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Induction of Oocyte Ovulation and Prostaglandin Synthesis by Gonadotropin and Phorbol Ester *in vitro* in Amphibian (*Rana nigromaculata*) Ovarian Follicles

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Experiments were carried out to ascertain whether gonadotropin or a phorbol ester (12-O-tetradecanoyl phorbol-13-acetate, TPA) induces oocyte ovulation and stimulates prostaglandin synthesis by *Rana* ovarian follicles *in vitro*. *Rana nigromaculata* collected from underground in spring were utilized for the present experiment. Treatment of frog pituitary homogenate (FPH) or TPA to ovarian fragments in culture induced oocyte ovulation in a dose dependent manner and stimulated prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) synthesis. Both treatments were more effective in inducing the ovulation and $PGF_{2\alpha}$ secretion by the follicles obtained in May than those in April. A Protein kinase C inactivator, 1-(5-isoquinolinyl-sulfonyl)-2-methyl-piperazine (H-7), or cyclooxygenase inhibitor, indomethacin (IM) suppressed the FPH- or TPA-induced $PGF_{2\alpha}$ production, but IM failed to suppress the FPH- or TPA-induced ovulation. Time course of oocyte ovulation and $PGF_{2\alpha}$ secretion by FPH and TPA treatments were very similar to each other. FPH stimulated progesterone secretion by the follicle but TPA failed to do so. Taken together, the data presented here suggest that protein kinase C (PKC) in follicle play a role in the ovulation process of *Rana nigromaculata*, probably via prostaglandin synthesis.

KEY WORDS: Amphibian, Frog, Ovulation, Prostaglandin, Phorbol Ester

Evidences accumulated so far support the view that various steroids and prostaglandins produced by ovarian follicles in response to gonadotropin play some roles in the ovulation process in various vertebrates (reviewed by Jones, 1987; Schuetz, 1985, 1986). Many investigators also reported that protein kinase C (PKC) is involved in the ovulation of fish (Ranjan and Goetz, 1987; Berndtson *et al.*, 1989; Goetz *et al.*, 1991) and mammals (Kaufman *et al.*, 1992; Shimamoto *et al.*, 1993), and activation of PKC stimulates ovarian prostaglandin synthesis animals (Goetz *et al.*, 1991; Kaufman *et al.*, 1992). Previously, we had also shown that PKC activation of ovarian follicles of *Rana dybowskii* with TPA (12-O-tetradecanoylphorbol-13-acetate) induced oocyte ovulation and stimulated prostaglandin synthesis *in vitro* (Kwon *et al.*, 1992; Chang *et al.*, 1995). *R. dybowskii* has a unique pattern of reproduction when compared to other Korean frogs, *R. nigromaculata* or *R. rugosa*. For example, they breed in winter (late February) while other frogs breed in late spring (May) and their ovarian follicles obtained in late winter exhibit spontaneous oocyte maturation and ovulation *in vitro* without any hormone treatment. Present experiments were carried out to know whether PKC is also involved in the ovulation process of other frogs which have

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typical breeding season. Moreover, it was needed to elucidate the relationship between prostaglandin and progesterone synthesis and induction of ovulation in amphibians.

Materials and Methods

Animals

Hibernating frogs (*R. nigromaculata*) were collected during spring (April - May) from underground in the field of Chonnam area. Animals were kept at room temperature and sacrificed within three days after collection.

Hormones and reagents

An active PKC activator, 12-O-tetradecanoyl-phorbol- 13-acetate (TPA), an inactive phorbol ester, 4α-phorbol-12,13-didecanoate (4α -PDD), or a PKC antagonist, 1-(5-isoquinolinylsulfonyl)-2-methyl-piperazine (H-7), was dissolved in dimethyl sulfoxide (DMSO) as 8 mM, 12.8 mM or 40 mM stock solutions, respectively. IM, a potent cyclooxygenase inhibitor, was dissolved in absolute ethanol (2 mg/ml). Frog pituitary homogenate (FPH) was prepared from female frogs collected during hibernation. Glands were homogenized in amphibian Ringer (AR) at 4°C using a glass homogenizer. Homogenate was centrifuged (4°C, 15,000 g, 20 min) to remove debris, and the supernatant was frozen (–20°C) in aliquots until needed.

