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The *in vitro* effect of theophylline on 17α, 20ß-dihydroxy-4-pregnen-3-one-induced germinal vesicle breakdown in the catfish, *Clarias batrachus*

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Abstract. The effects of theophylline (a phosphodiesterase inhibitor) and cAMP on 17α, 20ß-dihydroxy-4-pregnen-3-one-induced germinal vesicle breakdown was investigated *in vitro* in catfish (*Clarias batrachus*) oocytes. Folliculated oocytes incubated with 17α, 20ß-dihydroxy-4-pregnen-3-one at the concentration of 1 µg/ml induced 93·2 ± 2·23% germinal vesicle breakdown. When the oocytes were prestimulated with 17α,20ß-dihydroxy-4-pregnen-3-one for 6 h and then treated with different concentrations of theophylline, there was a significant drop in the frequency of germinal vesicle breakdown at the concentrations 2·0, 1·5 and 1·0 mM. However, theophylline was found to be incapable of inhibiting germinal vesicle breakdown at its lowest concentration (0·5 inM). In the time course study, significant inhibition of germinal vesicle breakdown was recorded when 1 mM theophylline was added up to 30 h of 17α,20ß-dihydroxy-4-pregnen-3-one Stimulation but the inhibitory effect of theophylline gradually (time dependent manner) declined if the stimulatory time of 17α,20ß-dihydroxy-4-pregnen-3-one was increased. A similar inhibition of germinal vesicle breakdown was also recorded with various concentrations of cAMP. Except 0·5 mM, all the higher concentrations of cAMP significantly inhibited 17α,20ß-dihydroxy-4-pregnen-3-one induced germinal vesicle breakdown.

Keywords. Oocyte maturation; catfish; phosphodiesterase inhibitor; 17α,20ß-dihydroxy-4-pregnen-3-one; cAMP.

1. Introduction

Resumption of meiosis (maturation) in fish oocytes is induced by maturation-inducing steroid (MIS) following stimulation by gonadotropic hormone. One particular steroid, 17α,20ß-dihydroxy-4-pregnen-3-one (17α,20ß-DP) has been implicated in the induction of oocyte maturation in a wide variety of teleosts (Goetz 1983; Nagahama 1987; Scott and Canario 1987; Jalabert *et al* 1991). Our recent studies have also suggested that this steroid is the most potent inducer of germinal vesicle breakdown (GVBD) among many steroids tested *in vitro* (Rao and Haider 1992) and is the naturally occurring physiologically active MIS in the catfish, *Clarias batrachus* (Haider and Rao 1992). Enhanced synthesis of this steroid and elevated plasma levels have also been demonstrated following human chorionic gonadotropin treatment during ovulation in this species (Zairin *et al* 1992). However, the mechanism of action of 17α,20ß-DP in the regulation of oocyte maturation in fish is still obscure.

A few *in vitro* studies have demonstrated that phosphodiesterase (PDE) inhibitors such as theophylline, 3-isobutyl-l-methyl-xanthine (IBMX), SQ 20,006 or cAMP inhibit 17α,20ß-DP-induced meiotic maturation of fish oocytes (DeManno 1983; Goetz and Hennessy 1984; Jalabert and Finet 1986; DeManno and Goetz 1987a,b).

A decrease in oocyte cAMP has also been observed following 17α,20ß-DP stimulation (Jalabert and Finet 1986; Finet *et al* 1988). Thus, it appears that in fish oocyte MIS may stimulate meiotic maturation by lowering cAMP and this decrease is most likely a result of inhibition of adenylate cyclase activity (Jalabert *et al* 1991). To our knowledge, no reports have been published concerning the effect of PDE inhibitors or cAMP on GVBD in any Indian fish species. In view of the interesting mode of action that 17α,20ß-DP have in the induction of oocyte maturation in rainbow trout, *Salmo gairdneri* (Jalabert and Finet 1986; Finet *et al* 1988), yellow perch, *Perca flavescens* (DeManno and Goetz 1987a) and brook trout, *Salvelinus fontinalis* (DeManno and Goetz 1987b), it seemed necessary for us to find out whether a similar mechanism exists in Indian catfish also. The present paper demonstrates that the PDE inhibitor, theophylline inhibits or delays 17α,20ß-DP-induced GVBD in *C. batrachus* which tends to suggest the involvement of PDE in cAMP associated resumption of meiosis in the primary oocyte. Observations were also made to find out the effect of exogenous cAMP on 17α,2Oß-DP-induced GVBD.

2. Materials and methods

2.1 *Fish and incubation medium*

Gravid female *C. batrachus* were obtained from local fish market during June and July and were maintained in aquaria for 7 days. During this period germinal vesicle (GV) was centrally located in a full-grown oocyte when observed under a dissecting microscope. The incubation medium (Upadhyaya and Haider 1986) was freshly prepared, autoclaved and stored in a refrigerator below 10°C.

