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2-hydroxyestradiol-17β-induced oocyte maturation: involvement of cAMP–protein kinase A and okadaic acid-sensitive protein phosphatases, and their interplay in oocyte maturation in the catfish *Heteropneustes fossilis*

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Summary

In *Heteropneustes fossilis*, *in vitro* incubation of postvitellogenic follicles with 2-hydroxyestradiol-17β (2-OHE$_2$, 5 μmol l^{-1}) decreased significantly the total cAMP level, concomitant with germinal vesicle breakdown (GVBD). The incubation of the follicles with cAMP or cAMP-elevating drugs [phosphodiesterase (PDE) inhibitors], such as IBMX (3-isobutyl-1-methyl-xanthine), theophylline and caffeine, inhibited the 2-OHE$_2$-induced GVBD in a concentration-dependent manner. The magnitude of the response varied: both cAMP and IBMX were effective at all concentrations (0.1–2.0 mmol l^{-1}), followed by theophylline (0.5–2.0 mmol l^{-1}) and caffeine (1–2.0 mmol l^{-1}). The protein kinase A (PKA) inhibitor H89 stimulated oocyte maturation in a concentration-dependent manner. However, when co-incubated with 2-OHE$_2$ for 24 h it produced a biphasic effect: low concentrations (0.1 and 1.0 μmol l^{-1}) did not alter the 2-OHE$_2$-induced GVBD, but high concentrations (5 and 10 μmol l^{-1}) inhibited it. The incubation of the follicles with H89 lowered the inhibitory effect of IBMX on the 2-OHE$_2$-induced GVBD. The incubation of the follicles with okadaic acid (OA), a protein phosphatase 1 and 2A inhibitor did not affect GVBD but when co-incubated with 2-OHE$_2$, it enhanced the GVBD response. OA reversed the inhibitory effect of IBMX. The results suggest that OA may overcome the inhibition of 2-OHE$_2$-induced GVBD by IBMX at a step distal to the cAMP–PKA pathway.

Key words: catfish, 2-hydroxyestradiol, GVBD, cAMP, protein kinase A, protein phosphatases, okadaic acid.

Introduction

In vertebrates, resumption of meiosis after prophase I arrest in oocytes is triggered by a pituitary luteinizing hormone (LH) surge, which stimulates the synthesis of a maturation-inducing substance (MIS) in the ovary (Jalabert et al., 1991; Kagawa, 1994; Nagahama, 1997; Yamashita, 1998; Thomas, 1994; Thomas, 1999; Yoshida et al., 2000; Patino et al., 2001). In teleosts, chemically different molecules have been demonstrated to elicit MIS activity and they include 17,20β-dihydroxy-4-pregnen-3-one (17,20β-DP), 17,20β,21-trihydroxy-4-pregnen-3-one (17β-S), corticosteroids, insulin, insulin-like growth factors and activin (Scott and Canario, 1987; Nagahama, 1997; Tyler et al., 1999). Estrogens are poor inducers of oocyte maturation but a recent report (Tokumoto et al., 2004) shows that the synthetic oestrogen diethylstilbestrol induces oocyte maturation in a manner similar to17,20β-DP. Recently, we have shown that the hydroxy- and methoxy-metabolites of estrogens along with their synthesizing enzymes (estrogen-2-hydroxylase and catechol-O-methyltransferase, respectively) occur in catfish ovary and that the hydroxyestrogens elicit varying degrees of maturational activity (Senthilkumaran and Joy, 2001; Mishra and Joy, 2006a; Mishra and Joy, 2006b). The MIS-like activity of 2-hydroxyestradiol-17β (2-OHE$_2$) has been attributed to an indirect action through follicular steroidogenesis notably by the production of 17,20β-DP (Mishra and Joy, 2006c). The MIS, in turn, initiates a series of phosphorylations and dephosphorylations catalyzed by protein kinases such as cAMP–protein kinase A (PKA), protein kinase C (PKC), mitogen-activated protein kinase (MAPK), cAMP-independent phosphatidylinositide 3-kinase (PI 3-kinase)-serine/threonine kinase Akt, and protein phosphatases (PP), respectively, leading to the activation of a cytoplasmic maturation-promoting factor (MPF), which induces germinal vesicle breakdown (GVBD) and progression of meiosis up to the second metaphase arrest (Nagahama et al., 1994; Balamurugan and Haider, 1998; Yamashita, 1998; Pace and Thomas, 2005).