Culture of ovarian fragments

Animals were sacrificed by decapitation and the ovaries were removed, and placed in AR. Ovarian fragments containing about 20 follicles each were dissected from the ovary using watchmaker's forceps. All experimental manipulations were conducted at room temperature (18 – 22°C). Routine *in vitro* cultures were carried out using multiwell culture dishes (24 wells/dish; Nunc) with one ovarian fragment (containing approximately 20 follicles). Cultures were maintained in a shaking incubator (Newbrunswick Scientific Edison, N. J., U. S. A.) at 24°C and agitated at 80 oscillations per min for various periods of time. After culture, oocytes liberated from the ovarian fragment were fixed with 5% trichloroacetic acid (TCA) and their surfaces were carefully examined under a stereomicroscope. Only those oocytes having a smooth surface and devoid of follicle cells were counted as ovulated oocytes. The duration of culture (24 hrs)and the dose of FPH (0.005-0.05 gland/ml) and TPA (0.1-10 μM) were chosen on the basis of our previous work (Kwon *et al.*, 1992; Chang *et al.*, 1995). Medium samples were stored frozen at –20°C until assayed.

Prostaglandin and progesterone radio-immunoassay (RIA)

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) or progesterone (P_4) secreted into medium by ovarian follicles during culture was measured by RIA. General assay procedure for $PGF_{2\alpha}$ was adopted from those described by Cetta and Goetz (1982) and our previous work(Chang *et al.*, 1995). Progesterone was assayed according to the method described elsewhere (Kwon *et al.*, 1991). Medium samples were assayed directly without extraction. Prostaglandin $F_{2\alpha}$ antiserum (Sigma) was reported to crossreact 60% with prostaglandin $F_{1\alpha}$, less than 0.1% with prostaglandin E_1 and E_2, and less than 0.01% with prostaglandin A_1, A_2, B_1. The sensitivity for $PGF_{2\alpha}$ RIA was 5 pg $PGF_{2\alpha}$/tube. Labeled $PGF_{2\alpha}$ (5,6,8,9,11,12,14,15[n]-^{3}H-prostaglandin$F_{2\alpha}$; 209Ci/mmole) was obtained from Amersham (Buckinghamshire, U.K.). Each sample was quantified for tritium using a Packard Tri-Carb 1500 liquid scintillation analyzer. Duplicate hormone standards (5-1000 pg) were included in each assay. The between and within assay coefficients of variation for $F_{2\alpha}$ were 7.3 and 6.5%, and those for progesterone were 11.6 and 7.7%, respectively.

Statistical analysis

Ovulation data (percent) were transformed using arcsin transformation before statistical analysis. Ovulation data and $PGF_{2\alpha}$ or progesterone levels were analyzed by one- or two-way analysis of variance (ANOVA) or Student's t-test.

Results

Effect of FPH, TPA, or progesterone on the oocyte ovulation during ovarian fragment culture: dose response and time course study

Experiments were carried out to ascertain whether treatment of FPH or TPA to cultured ovarian follicles of *R. nigromaculata* induces oocyte ovulation. Ovarian fragments obtained in April and May were cultured for 24 hr in AR in the presence or absence of various doses of FPH (0.005 - 0.05 gland/ml), TPA (0.1 -10 mM), or progesterone (1 μg/ml), and examined for ovulation after culture (Fig. 1). All the three agents treated in high doses induced ovulation from the ovarian follicles obtained in April, but the ovulation rate was less than 50% except progesterone treated group (about 75%)(Fig. 1A). However, FPH or TPA treatment to the follicles obtained in May induced ovulation very effectively in a dose dependent manner (Fig. 1B). Progesterone was also very effective in inducing ovulation.

Time course of ovulation induced by FPH or TPA was examined with ovarian fragments obtained in May. Ovarian fragments containing 20 follicles were cultured in wells containing 2 ml of AR for up to 24 hr in the presence or absence of FPH (0.05 gland/ml) or TPA (10 μM) or 4α-PDD (1.6 μM), and at designated time points (3, 6, 9, 12, and 24 hr), ovulated oocytes were counted. FPH and TPA increased the incidence of ovulation (about 40%) from 6 hr of culture ($P < 0.01$ when compared to control) (Fig. 2). Ovulation rate induced by TPA was significantly lower than that by FPH during 24 hr culture ($P < 0.01$ by Two-way ANOVA). In contrast, 4α-PDD, a PKC inactivator, was totally ineffective in inducing the ovulation. The overall time course of TPA-induced ovulation was very similar to that of FPH-induced ovulation (Fig. 2).

