International Journal of Entomology Research
www.entomologyjournals.com
ISSN: 2455-4758
Received: 11-03-2023, Accepted: 27-03-2023, Published: 12-04-2023
Volume 8, Issue 4, 2023, Page No. 20-23

Induction of oocyte maturation in common toad, *Bufo melanostictus*: Effects of maturation inducing/inhibiting drugs

Laishram Sanahanbi Devi[1*], Hijam Anita Devi[2], R K Geetanjali Devi[2], Ibemhal Devi Asem[2], Pardeshi Lal[3]
[1] Department of Zoology, Lilong Haoreibi College, Thoubal District, Manipur, India
[2] Department of Botany, Shree Shree Gourgobind Girls' College, Khurai, Imphal East, Manipur, India
[3] Nagaland University, Mokokchung, Nagaland, India

Abstract
Peripheral migration and the breakdown of the germinal vesicle occur during the penultimate stage of oocyte maturation, which follows the conclusion of the vitellogenic growth phase (GVBD). The effects of cynoketone, actinomycin D, cycloheximide and froskolin on spontaneous and progesterone- induced and of the ophyllins on toad-pituitary extracts induced oocyte maturation were investigated during the study. The addition of TPA to the incubation media at concentrations of 0.01 - 10µM induced GVBD in a dose dependent manner. At the end of 48 hrs of incubation 100% GVBD had occurred both in intact and denuded oocytes only in the group incubated with TPA at 10 µM and the percent GVBD in intact and denuded oocytes were 41.43 ± 0.71 and 43.00 ± 2.50 respectively. Follicles cultured with 10 µM of TPA induced GVBD in appropriately 50% of both, intact and denuded oocytes. Around 50 % intact as well as denuded oocytes underwent GVBD after 18 hrs of incubation in the presence of 1µg/ml of progesterone in the culture media. However, by the end of culture period (48 hrs) both, TPA (10 µM) had induced 100% GVBD in intact and denuded oocytes.

Keywords: *Bufo melanostictus*, mammalian and non-mammalian, various hormones/drugs

Introduction
During prophase of the first mitotic division, the germinal vesicle (nucleus) of an egg stays in the centre until further maturation. Peripheral migration and the breakdown of the germinal vesicle occur during the penultimate stage of oocyte maturation, which follows the conclusion of the vitellogenic growth phase (GVBD). Meiotic maturation in ovarian follicles of mammalian and non-mammalian vertebrates occurs spontaneously in *in vitro* cultures (Epping and Downs, 1984; Schuetz, 1985) [17]. In amphibians and fishes follicle cells are known to produce maturation-inducing steroids that cause oocyte maturation in response to gonatropin stimulation (Nagahama and Adachi, 1985; Petrino and Schuetz, 1986; Lin *et al*. 1987) [15, 8].
Ca^{2+} activated phospholipid dependent protein kinase (Protein kinase C) may be involved in regulating the resumption of meiosis in amphibian oocytes. The phorbol ester TPA, which is known to stimulate protein kinase C (PKC) acting in other tissues induces the resumption of meiosis in *Xenopus laevis* (Stith and Maller, 1987) [18]; *Rana oupiens* oocyte (Kleis-San Francisco and Schuetz, 1988) [4] and *Rana dybowskii* (Kwon and Lee, 1991) [6]. PKC appears to play a role in regulating the activation of cells and cell proliferation in a number of different cell types stimulated by hormones and growth factors (Nishizuka 1984 a,b) [13].
In the case of *Bufo melanostictus*, no information is available on the process and mechanism of oocyte maturation. In the present study, an attempt was made to investigate the possible role of follicular constituents in hormone-induced maturation of toad oocyte, in vitro. Experiments were undertaken to examine the oocyte responsiveness to PKC activations. In addition, the effects of cynoketone, actinomycin D, cycloheximide and froskolin on spontaneous and progesterone- induced and of the ophyllins on toad-pituitary extracts induced oocyte maturation were also investigated.

Materials and Methods
Female *Bufo melanostictus* were collected from the University's (Manipur University) campus between October/November and December/January. They were sacrificed. Ovaries were put in amphibians-ringer immediately after the removal from the gravid females. Fresh ovaries were used for each experiment.

Hormones and reagents
Froskolin, PKC, phorbol 12-myristate 13-acetate (TPA) were dissolved in ethanol and glycerol (7:3) in a stock of 10 mM and 1 mM. Cycloheximide, cynoketone, actinomycin D and theophylline were dissolved in amphibian ringer to give a stock solution of 0.1 mg/ml, 0.1 mg/ml, 2mg/ml, and 1 mM, respectively. All hormones and drugs were purchased from Sigma chemical Co., USA. Required concentrations of hormones and drugs were obtained by diluting stock solutions and were added to culture tubes.