Investigations in starfish, fish, amphibians and mammals support the contention that the cAMP-PKA pathway is widely held responsible for the maintenance of the first meiotic arrest and its resumption is attributed to a decrease in intra-oocyte

cAMP level under the influence of a maturation inducing substance or hormone (MIS or MIH) (Meijer et al., 1989; Jalabert et al., 1991; Chaube and Haider, 1997). The MIS decreases cAMP by inhibiting adenylyl cyclase (Yoshikuni and Nagahama, 1994; Pace and Thomas, 2005) or increasing phosphodiesterase (PDE) (Chaube and Haider, 1997) activity. In teleosts, cAMP, adenylyl cyclase stimulators or PDE inhibitors have been reported to inhibit the MIS-mediated oocyte maturation (Jalabert et al., 1991; Haider and Chaube, 1995; Chaube and Haider, 1997; Haider and Baqri, 2000). It has been widely held that a threshold level of cAMP-PKA activity maintains putative initiator protein(s) in a phosphorylated (inactive) state inhibiting oocyte maturation and the activation of the initiator protein (active) dephosphorylation by some unknown mechanisms would induce maturation-promoting factor (MPF) activity. Recently, the importance of cAMP-independent signaling pathways in the regulation of oocyte maturation has been demonstrated (Schmitt and Nebreda, 2002; Pace and Thomas, 2005).

An involvement of okadaic acid (OA)-sensitive PP has been demonstrated during oocyte maturation in starfish, *Xenopus* and mammals (Huchon et al., 1981; Bornslaeger et al., 1986; Goris et al., 1989; Pondaven et al., 1989; Rime and Ozon, 1990; Schwartz and Schultz, 1991; Lu et al., 2001; Wang et al., 2004). Evidence for a crosstalk between cAMP-PKA and OA-sensitive PP was presented as well (Schwartz and Schultz, 1991; Lu et al., 2001). To our knowledge, studies on the role of PP in oocyte maturation in fish are not available.

Heteropneustes fossilis (Bloch) (Order Cypriniformes, Suborder Siluroidei, Family Saccobranchidae) is a freshwater, air-breathing species found in ponds, pools, swamps and rivers of India, Sri Lanka, Burma, Laos, Thailand and Vietnam (see Dutta-Munshi and Hughes, 1992). The catfish is interesting in that it possesses a pair of long tubular accessory respiratory organ, which extends through the dorsal muscle of the body on either side of the vertebral column. The air-breathing adaptation enables it to survive in waters of low oxygen content and also out of water on wet grounds. The meat is rich in protein and iron but poor in fat content and, therefore, esteemed for its invigorating qualities. This species is cultured intensively and is also ideal for wastewater aquaculture. The catfish has been used as a model for studying various aspects of reproductive physiology (see Sundararaj, 1981) and has been one of the species studied in detail for understanding the hormonal mechanisms of oocyte maturation.

The objectives of the present study were to demonstrate (1) the involvement of both cAMP-PKA and protein phosphatases in 2-OHE$_2$-induced oocyte maturation and (2) to demonstrate the possible interactions between these two systems in the catfish.

Materials and methods

Chemicals

2-hydroxyestradiol-17β (2-OHE$_2$), adenosine-3′,5′-cyclic monophosphate (cAMP), 3-isobutyl-1-methyl-xanthine (IBMX), theophylline, caffeine, *N*-(2-[p-bromocinnamylamino]ethyl)-5-isoquinoline sulfonamide hydrochloride (H89) and okadaic acid (OA) were purchased from Sigma Chemical Company (St Louis, MO, USA). A cyclic AMP EIA kit was purchased from Cayman Chemical Co., Ann Arbor, MI, USA. All other chemicals were of analytical grade and purchased locally.

Animal collection and maintenance

The experiments were performed in accordance with local/national guidelines for experimentation in animals and all care was taken to prevent cruelty of any kind.

Gravid female *Heteropneustes fossilis* Bloch (30–40 g) were obtained from a local fish market in prespawning phase (June) of the reproductive cycle. They were maintained in the laboratory under normal photoperiod (13 h:11 h L:D) and temperature (25±2°C) until used for experiments. The fish were fed goat liver daily *ad libitum*. A few fish were randomly checked for spontaneous ovulation and sacrificed to determine the maturation of ovary. The fish containing ovaries filled with dark green, postvitellogenic follicles were used in the study.

Preparation of incubation medium and test compounds

The incubation medium was prepared as follows (quantities given are in g): NaCl 3.74, KCl 0.32, $CaCl_2$ 0.16, $NaH_2PO_4.2H_2O$ 0.10, $MgSO_4.7H_2O$ 0.16, glucose 0.40 and Phenol Red 0.008 were dissolved in 1 l of triple distilled water. The pH was adjusted to 7.5 with 1 mol l^{-1} sodium bicarbonate and autoclaved. Penicillin (2×10^5 i.u.) and streptomycin sulphate (200 mg) were added and filtered. The medium was stored at 4°C and prepared fresh every week.

Stock solutions of cAMP, IBMX and caffeine were prepared by dissolving known amounts in the incubation medium. Theophylline, OA and 2-OHE$_2$ were dissolved in the medium containing 50 μl ethanol. H89 was dissolved in acidic incubation medium (10 μl, 5 mmol l^{-1} HCl). The stock solutions were prepared on the day of the experiment and kept at 0°C. Just before the incubation, they were diluted with the incubation medium to give different working concentrations.