2.2 *In vitro incubations*

Fish were sacrificed by decapitation and their ovaries were transferred to a Petri dish containing cool-incubating medium and then the oocytes were separated from each other without causing any mechanical injury to them. Thirty to thirtyfive oocytes were transferred to each cavity block containing 3 ml of medium. Three different experiments were conducted in an air conditioned laboratory where the temperature was maintained at 23±2°C.

In the first set of experiment, oocytes were stimulated with 1 μg/ml 17 α,20ß-DP (Sigma) for 6 h (this was used for 6 h treatment because result of preliminary experiments indicated that this dose and time was sufficient to induce GVBD) and then treated with different concentrations (0·5, 1·0, 1·5 and 2·0 mM) of theophylline (Sigma). In the second set of experiment, oocytes were incubated in the presence of 1 μg/ml 17α,20ß-DP and different concentrations (0·5, 1·0, 2·0, 4·0 and 8·0 mM) of cAMP (Sigma). Control incubations with or without 17α,20ß-DP or with different concentrations of theophylline and cAMP were also maintained. All the incubations were maintained up to 36 h after which the oocytes were cleared in a clearing solution (Trant and Thomas 1988) and examined under a microscope for the incidence of the GVBD.

To find out the effect of time of theophylline addition on GVBD in 17 α,20ß-DP-stimulated oocytes, oocytes of third set of experiment were prestimulated

with 1 µg/ml 17 α,20ß-DP for various times (0–6, 0–12, 0–18, 0–24, 0–30 and 0–36 h). After each prestimulatory period, oocytes were removed, washed and treated with 1 mM of theophylline for another 6 h after which they were kept in medium for remaining period (up to 36 h) except for the last incubation which was maintained up to 42 h. Control incubations with (stimulated) or without (unstimulated) 17α,20ß-DP were also maintained simultaneously. In addition, oocytes were treated with 1 mM theophylline only for a given time (during 0–6, 6–12, 12–18, 18–24, 24–30 and 30–36 h) and were removed, washed and kept in medium. The incubations were then continued and all oocytes were assayed for GVBD at 36 h.

2.3 *GVBD data analysis*

Rate of GVBD was expressed as the mean percentage value of three replicate incubations. Data on GVBD percentage for all replicates of the experiment were transformed using arcsine-square root transformation. Arcsine-transformed data were analysed by one-way analysis of variance (ANOVA) or by Student's *t* test.

3. Results

Table 1 shows the effect of theophylline on 17α ,20ß-DP-induced GVBD in catfish. When the oocytes were cultured in the medium alone, only 4·1 ±0·08% of oocytes underwent GVBD. A comparable rate of GVBD was also observed when the oocytes were treated with different concentrations (0·5, 1·0, 1·5 and 2· 0 mM) of theophylline alone. A marked induction of GVBD (93·2 ± 2·23%) was recorded when the oocytes were incubated in the presence of 1 µg/ml 17α,20ß-DP. Oocytes when prestimulated with 1 µg/ml 17α,20ß-DP for 6h and then treated with different concentrations of theophylline, a significant inhibition of GVBD was observed at l·0 mM or above this concentration (2·0 and 1·5 mM). However, theophylline at its lowest concentration (0·5 mM) did not inhibit 17α,20ß-DP-induced GVBD (89·4 ± 2·17%). Having confirmed the inhibitory effect of theophylline, oocytes were prestimulated with 1 µg/ml 17α,20ß-DP for various times and then treated with 1 mM theophylline to observe the effect of time of theophylline addition on 17α,20ß-DP-induced oocyte maturation. The results are given in table 2. Briefly, theophylline (1 mM) significantly inhibited 17α,20ß-DP-induced GVBD when added up to 30 h of stimulation. The

Table 1. Effect of theophylline treatment on l7α,20ß-DP-stimulated GVBD in catfish oocytes *in vitro*.

	Percentage of GVBD[a] Concentrations of theophylline (mM)				
Treatments	0	0·5	1·0	1·5	2·0
Theophylline	4·1 ± 0·88	3·6 ± 0·54	3·4 ± 0·42	3·5 ± 0·45	3·8 ± 0·54
17α,20β-DP (1 µg/ml) + theophylline	93·2 ± 2·23	89·4 ± 2·17	4·9 ± 0·96*	4·8 ± 1·10*	3·3 ± 0·13*

[a]Values expressed as % are mean ± SEM of three replicate incubations.
*$P < 0·001$.

inhibitory effect of theophylline gradually (time dependent manner) declined if the stimulatory time of 17α,20ß-DP was increased (one-way ANOVA, $F = 467{\cdot}65$, $P < 0{\cdot}001$). A similar inhibition of GVBD was also recorded with different concentrations of cAMP; except 0·5 mM, all the higher concentrations (1·0, 2·0, 4·0 and 8·0 mM) significantly inhibited 17α,20ß-DP-induced GVBD when compared to 17α,20ß-DP alone (94·3 ± 0·6%). The oocytes treated with different concentrations of cAMP or cultured in medium alone did not mature (table 3).