Follicle and oocyte cultures *in vitro*
Few oocytes were denuded. Both intact and denuded oocytes were incubated *in vitro.* Incubation was done with different hormones/drugs. Cultures were maintained at 25°C in a water bath with shaking and agitation is done at 80 oscillations per minute.
Ovarian fragments of female *Bufo melanostictus* from the amphibian – ringer were used. Fully grown (stage V and VI) oocytes were isolated by the help of fine forceps. For few experiments these oocytes were made denuded for the somatic components. This was done by the removal of the follicular envelop manually which was continued by treating two hours in Ca2+ free media for removing the adherent follicle cells (Linn and Schuetz, 1985a). Intact and denuded oocytes were incubated *in vitro* with the application various hormones/drugs taken for the study. Cultures were kept in a

water bath (shaking) at 25°C and carried on agitation at 80 oscillations/minute. Under atmospheric air for various length of time (6, 12, 18, 24 and 48 hours). Each sample contained a minimum of 40 oocytes. Four samples from different female toads were taken for each treatment.

Treatments with oocyte maturation –inhibiting drugs in vitro

A decrease in oocyte cAMP levels and protein synthesis is known to be important for - oocyte maturation induced by progesterone (Maller, 1983; Kwon and Lee, 1991). Present experiment was performed to study the effects of preventing decrease of cAMP, protein synthesis, RNA synthesis and 3-βHSD (3-β hydroxyl steroid dehydrogenase) by using forskolin, an adenyl cyclise stimulator; cycloheximide, an inhibitor of protein synthesis, actinomycin D, a transcription inhibitor and cynoketone, an inhibitor of 3 β HSD on progesterone-induced oocyte maturation at various concentrations of inhibitors for 48 hrs. This study was extended by observing the effect of theophyllins, an inhibitor of phosphodiesterase on TPE -induced oocyte maturation at different concentration for 48 hours.

At the end of incubation, oocytes were fixed in 5 % TCA (trichloro acetic acid). After 3 hrs oocytes wre cut by a sharp razor blade and checked under dissecting microscope for general vesicle.

Result and Discussion

Effect of TPA on GVBD

Addition of TPA to the incubation media at concentrations of 0.01 - 10µM induced GVBD in a dose dependent manner (Table 1). The response of denuded oocytes to 0.1 – 1.0 µM between 18- 48 hrs had similar effects in both intact and denuded oocytes (Table 1). At the end of 48 hrs of incubation 100% GVBD had occurred both in intact and denuded oocytes only in the group incubated with TPA at 10 µM. By this time percent GVBD in intact and denuded oocytes were 41.43 ± 0.71 and 43.00 ± 2.50 respectively (Table 1).

Table 1: Effect of Prorbol 12-myristate 13 acetate (TPA) on *in vitro* Germinal Vesicle break down (GVBD) of *Bufo melanostictus* oocytes with intact and denuded follicles.

Treatment TPA	Incubation time (Hrs)				
	6	12	18	24	48
	% GVBD	% GVBD	% GVBD	% GVBD	% GVBD
Intact					
0.01 µM	0.00 ±0.00	7.14 ±3.57	23.33 ±1.36	39.81 ±1.89	52.38 ±1.94
0.1 µM	0.00 ±0.00	17.86±5.92	36.67±7.20	54.23±1.77	64.45±1.82
1.0 µM	12.50±5.89	26.19±2.55	45.24±1.94	76.11±3.17	93.33±5.44
10.0 µM	26.78±5.52	52.38±4.37	65.08±3.43	91.67±4.56	100.00±0.00
Control	0.00 ±0.00	4.81±2.41	15.00±2.50	22.22±3.93	41.43±0.71
Denuded					
0.01 µM	4.81±2.41	12.32±0.76	23.35±2.22	38.65±0.59	49.99±2.62
0.1 µM	9.37±5.18	26.44±1.52	36.50±2.59	49.66±2.38	60.57±2.76
1.0 µM	21.27±2.56	34.58±1.57	50.00±3.37	70.93±4.51	87.78±5.05
10.0 µM	32.29±2.27	57.40±2.34	65.94±2.64	90.28±4.09	100.00±0.00
Control	1.00±0.05	10.00±2.40	20.00±2.50	28.00±1.80	43.00±2.50

Each value represents the mean ± SEM of four replicate incubations.