Collection, selection and incubation of ovarian follicles

All instruments and glassware were sterilized. The acclimatized, gravid female *H. fossilis* were sacrificed by decapitation and ovaries were transferred to a Petri dish containing fresh cooled incubation medium. Rounded dark green postvitellogenic follicles were separated from each other with the help of a fine brush and watchmaker's forceps. The follicles were incubated in embryo cups containing 3 ml of the incubation medium or the medium containing various test compounds, at 25±2°C. As controls, follicles were incubated in plain medium (control) or the medium containing vehicle (vehicle control). At the end of the incubations, the follicles were cleared in ethanol:acetic acid:formalin to determine germinal vesicle (GV) breakdown (GVBD) as an index of oocyte maturation. Translucent follicles without GVs and opaque follicles containing GVs were counted separately. The

percentage of GVBD was determined from the total number of the follicles incubated.

Effects of 2-OHE$_2$ on oocyte maturation

About 30–40 follicles in triplicate from each fish (*N*=3) were incubated in the medium containing 5 μmol l^{-1} 2-OHE$_2$ (Mishra and Joy, 2006b) for 0, 3, 6, 12, 24 and 30 h. As controls, the follicles were incubated in the incubation medium alone or in the medium containing the same volume of vehicle. At the end of each time interval, the follicles were transferred to fresh medium to complete a total of 30 h incubation (except the 30 h group). The follicles were cleared and scored for GVBD.

Effect of 2-OHE$_2$ incubation on total follicular cAMP level

About 70–80 follicles in duplicate from 5 fish each were incubated for different time intervals (0, 3, 6, 12, 24 and 30 h) in the medium containing 5 μmol l^{-1} 2-OHE$_2$. The incubations continued up to 30 h in plain medium for all groups except the 30 h group. For the controls, the follicles were incubated in the vehicle medium for similar durations. The follicles were harvested in groups; they were homogenized in 200 μl trichloroacetic acid (TCA, 5%), centrifuged at 1500 ***g*** at 4°C for 10 min and the supernatant was collected. TCA was extracted from the supernatant with water-saturated ether (5 volumes, two times), the ether layer was discarded and the supernatant was collected. The residual ether was removed with a N_2 stream. Extracted samples were kept at –20°C for cAMP estimation.

Cyclic AMP concentration was measured using an EIA kit, following the method of Pradelles et al. (Pradelles et al., 1989). On the day of the analysis, the samples were diluted with assay buffer (provided with the kit). Samples (50 μl) were reacted with 50 μl each of cAMP–acetycholine esterase (AchE) tracer and cAMP antibody in mouse monoclonal anti-rabbit IgG precoated wells, followed by 18 h incubation at 4°C. After the incubations, the solutions were decanted and the wells rinsed five times with the wash buffer. 200 μl Ellman's Reagent (provided with the kit) was added to the wells and incubated in dark for 90–120 min for optimum color development (0.3–0.8 a.u., after blank subtraction). The developed plate was read at 405 nm in a Multiskan EX (Thermo Labsystems, Milford, CA, USA) ELISA reader. The concentration of total cAMP was calculated as pmol mg^{-1} oocyte. The minimum detection limit with the kit was 4 pmol ml^{-1}. The intra- and inter-assay coefficients of variation were 4.5% and 7.4%, respectively.

Effects of cAMP and cAMP-elevating drugs on 2-OHE$_2$-induced-oocyte maturation

Concentration-response study

About 30–40 follicles in triplicate (*N*=3 fish) were incubated with different concentrations of cAMP, IBMX, theophylline or caffeine (0.1, 0.5, 1 and 2 mmol l^{-1}) alone or in combination with 5 μmol l^{-1} 2-OHE$_2$ for 24 h. Control groups were maintained concurrently. After the completion of the incubations, the follicles were cleared and counted for the percentage of GVBD.

Pre-incubation study

About 30–40 follicles in triplicate (*N*=3 fish) were incubated with 1 mmol l^{-1} each of cAMP, IBMX, theophylline and caffeine for 2, 4 or 6 h. After the respective intervals, the follicles were rinsed in fresh incubation medium and transferred to the medium containing 5 μmol l^{-1} 2-OHE$_2$ for 6 h (this duration elicited ~50% GVBD). Subsequently, the follicles were briefly rinsed in fresh incubation medium and maintained in the plain medium for up to 30 h (total incubation time). Control incubations were also conducted side by side. At the end of the incubations, the follicles were cleared and scored for the percentage of GVBD.