4. Discussion

The induction of oocyte maturation is followed by a transient decrease in oocyte cAMP. Initial reduction in oocyte cAMP in response to MIS may also be due to an increase in PDE activity. On the other hand, PDE inhibitors such as theophylline, IBMX or SQ 20,006 inhibit MIS-induced oocyte maturation by promoting accumulation of cAMP in oocytes (Jalabert *et al* 1991). Theophylline at 1 mM or above this concentration completely blocked 17 α,20ß-DP-induced GVBD in rainbow

Table 2. Effect of time of theophylline addition on 17 α,20ß-DP-induced GVBD in catfish oocytes *in vitro*.

	Percentage of GVBD[a] Time (h)					
Treatments	6	12	18	24	30	36
Unstimulated control (plain medium)	2·7 ± 1·36	4·0 ± 0·34	3·1 ± 1·57	3·7 ± 0·80	3·7 ± 0·65	3·9 ± 0·70
Stimulated control (1 µg/ml 17α,20β-DP)	83·2 ± 1·50	86·7 ± 2·23	89·8 ± 2·04	92·7 ± 0·63	92·2 ± 0·77	93·4 ± 0·99
Drug control (1 mM theophylline)	3·9 ± 0·29	4·3 ± 2·20	5·3 ± 0·70	3·6 ± 1·81	4·8 ± 0·45	4·9 ± 2·46
1 µg/ml 17α,20β-DP + 1 mM theophylline	3·6 ± 0·15*	4·1 ± 0·42*	21·1 ± 1·51*	33·4 ± 1·40*	82·5 ± 0·55*	89·6 ± 1·90

[a]Values expressed as % are mean ± SEM of three replicate incubations.
*$P < 0{\cdot}001$ (compared with respective stimulated control).

Table 3. Effect of cAMP on 17 α,20ß-DP-stimulated GVBD in catfish oocytes *in vitro*.

	Percentage of GVBD[a] Concentrations of cAMP (mM)					
Treatments	0	0·5	1·0	2·0	4·0	8·0
cAMP	3·0 ± 0·57	4·0 ± 0·03	4·1 ± 0·07	4·2 ± 0·10	4·2 ± 0·03	3·6 ± 0·56
1 µg/ml 17α,20β-DP + cAMP	94·3 ± 0·67	93·9 ± 0·96	83·2 ± 1·30*	62·5 ± 1·50**	41·7 ± 0·86**	8·4 ± 0·36**

[a]Values expressed as % are mean ± SEM of three replicate incubations.
*$P < 0{\cdot}01$: **$P < 0{\cdot}001$.

trout but lower concentrations were noninhibitory (Jalabert and Finet 1986). Similar inhibition has been observed for 17 α,20ß-DP-stimulated GVBD in brook trout and yellow perch when IBMX and/or SQ 20,006 was added to the incubations (Goetz and Hennessy 1984; DeManno and Goetz 1987a,b). In the present study, we also observed that theophylline at 1 mM or higher doses to this completely blocked 17α,20ß-DP-induced GVBD while the lower concentration (0.5 mM) was noninhibitory. This indicates that the action of MIS on the oocyte was countered by the presumed effect of theophylline.

Earlier report related to the time course for IBMX inhibition of 17 α,20ß-DP-induced GVBD in brook trout have shown that 1 mM IBMX significantly blocked oocyte maturation up to the end of incubation period i.e., 24 h (Goetz and Hennessy 1984). Later, using oocytes from the same fish, DeManno and Goetz (1987b) have observed that IBMX (1 mM) significantly inhibited GVBD when added up to 18 h after 17 α,20ß-DP-stimulation but at 30 h or more there was no inhibition of GVBD. In the present study, theophylline (1 mM) significantly inhibited GVBD when added up to 30 h after 17 α,20ß-DP-stimulation, however, the inhibitory effect of theophylline gradually declined if the stimulatory time was increased. Further, to observe a direct effect of cAMP on 17α,20ß-DP-induced GVBD, oocytes were incubated in the presence of l7 α,20ß-DP and at different concentrations of cAMP. Results obtained from this study indicated that 1 mM or above this concentration significantly inhibited 17 α,20ß-DP-induced GVBD. Similar inhibitory effect of cAMP on 17α,20ß-DP-induced GVBD was also reported in *S. gairdneri* (Jalabert and Finet 1986) and in *P. flavescens* oocytes (DeManno and Goetz 1987a).

The overall results of this study indicate that theophylline inhibits or delays 17α,20ß-DP-induced oocyte maturation in *C. batrachus* presumably via an increase in oocyte cAMP, and that the action of 17α,20ß-DP in the induction of oocyte maturation is accompanied by some transitory decrease in cAMP level as has been observed by Jalabert and Finet (1986) and Finet *et al* (1988) in rainbow trout. In an another study, we have also measured cAMP levels in 17α,20ß-DP-induced oocytes and observed a significant reduction in its level (Haider and Chaube 1995).

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