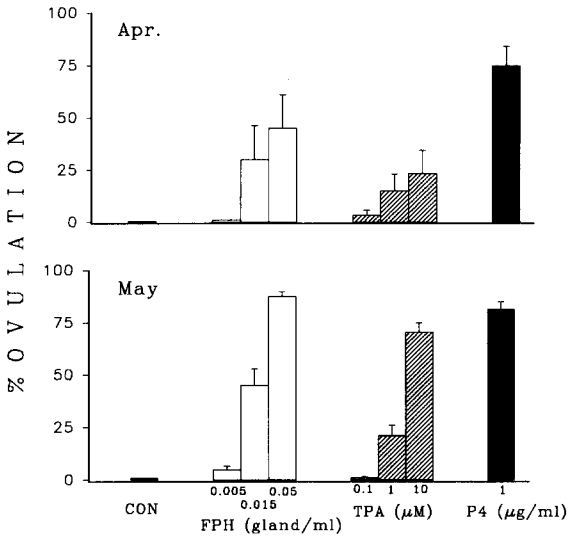

Fig. 1. Effect of FPH, TPA and progesterone on the ovulation of *R. nigromaculata in vitro*: dose response study. Ovarian fragments obtained from frogs collected in April (A) and May (B) were cultured for 24 hr in the presence or absence of various doses of FPH (0.005 - 0.5 gland/ml), TPA (0.1 - 10 μM) or progesterone (1 μg/ml). After culture, ovulation rates (%) were examined. Each bar in the figure represents average (mean ± SEM) % ovulation per fragment (n=6, triplicate incubation, 2 animals in A and n=10, duplicate incubation, 5 animals in B). The data from ovarian fragments obtained in April were depicted in A and those in May in B.

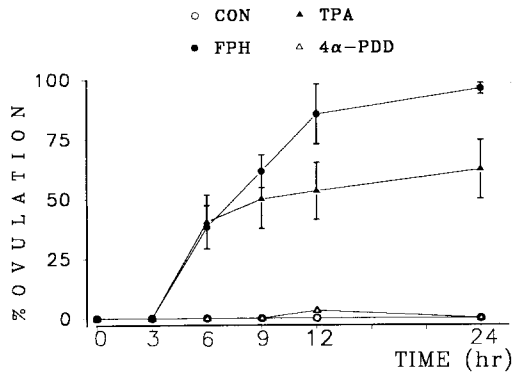

Fig. 2. Time course of ovulation by ovarian fragments in response to FPH or TPA. Ovarian fragments obtained in May were cultured in the presence or absence of FPH (0.05 gland/ml), TPA (10 μM) or 4α-PDD (1.6 μM) for up to 24 hr. At designated time points, ovulation rate was examined. Each point in the figure represents average(mean ± SEM) % ovulation per fragment (n=8, duplicate incubations, 4 animals).

Effects of FPH and TPA on the prostaglandin $F_{2\alpha}$ and progesterone secretion by the ovarian fragments *in vitro*: time course study

Using the same animals utilized in the above experiment (Fig. 2), the time course of $PGF_{2\alpha}$ and progesterone (P_4) secretion by the ovarian fragments in response to FPH and TPA were examined. After examining the ovulation rate culture medium was collected at designated time points and levels of $PGF_{2\alpha}$ and P_4 in the culture medium were measured by RIA. FPH markedly stimulated the secretion of $PGF_{2\alpha}$ by the follicles from 6 hr of culture (200 pg/follicle) and increased the level of $PGF_{2\alpha}$ consistently until 24 hr (518 pg/follicle) (Fig. 3A). Likewise, TPA stimulated the $PGF_{2\alpha}$ secretion from 6 hr of culture (81 pg/follicle) and increased the hormone level steadily until 24 hr (185 pg/follicle) while basal level of the hormone remained very low (<18 pg/follicle) during the culture period (Fig. 3A). In general, the time course of $PGF_{2\alpha}$ secretion produced by TPA stimulation was similar to that by FPH stimulation. The absolute level of $PGF_{2\alpha}$ produced by TPA stimulation was significantly lower than that produced by FPH stimulation ($P < 0.01$ by Two-way ANOVA). In contrast, 4α-PDD was totally ineffective in stimulating $PGF_{2\alpha}$ secretion by the fragments.