Post-incubation study

About 30–40 follicles in triplicate (*N*=3 fish) were incubated with 5 μmol l^{-1} 2-OHE$_2$ for 6 h. The follicles were then rinsed with fresh medium and transferred into the medium containing 1 mmol l^{-1} each of cAMP, IBMX, theophylline or caffeine for different time intervals (2, 4 or 6 h). After the respective intervals, the follicles were placed in plain incubation medium for the completion of 30 h incubation period. Control incubations were set up simultaneously. At the end of the incubations, the follicles were cleared and scored for GVBD.

Effect of H89 on 2-OHE$_2$-induced oocyte maturation

Concentration-response study

About 30–40 follicles in triplicate (*N*=3 fish) were incubated with different concentrations of H89 (0.1, 1, 5 and 10 μmol l^{-1}) alone or in combination with 5 μmol l^{-1} 2-OHE$_2$ for 24 h. Control groups were maintained concurrently. After the incubations, the follicles were cleared and scored for GVBD.

Effect of H89 on IBMX inhibition of 2-OHE$_2$-induced oocyte maturation

About 30–40 follicles in triplicate (*N*=3 fish) were incubated with 5 μM 2-OHE$_2$ and 1 mmol l^{-1} IBMX. After 1 h, the medium was replaced by the medium containing 2-OHE$_2$, IBMX and 10 μmol l^{-1} H89 for 7 h. Then, the follicles were briefly rinsed and maintained in plain incubation medium to complete the 30 h incubation. Control series consisted of incubation of the follicles with 2-OHE$_2$ (5 μmol l^{-1}, 8 h), IBMX (1 mmol l^{-1}, 8 h), H89 (10 μmol l^{-1}, 7 h), 2-OHE$_2$ and IBMX (8 h), 2-OHE$_2$ (8 h) and H89 (7 h) and plain incubation medium with or without the vehicle. At the end of the incubations, the follicles were cleared and scored for the percentage of GVBD.

Effect of okadaic acid on 2-OHE$_2$-induced oocyte maturation

Concentration-response study

About 30–40 follicles in triplicate (*N*=3 fish) were incubated with different concentrations of OA (0.5, 1 and 2 μmol l^{-1}) alone or in combination with 5 μmol l^{-1} 2-OHE$_2$ for 24 h.

Control groups were maintained concurrently. After the incubations, the follicles were cleared and scored for GVBD.

Effect of okadaic acid (OA) on IBMX inhibition of 2-OHE$_2$-induced oocyte maturation

About 30–40 follicles in triplicate (*N*=3 fish) were co-incubated with 5 μmol l^{-1} 2-OHE$_2$ and 1 mmol l^{-1} IBMX. After 1 h, the medium was replaced by fresh medium containing 2-OHE$_2$, IBMX and 1 μmol l^{-1} OA for 7 h. Then, the follicles were transferred to plain incubation medium to complete the 30 h incubation. Control series consisted of incubation of the follicles with 2-OHE$_2$ (5 μmol l^{-1}, 8 h), IBMX (1 mmol l^{-1}, 8 h), OA (1 μmol l^{-1}, 7 h), 2-OHE$_2$ and IBMX (8 h), 2-OHE$_2$ (8 h) and OA (7 h) and plain incubation medium with or without the vehicle. At the end of the incubations, the follicles were cleared and scored for the percentage of GVBD.

Statistical analysis

Data were expressed as means ± s.e.m. and were analyzed by one-way analysis of variance (ANOVA), followed by Newman–Keuls' test ($P<0.05$) for multiple group comparisons.

Results

Incubations of follicles in plain medium or with vehicle gave very low GVBD response and the values did not vary significantly between the groups.

Effect of 2-OHE$_2$ on GVBD and total follicular cAMP level

A significant GVBD was registered in the 2-OHE$_2$ groups, which increased from 3 h onwards (Fig. 1; F=680.10; $P<0.001$, one-way ANOVA). The response was significantly high at all intervals as compared to the control group ($P<0.05$, Newman–Keuls' test).

The incubation of the follicles with 2-OHE$_2$ decreased cAMP significantly in a duration-dependent manner (Fig. 1; F=137.32; $P<0.001$, one-way ANOVA). The decrease was significant at all intervals compared with the control groups ($P<0.05$, Newman–Keuls' test). The control values (plain medium and vehicle groups) were not significantly different.

Effect of cAMP and cAMP-elevating drugs on 2-OHE$_2$-induced oocyte maturation

Cyclic AMP, IBMX, theophylline and caffeine all produced a significant suppression of 2-OHE$_2$-induced GVBD (Fig. 2; cAMP, F=294.91; IBMX, F=364.14; theophylline, F=214.89; caffeine, F=267.67; $P<0.001$ one-way ANOVA) whereas when applied without 2-OHE$_2$ they did not produce any significant effect compared to the vehicle control. The inhibition due to cAMP and IBMX was concentration-dependent ($P<0.05$, Newman–Keuls' test). Only higher concentrations of theophylline (0.5, 1.0 or 2.0 mmol l^{-1}) and caffeine (1.0 or 2.0 mmol l^{-1}) inhibited GVBD significantly ($P<0.05$, Newman–Keuls' test). The magnitude of the inhibition was in the order, cAMP>IBMX>theophylline> caffeine.

