholipid N-methylation and sphingomyelin synthesis in the amphibian oocyte plasma membrane: a second source of the 1,2-diacylglycerol second messenger associated with the G_2/M transition. *Biochim Biophys Acta* 1224: 589-596.

Nebreda AR, Gannon JV, and Hunt T (1995) Newly synthesized proteins must associate with $p34^{cdc2}$ to activate MAP kinase and MPF during progesterone-induced maturation of *Xenopus* oocytes. *EMBO J* 14 : 5597-5607.

Sadler SE and Maller JL (1981) Progesterone inhibits adenylate cyclase in *Xenopus* oocytes: action on the guanine nucleotide regulatory protein. *J Biol Chem* 256: 6368-6373.

Sagata N, Oskarsson M, Copeland T, Brumbaugh J, and Vande Woude GF (1988) Function of c-*mos* protooncogene product in meiotic maturation in *Xenopus* oocytes. *Nature* 335: 519-525.

Sagata N, Daar I, Oskarsson M, Showwalter SD, and Vande Woude GF (1989) The product of the *mos* proto-oncogene as a candidate Initiator for oocyte maturation. *Science* 245: 643-646.

Schuetz AW (1967) Effects of steroids on germinal vesicle of oocytes of the frog (*Rana pipiens*) *in vitro*. *Proc Soc Exp Biol Med* 124: 1307-1310.

Smith LD (1989) The induction of oocyte maturation: transmembrane signaling events and regulation of the cell cycle. *Development* 107: 685-699.

Stith BJ, Jaynes C, Goalstone M, and Silva S (1992) Insulin and progesterone increase $^{32}PO_4$ labeling of phospholipid and inositol 1,4,5-triphosphate mass in *Xenopus* oocytes. *Cell Calcium* 13: 341-352.

Stith BJ, Kirkwood AJ, and Wohnlich E (1991) Insulin-like growth factor 1, insulin, and progesterone induce early and late increases in *Xenopus* oocyte sn-1,2-diacylglycerol levels before meiotic cell division. *J Cell Physiol* 149: 252-259.

Stith BJ and Maller JL (1987) Induction of meiotic maturation of *Xenopus* oocytes by 12-*O*-tetradecanoylphorbol-13-acetate. *Exp Cell Res* 169: 514-523.

Varnold RL and Smith LD (1990) Protein kinase C and progesterone-induced maturation in *Xenopus* oocytes. *Development* 109: 597-604.

Wasserman WJ, Freedman AB, and LaBella JJ (1990) sn-1,2-diacylglycerol levels increase in progesterone-stimulated *Xenopus* laevis oocytes. *J Exp Zool* 255: 63-71.

[Received October 2, 1997; accepted November 5, 1997]