The level of P_4 secreted into culture medium by the ovarian fragments increased in response to FPH but not to TPA or 4α-PDD (Fig. 3B). Thus, TPA was ineffective in stimulating P_4 production like 4α-PDD. In contrast, FPH markedly

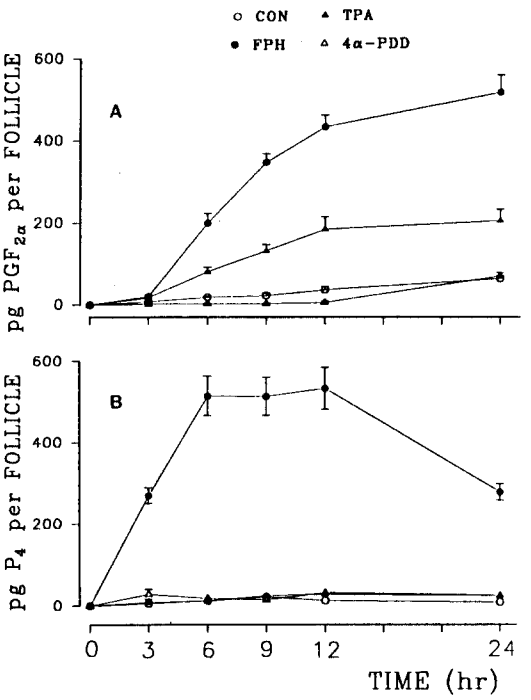

Fig. 3. Time course of $PGF_{2\alpha}$ and progesterone secretion by ovarian fragments in response to FPH or TPA. Ovarian fragments obtained from the same animals used in Figure 2 were cultured in the presence or absence of FPH (0.05 gland/ml), TPA (10 μM), and 4 -PDD (1.6 M) for up to 24 hr. At designated time points, levels of $PGF_{2\alpha}$ or progesterone in medium were measured. Each point in the figure represents average (mean ± SEM) pg $PGF_{2\alpha}$ or pg P_4 per follicle (n=12, triplicate incubations, 4 animals). Levels of P_4 were duplicated in A and that of $PGF_{2\alpha}$ in B.

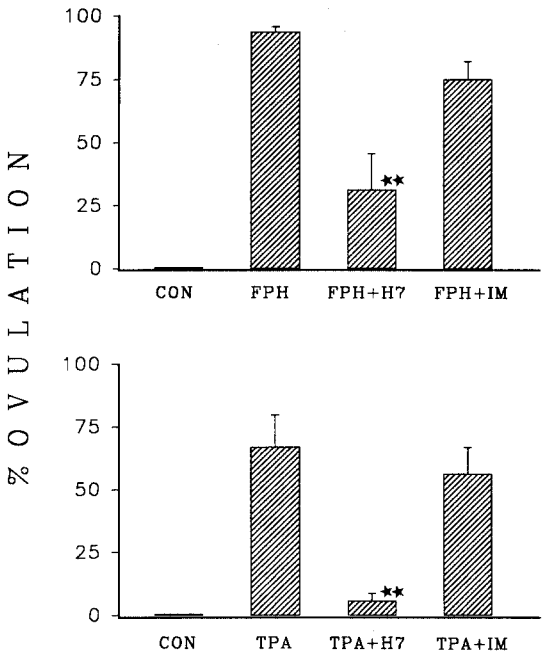

Fig. 4. Effect of H-7 or IM on the FPH- or TPA-induced ovulation by the fragments *in vitro*. Ovarian fragments obtained in May were cultured for 24 hr in the presence or absence of H-7 (100 μM) or IM(5 μg/ml) with FPH (0.05 gland/ml) or TPA (10 μM). Ovulation rate was examined after culture. Each bar in the figure represents average (mean ± SEM) % ovulation per fragment(n=8, duplicate incubations, 4 animals). (**P < 0.01).