The pre-incubation of the follicles with cAMP (1 mmol l^{-1}) for 2, 4 and 6 h and IBMX and theophylline for 4 and 6 h decreased the 2-OHE$_2$-induced GVBD significantly (data not shown). However, the post-treatment with cAMP for 4 and 6 h and IBMX and theophylline for 6 h only, inhibited the 2-OHE$_2$-induced GVBD response significantly (data not shown). Caffeine did not produce any significant effect either when used pre-incubation or post-incubation (data not shown).

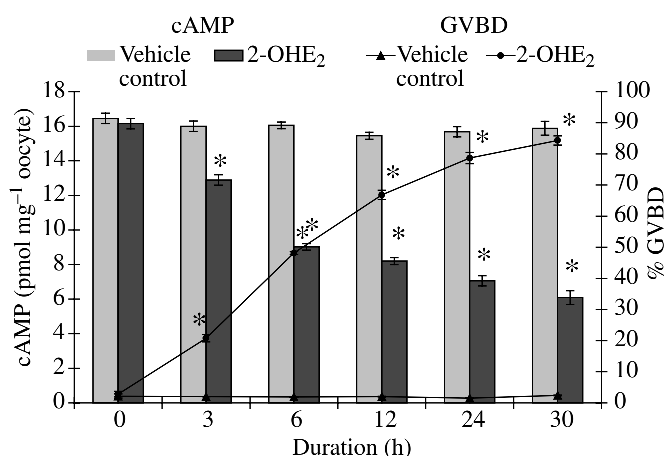

Fig. 1. Effects of 2-hydroxyestradiol-17β (2-OHE$_2$, 5 μmol l^{-1}) on germinal vesicle breakdown (GVBD, line curves) and cAMP level (bars) in *Heteropneustes fossilis*. Values are means ± s.e.m. (*N*=3 for GVBD assay; *N*=5 for cAMP assay). Data were analyzed by one-way ANOVA ($P<0.001$) and Newman–Keuls' test ($P<0.05$). Asterisks indicate significant difference from the respective vehicle control groups ($P<0.05$).

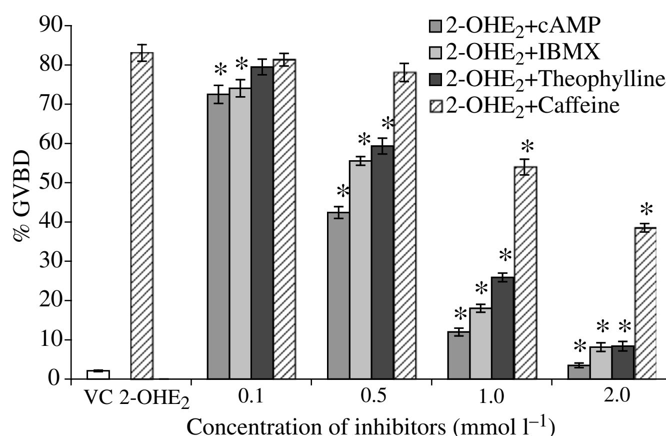

Fig. 2. *In vitro* effects of incubation of postvitellogenic follicles with 2-hydroxyestradiol-17β (2-OHE$_2$, 5 μmol l^{-1}) and different concentrations of cAMP, IBMX, theophylline or caffeine on the percentage of germinal vesicle breakdown (GVBD) in *Heteropneustes fossilis*. Values are means ± s.e.m. (*N*=3). Data were analyzed by one-way ANOVA ($P<0.001$) and Newman–Keuls' test ($P<0.05$). All the groups are significantly different from the vehicle control (VC) group. *Significant difference from the 2-OHE$_2$ group ($P<0.05$).

Effect of H89 on 2-OHE$_2$-induced GVBD

Concentration effect

The incubation of the follicles with H89 alone produced an overall significant increase in the percentage of GVBD (Fig. 3A; F=237.21; P<0.001, one-way ANOVA) as compared to the control groups. The percentage of GVBD increased significantly in a concentration-dependent manner (P<0.05, Newman–Keuls' test). The co-incubation of the follicles with H89 and 2-OHE$_2$ produced varied effects (Fig. 3A; F=775.52; P<0.001, one-way ANOVA). The low concentrations of the drug (0.1 and 1 μmol l^{-1}) did not affect the GVBD in response to 2-OHE$_2$ but higher concentrations (5 and 10 μmol l^{-1}) inhibited the response of significantly (P<0.05, Newman–Keuls' test).