Time course of progesterone / TPA induced maturation of oocyte *in vitro*

Follicles cultured with 10 µM of TPA induced GVBD in appropriately 50% of both, intact and denuded oocytes (Table 1). Nearly 50 % intact as well as denuded oocytes underwent GVBD after 18 hrs of incubation in the presence of 1µg/ml of progesterone in the culture media. However, by the end of culture period (48 hrs) both, TPA (10 µM) had induced 100% GVBD in intact and denuded oocytes (Table 1). By this time control groups had GVBD in appropriately 40% of oocytes only.

Influence of GVBD inhibiting drugs on progesterone induced oocyte maturation

Progesterone of (1µg/ml) level induced GVBD in 100% oocytes at the end of the cultures (48 hrs) period. Cycloheximide, cynoketone and actonomycin D had no significant effects at 0.01-100 µg/ml inhibited GVBD in a concentration dependent manner (Table 2). Similarly, froskolin at dose levels from 1 to 20 µM inhibited GVBD in dose dependent (Table 3).

Table 2: *In vitro* effect of Cycloheximide, Cynoketone and Actinomycin D on Progesterone induced Germinal Vesicle break down (GVBD) in the oocytes of *Bufo melanostictus*

Treatment	% GVBD				
	0	0.01µg/ml	0.1µg/ml	1.0 µg/ml	10.0 µg/ml
Cycloheximide + Progesterone (1.0 µg/ml)		100.00±0.00	77.55 ±3.19	46.67 ±7.63	23.50 ±3.95
Cycloheximide				2.06±1.24	
Cynoketone + Progesterone (1.0 µg/ml)		100.00±0.00	93.75±3.75	58.25±5.45	28.75±4.27
Cynoketone				10.04±1.51	
Actinomycin D + Progesterone (1.0 µg/ml)		9.00±5.77	69.84±3.99	50.35±1.64	19.02±3.46
Actinomycin D				10.58±0.98	
Control	39.62±2.08				

Each value represents the mean ±SEM of four replicate incubations.

Table 3: *In vitro* effect of Froskolin on Progesterone induced Germinal Vesicle break down (GVBD) in the oocytes of *Bufo melanostictus*

Treatment	% GVBD (48 hrs)
Froskolin + Progesterone	61.00±2.50
1.0µM + 1.0 µg/ml	40±37±4.40
5 µM + 1.0 µg/ml	21.73±2.35
10 µM + 1.0 µg/ml	3.00±1.13
20 µM + 1.0 µg/ml	39.62±2.08
Control	

Each value represents the mean ± SEM of four replicate incubations.

Data presented here intricate that PKC activation induced oocyte maturation during *invitro* cultures. TPA appeared to act directly on the oocyte. Its effects neither required follicle cells nor the stimulated follicular progesterone accumulation. Earlier it was reported that other PKC activators such as OAG (1-oleoyl-2-acetyl-rac-glycerol) and DAG (1,2 dicargloyl-rac-glycerol) were observed to induce oocyte GVBD of *Rana dybowski* oocytes (Kwon *et al*. 1991; Kwon and Lee, 1991) [6]. It is suggested that TPA acts via PKC action for maturation induction. The rate of maturation in response to PKC activation varies more depending upon the type of eggs (Kwon *et al*. 1991; Kwon and Lee, 1991) [6]. It was suggested that the protein phosphorylation is related with the formation or activation of maturation promoting factor, MPF (Eckberg *et al*. 1987; Eckberg 1988; Kwon and Lee, 1991) [6]. Forskilin, cAMP stimulator is used as an inhibitor of oocyte maturation in *Rana dybowskii* (Kwon *et al*. 1990) [5]. Cynoketone, cycloheximide, actinomycin D prevent HCG-induced oocyte maturation in Atlantic croaker (Patino and Thomas, 1990) [5].

Adenyl cyclase stimulator, forskolin suppressed progesterone induced oocyte maturation in *Bufo melanostictus*. As much as actinomycin D inhibition of RNA synthesis as induced maturation showing RNA synthesis as essential event for oocyte maturation in this species. Furthermore, inactivation of 3-β HSD with cynoketone suppressed the progesterone-induced oocyte maturation in *Bufo melanostictus*. All these treatments also suppressed TPA stimulated oocyte GVBD. Thus, similar effects of various drugs in response of follicular oocytes of *Bufo melanostictus*. All these treatments also suppressed TPA stimulated oocyte GYBD. Thus, similar effects of various drugs in response to follicular oocytes of *Bufo melanostictus* to PKC activation and hormonal stimulation imply that TPA use pathway for GVBD induction in oocytes. The possibility remains to be whether TPA occupied different pathway than that triggered by progesterone remains to be ascertained. Practically, no information is available to explain as how PKC activation is linked to MPF formation, activation and eventual GYBD.