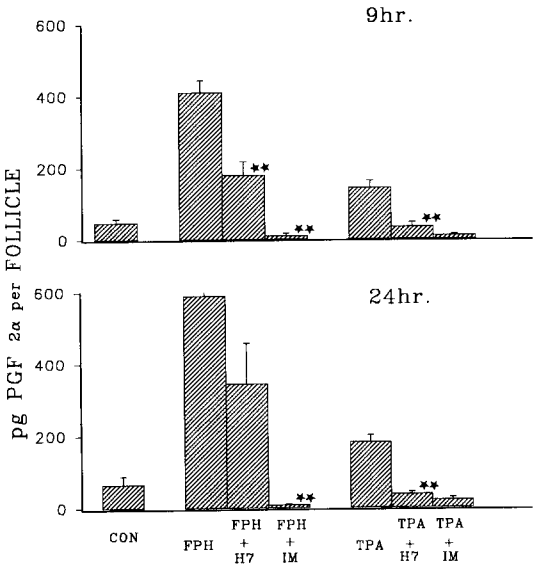

Fig. 5. Effect of H-7 or IM on the $PGF_{2\alpha}$ secretion by the fragments in response to FPH or TPA *in vitro*. Ovarian fragments obtained in May were cultured for 9 or 24 hr in the presence or absence of H-7 (100 μM) or IM (5 μg/ml) with FPH (0.05 gland/ml) or TPA (10 μM). Levels of $PGF_{2\alpha}$ in medium by 9 (A) or 24 hr (B) of culture were measured. Each bar in the figure represents average (mean ± SEM) pg per follicle (n=12, triplicate incubations, 4 animals). (**P < 0.01).

stimulated P_4 production by the follicle from 3 hr (270 pg/follicle) and the P_4 level reached maximum levels by 6 hr of culture (543 pg/follicle)(Fig. 3B).

Effects of H-7 and IM on the FPH- or TPA-induced ovulation and prostaglandin synthesis by the ovarian fragments *in vitro*

Experiments were carried out to ascertain whether inactivation of PKC with H-7 or suppression of prostaglandin synthesis of the ovarian follicles by IM negated TPA- or FPH-induced oocyte ovulation and $PGF_{2\alpha}$ production by the follicles. Ovarian fragments were cultured for 9 or 24 hr in the presence or absence of H-7 (100 μM) or IM (5 μg/ml) with FPH or TPA. Levels of $PGF_{2\alpha}$ in culture medium were measured by 9 or 24 hr of culture and ovulation rates were examined after 24 hr of culture. FPH-induced ovulation (95%) was markedly suppressed by the presence of H-7 (38%)($P< 0.01$ by t-test), but the ovulation was not suppressed by the presence of IM (75%) (Fig. 4). TPA-induced ovulation (67%) was also significantly suppressed by the presence of H-7 (6%) ($P< 0.01$ by t-test) while not suppressed by the presence of IM (56%). FPH-stimulated $PGF_{2\alpha}$ secretion by the fragments was also significantly suppressed by the presence of H-7 or IM during 9 hr but FPH-stimulated $PGF_{2\alpha}$ secretion was not suppressed by H-7 by 24 hour of culture. H-7 was less effective than IM in suppressing FPH-induced $PGF_{2\alpha}$ production by the fragments during culture (Fig. 5). Likewise, TPA-stimulated $PGF_{2\alpha}$ production by the fragments was strongly suppressed by the presence of H-7 or IM (Fig. 5). $PGF_{2\alpha}$ content secreted by the fragments in response to TPA after 24 hr of culture were 181 pg/ml while it was and 37 pg/ml in the presence of H-7, and 22 pg/ml in the presence of IM (Fig. 5). Taken together, it appeared that H-7 and IM suppressed FPH or TPA stimulated prostaglandin synthesis.

Discussion

The present study clearly demonstrated that gonadotropin and TPA, a PKC activator, induced oocyte ovulation and stimulated prostaglandin synthesis by the ovarian fragment of *R. nigromaculata in vitro*. Thus, this study supports the idea that PKC in ovarian follicles plays an important role in the ovulation of amphibians as proposed in our previous study with *R. dybowskii* (Chang *et al.*, 1995).

Although the time course of TPA-induced ovulation and $PGF_{2\alpha}$ production by ovarian fragments was similar to that of gonadotropin (FPH) stimulation, TPA was consistently less efficient than FPH in inducing ovulation and stimulating $PGF_{2\alpha}$ synthesis by the ovarian fragments (Figs. 1 and 3). But, it was found that indomethacin failed to block the FPH- and TPA-induced ovulation though $PGF_{2\alpha}$ production was substantially suppressed (Figs. 4 and 5). These facts imply that $PGF_{2\alpha}$ is not a sole mediator for ovulation. Possibly, there may be other eicosanoids that are less sensitive to IM inhibition

than $PGF_{2\alpha}$ or other unidentified mediators of ovulation (Espey *et al.*, 1986). These data indicate that $PGF_{2\alpha}$ acts in combination with other factors and events to ensure a maximum ovulation. In addition, follicular prostaglandins may also play a role in extrafollicular aspects of amphibian reproduction such as spawning or sexual receptivity as occurs in many other species (reviewed by Guillette *et al.*, 1991). This idea is consistent with *in vitro* studies in fish which demonstrated that plasma prostaglandin levels are not significantly elevated until the end of natural or induced ovulation and remained higher than controls in the post-ovulation period (Cetta and Goetz, 1982; Goetz and Cetta, 1983).