Effect of H89 on the IBMX inhibition of GVBD response induced by 2-OHE$_2$

Priming of the follicles with 2-OHE$_2$ and IBMX for 1 h and continuation of the incubation in the presence of H89 for 7 h lowered the inhibitory effect of IBMX (1.9% *vs* 14.5%) significantly (Fig. 3B; F=231.90; P<0.001, one-way ANOVA; P<0.05, Newman–Keuls' test). In control studies, the incubation of the follicles with 2-OHE$_2$ (8 h), H89 (7 h) or 2-OHE$_2$ + H89 stimulated GVBD response significantly; the response in the 2-OHE$_2$ and 2-OHE$_2$ + H89 groups did not vary significantly. The incubation of the follicles with IBMX inhibited the stimulatory effect of 2-OHE$_2$ on GVBD, as described earlier.

Effect of okadaic acid on 2-OHE$_2$-induced oocyte maturation

Concentration-response study

The incubation of the follicles with OA alone did not alter the GVBD response but when co-incubated with 2-OHE$_2$, GVBD was significantly higher than with 2-OHE$_2$ alone (Fig. 4A; F=824.13; P<0.001, one-way ANOVA; P<0.05, Newman–Keuls' test).

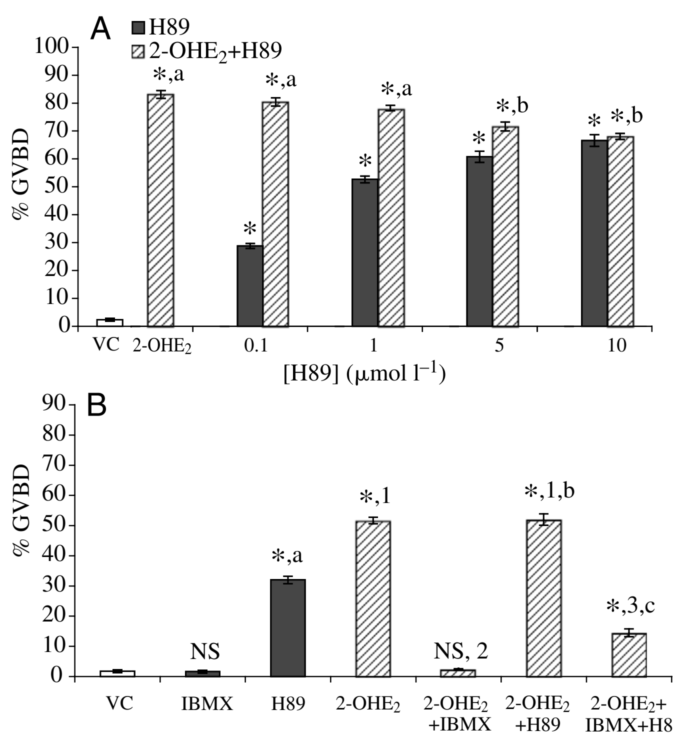

Fig. 3. (A,B) *In vitro* effects of different concentrations of H89 on the percentage germinal vesicle breakdown (GVBD) in *Heteropneustes fossilis.* (A) H89 alone or co-incubated with 2-hydroxyestradiol-17β (2-OHE$_2$, 5 μmol l^{-1}) for 24 h. Values are mean ± s.e.m. (N=3). (B) The effect of H89 on IBMX inhibition of 2-OHE$_2$-induced GVBD. Data were analyzed by one-way ANOVA (P<0.001) and Newman–Keuls' test (P<0.05). Asterisks indicate significant difference from the vehicle control (VC) group. Letters indicate comparisons with the 2-OHE$_2$ (A) or H89 (B) group. Numbers show comparisons with 2-OHE$_2$ (B) group. Groups marked with the same letter or number are not significantly different and that with different letters or numbers are significantly different. NS, not significant from the vehicle control (VC) group.