References

1. Eckberg WR, Ete Z Szuts, Alan G Carroll. Protein kinase C activity, protein phosphorylation and germinal vesicle breakdown in *Spisula oocytes*. Developmental Biology,1987:124(1):57-64. https://doi.org/10.1016/0012-1606(87)90459-3.
2. Eckberg WR. Intracellular Signal Transduction and Amplification Mechanisms in the Regulation of Oocyte Maturation. Biological Bulletin,1988:174(2):95-108. https://doi.org/10.2307/1541777.
3. Eppig JJ, Downs SM. Chemical signals that regulate mammalian oocyte maturation. Biol Reprod,1984:30(1):1-11. doi: 10.1095/biolreprod30.1.1. PMID: 6320914.
4. Kleis-San Francisco S, Schuetz AW. 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,1988:21(3):323-34. doi: 10.1002/mrd.1120210313. PMID: 2854534.
5. Kwon HB, Park HJ, Schuetz AW. Induction and inhibition of meiotic maturation of amphibian (*Rana dybowskii*) follicular oocytes by forskolin and cAMP in vitro. Mol. Reprod. Dev,1990:25:147-154. https://doi.org/10.1002/mrd.1080250207.
6. Kwon HB, Lee WK. Involvement of protein kinase C in the regulation of oocyte maturation in amphibians (*Rana dybowskii*). J. Exp. Zool,1991:257:115-123. https://doi.org/10.1002/jez.1402570115.
7. Laurent AM, Basset M, Dorée CJ, Le Peuch. Involvement of a calcium-phospholipid-dependent protein kinase in the maturation of *Xenopus laevis* oocytes. FEBS Letters,1988:226(20):324-330 https://doi.org/10.1016/0014-5793(88)81448-0
8. Lin YW P, Lamara MJ, Wallace RA. *Fundulus heteroclitus* Gonadotropin (S)1. Homologus bioassay using oocyte maturation and steroid production by isolated ovarian follicles. Gen. Comp. Endrocrinol,1987:67:126-141.
9. Lin YW, Schuetz AW. Intrafollicular action of estrogen in regulating pituitary-induced ovarian progesterone synthesis and oocyte maturation in Rana pipiens: temporal relationship and locus of action. Gen Comp Endocrinol,1985:58(3):421-35. doi: 10.1016/0016-6480(85)90115-7. PMID: 3874115.
10. Maller JL. Interaction of steroids with cyclic nucleotide system in amphibian oocytes. Adv. Cyclic Nucleotide Res,1983:15:295-336.
11. Masui Y, Clarke HJ. Oocyte maturation. International review of cytology,1979:57:185-282.
12. Nagahama Y, Adachi S. Identification of maturation-inducing steroid in a teleost, the amago salmon (*Oncorhynchus rhodurus*). Dev Biol,1985:109(2):428-35. doi: 10.1016/0012-1606(85)90469-5. PMID: 3996758.
13. Nishizuka Y. The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature,1984a:308(5961):693-8. doi: 10.1038/308693a0. PMID: 6232463.
14. Nishizuka Y. Turnover of inositol phospholipids and signal transduction. Science,1984b:21;225(4668):1365-70. doi: 10.1126/science.6147898. PMID: 6147898.
15. Petrino TR, Schuetz AW. Protein synthesis involvement in regulating pituitary-induced progesterone levels in ovarian follicles of Rana pipiens. J. Exp. Zool.,1986:239:411-421. https://doi.org/10.1002/jez.1402390312
16. Patiño R, Thomas P. Induction of maturation of Atlantic croaker oocytes by 17 alpha,20 beta,21-trihydroxy-4-pregnen-3-one in vitro: consideration of some biological and experimental variables. J Exp Zool,1990:255(1):97-109. doi: 10.1002/jez.1402550113. PMID: 2391470.

17. Schuetz AW. Local control mechanisms during oogenesis and folliculogenesis. Dev Biol,1985:1:3-83. doi: 10.1007/978-1-4615-6814-8_1. PMID: 3917203.
18. Stith BJ, Maller JL. Induction of meiotic maturation in Xenopus oocytes by 12-O-tetradecanoylphorbol 13-acetate. Exp Cell Res,1987:169(2):514-23. doi: 10.1016/0014-4827(87)90211-4. PMID: 3549336.
19. Wasserman WJ, Penna MJ, Houle JG. The regulation of *Xenopus laevis* oocyte maturation. In: Gametogenesis and the Early Embryo. 44thsymposium of the society for Developmental Biology. J G Gall, ed. Alan R. Liss, Inc New York, 1986, 111-130.