Significantly, ovulation rate and levels of $PGF_{2\alpha}$ produced by the ovarian fragments obtained in April in response to FPH or TPA were much lower than those produced by fragments obtained in May (Figs. 1 and 3). Thus, responsiveness of the follicles to gonadotropin or PKC activation varied at different hibernation periods and possibly, several types of differentiation seem to occur in ovarian follicles as breeding season approached. It is also of interest that ovarian follicles of this frog in culture do not exhibit spontaneous ovulation and $PGF_{2\alpha}$ secretion when examined in breeding season as observed in *R. dybowskii* (Chang *et al.*, 1995).

Interestingly, progesterone was very efficient in inducing ovulation from the ovarian fragments. Moreover, progesterone peaks came earlier than that of $PGF_{2\alpha}$ peak or the onset of ovulation (Fig. 3). The data suggest that progesterone is one of mediators of ovulation in amphibian ovarian follicles and directly involved in ovulation process, or it acts on oocyte and play a supportive role in ovulation through inducing oocyte maturation.

Since TPA stimulates $PGF_{2\alpha}$ secretion and induces oocyte ovulation, it is likely that PKC in follicle wall plays a role in ovulation process. However, Kaufman *et al.*, (1992) has suggested that PKC plays a role in ovulation independent of prostaglandin synthesis. PKC stimulation of tissue type plasminogen activator, which is involved in ovulatory processes, has also been reported (Shimamoto *et al.*, 1993). H-7, a PKC inactivator, was found to suppress TPA and FPH stimulated $PGF_{2\alpha}$ secretion. However, it is important to remember that H-7 is a non-specific inhibitor of protein kinase. Consequently, its inhibitory effects may occur through a number of mechanisms. Alternatively, TPA may act on oocyte and induce oocyte maturation (Kwon and Lee, 1991), which is prerequisite for oocyte ovulation.

In summary, present result confirmed that PKC activation induced oocyte ovulation and prostaglandin $F_{2\alpha}$ secretion simultaneously. However, present study demonstrated that $PGF_{2\alpha}$ is not a sole mediator for ovulation and there are other eicosanoids or mediators for ovulation in amphibians. Further studies are needed to find out these mediators for ovulation in amphibians.

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뇌하수체 호르몬과 포르볼에스터에 의한 참개구리 난자의 배란과 프로스타글란딘 합성유도

장경자 · 나철호 · 소재목 · 이원교† · 권혁방(전남대학교 자연대 생물학과, 호르몬연구센터, †여수수산대학교 양식학과)

봄에 채집한 참개구리의 난소조각 배양계를 사용하여 난자의 배란과정에 프로스타글란딘과 protein kinase C(PKC)가 관여하는 지를 조사하였다. 난소조각을 배양하면서 뇌하수체추출물(PPH) 혹은 PKC의 활성제인 12-O-tetradecanoyl phorbol-13-acetate(TPA)를 처리한 후 배란율과 프로스타글란딘의 생성량을 방사면역측정법으로 조사한 결과 농도에 의존하여 난자의 배란이 유도되었으며 프로스타글란딘의 생성이 촉진되었다. FPH와 TPA에 의한 배란과 프로스타글란딘 생성은, 4월 보다는 5월에 채집한 개구리에서 훨씬 더 효과적이었다. FPH처리는 프로스타글란딘과 함께 progesterone의 생성을 촉진하였으나 TPA는 progesterone의 생성을 촉진하지 못하였다. FPH와 TPA에 의한 배란과 프로스타글란딘의 생성은 PKC 저해제인 H-7에 의해 현저히 억제되었으나 프로스타글란딘 생성 억제제인 indomethacin에 의해서는 난자의 배란이 억제되지 않았다.

이러한 결과들은 참개구리 난자의 배란 과정에 PKC의 활성화가 중요한 역할을 하며, 프로스타글란딘의 생성이 매개할 것으로 생각된다.