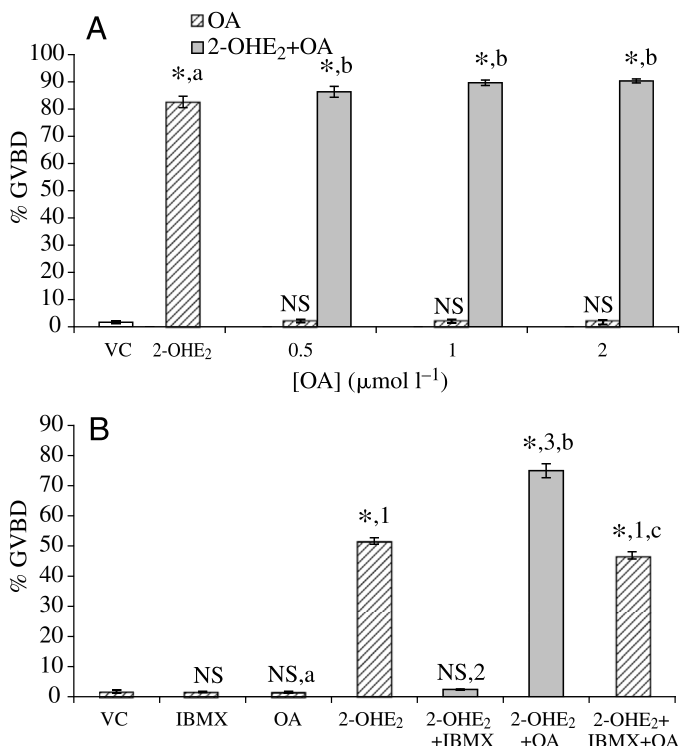

Fig. 4. (A,B) *In vitro* effects of different concentrations of okadaic acid (OA) on the percentage of 2-OHE$_2$-induced germinal vesicle breakdown (GVBD) in *Heteropneustes fossilis.* (A) Effect of OA alone or co-incubated with 2-hydroxyestradiol-17β (2-OHE$_2$, 5 μmol l^{-1}) for 24 h. (B) The effects of OA on IBMX inhibition of 2-OHE$_2$-induced GVBD. Values are mean ± s.e.m. Data were analyzed by one-way ANOVA (P<0.001) and Newman–Keuls' test (P<0.05). Asterisks indicate significant difference from the vehicle control (VC) group. Letters indicate comparisons with the 2-OHE$_2$ (A) or OA (B) group. Numbers show comparisons with 2-OHE$_2$ (B) group. Groups marked with the same letter or number are not significantly different and those with different letters or numbers are significantly different. NS, not significant from the vehicle control (VC) group.

Effect of OA on the IBMX inhibition of 2-OHE$_2$-induced GVBD

The incubation of the follicles with 2-OHE$_2$, IBMX and OA for 7 h reversed the inhibitory effect of IBMX on the 2-OHE$_2$-induced GVBD response, elevating it to that of the 2-OHE$_2$ group (Fig. 4B; F=454.54; P<0.001, one-way ANOVA; P<0.05, Newman–Keuls' test).

Discussion

The data of the present study demonstrate the involvement of both cAMP–PKA and OA-sensitive protein phosphatases (PP) in 2-OHE$_2$-induced oocyte maturation in the catfish.

Total oocyte cAMP levels decreased significantly and persistently concomitant with the increase in GVBD. The time-course study showed that the effect was evident at 3 h (the first sampling interval) and was maximal at 30 h (the end sampling time). The inhibition varied from 20.18% at 3 h to 62.29% at 30 h. The changes were similar to those reported in catfish (*Clarias batrachus*) oocytes after 17,20β-DP treatment (Haider and Chaube, 1995; Haider and Baqri, 2000). These workers correlated the changes in cAMP level with the morphological stages of the oocytes; it was high in centrally located GV oocytes, decreased in relation to GV migration and lower still in oocytes that had undergone GVBD. A 10–21% decrease in cAMP level was sufficient to induce GVBD in *Xenopus* (Huchon et al., 1981; Cicirelli and Smith, 1985).

A negative correlation of cAMP level with the GVBD response was further obtained from the results of the cAMP supplementation experiment. In the presence of cAMP, the 2-OHE$_2$-induced GVBD was inhibited in a concentration-dependent manner in the co-incubation (24 h), pre-stimulation (2, 4 and 6 h) and post-stimulation (4 and 6 h) studies. In the catfish, 0.1 mmol l^{-1} cAMP inhibited GVBD, whereas in yellow perch, concentrations of 1.0 and 0.5 mmol l^{-1} were not inhibitory (DeManno and Goetz, 1987a). Further, the PDE inhibitors IBMX, theophylline and caffeine also inhibited the 2-OHE$_2$-induced GVBD. The response of the PDE inhibitors appears to vary with species, inhibitor type and concentration and duration (DeManno and Goetz, 1987a; DeManno and Goetz, 1987b; Chaube and Haider, 1997; Haider and Baqri, 2000; Pace and Thomas, 2005). In the present study, the inhibitory response was evident at >0.1 mmol l^{-1} for IBMX, >0.5 mmol l^{-1} for theophylline and at >1.0 mmol l^{-1} for caffeine in the co-incubation studies. At 2.0 mmol l^{-1}, both IBMX and theophylline inhibited ~90% of GVBD, whereas caffeine blocked only about 65%. In *C. batrachus* >1.0 mmol l^{-1} theophylline or IBMX completely blocked the 17,20β-DP-induced GVBD (Chaube and Haider, 1997; Haider and Baqri, 2000). In *H. fossilis*, a prior 4 h incubation of the follicles with IBMX or theophylline inhibited the GVBD response to 2-OHE$_2$. In the post-stimulation experiment, only a 6 h treatment of IBMX or theophylline could inhibit the GVBD response. The post-treatment with cAMP or cAMP-elevating drugs produced a low inhibition of GVBD unlike the pre-treatment. This may be due to the prior exposure of the follicles to a high level of cAMP, which strongly countered the steroid's effect, resulting in higher inhibition than in the post-treatment groups. In the post-treatment groups, the follicles were first exposed to 2-OHE$_2$, which lowered the endogenous cAMP level initiating the GVBD response normally. After the stimulation, maintaining the follicles at elevated cAMP level (post-treatment) might have suppressed only the subsequent maturational activity.

A comparison of the inhibitory effect of cAMP on the one hand and IBMX and theophylline on the other showed that cAMP acted faster (2 h) and relatively at a lower concentration (0.1 mmol l^{-1}) to inhibit the GVBD response. The slower rate of response to IBMX and theophylline may be due to their indirect action through PDE inhibition. The inhibitory effect of caffeine on the GVBD response was relatively low and slow, unlike IBMX or theophylline. This may be due to the fact that the potency of caffeine to inhibit PDE activity is low, with IC_{50} values in mmol l^{-1} range (Vadziuk and Kosterin, 2003), compared with IBMX and theophylline, which have lower IC_{50} values (in μmol l^{-1} range) (Lee et al., 2002; Yuasa et al., 2005).

It has been reported that the MIS decreases cAMP by inhibiting adenylate cyclase or cAMP-dependent PDE activity (Finidori-Lepicard et al., 1981; Chaube and Haider, 1997; Haider and Baqri, 2000). The adenylyl cyclase activator forskolin, which stimulates cAMP, has been demonstrated to inhibit MIS-induced maturation in fish (see Jalabert et al., 1991; Haider and Chaube, 1995). Likewise, the PDE inhibitors, such as IBMX and theophylline that elevate cAMP, have been shown to inhibit the MIS-induced maturation (DeManno and Goetz, 1987a; DeManno and Goetz, 1987b; Chaube and Haider, 1997; Haider and Baqri, 2000). Haider and Baqri (Haider and Baqri, 2000) showed a correlation between PDE activity, cAMP level and GVBD response. Hydroxyestrogens have been shown to decrease cAMP level in other tissue systems (Paul and Skolnick, 1977). By contrast, we have shown that the steroid-induced GVBD is associated with the production of ovarian MIS such as 17,20β-DP (Mishra and Joy, 2006a), which, in turn, might have inhibited the follicular cAMP level. The significant and persistent decrease in the cAMP level during the maturation of the catfish oocytes might be due to the combined action of all these mechanisms.

The available evidence indicates that cAMP maintains meiotic arrest or inhibits the MIS-induced GVBD response by stimulating PKA activity (Huchon et al., 1981; Bornslaeger et al., 1986; Jalabert et al., 1991; Rime et al., 1992; Matten et al., 1994). The involvement of PKA in oocyte maturation has been demonstrated in *Fundulus heteroclitus* (Cerda et al., 1997) and *C. batrachus* (Haider and Baqri, 2002). In both species, the PKA inhibitor H-8 or H89 stimulated GVBD, bypassing the MIS. In *F. heteroclitus*, the response was similar to that of 17,20β-DP but with a time lag of several hours (Cerda et al., 1997). In *C. batrachus,* the response was significantly low compared to the MIS effect but the time lag of the response was shorter than that reported in *F. heteroclitus* (Haider and Baqri, 2002). These workers have demonstrated a negative

correlation between H89, 17,20β-DP-induced GVBD response and PKA activity. In *H. fossilis*, H89 stimulated GVBD in a concentration-dependent manner (66.5% at 10 $\mu mol\ l^{-1}$) but was less effective than 2-OHE$_2$ (80.4% at 5 $\mu mol\ l^{-1}$). However, in Atlantic croaker, PKA inhibitors (Rp-cAMP and KT5720), did not stimulate GVBD in the absence of the MIS (20β-S) nor did they enhance the efficacy of the MIS (Pace and Thomas, 2005).

An interesting observation of the present study was that H89 produced concentration-dependent differential effects on the 2-OHE$_2$-induced GVBD when co-incubated for 24 h: high concentrations (5 and 10 $\mu mol\ l^{-1}$) were inhibitory, and low concentrations (0.1 and 1 μM) were ineffective. The underlying mechanism of this dual effect is not clear. The H89 treatment also partially reversed the inhibitory effect of IBMX on the 2-OHE$_2$-induced GVBD. The differential effects can be attributed to the compartmentalization of PKA isoforms or functions. The PKA isoforms exert opposite effects in mouse oocyte maturation; PKA I within the oocytes suppresses GVBD, whereas PKA II within the somatic cell stimulates maturation (Downs and Hunzicker-Dunn, 1995; Rodriguez et al., 2002). The inhibition of 2-OHE$_2$-induced GVBD at higher concentrations of H89 and partial release of the inhibitory effect of IBMX on the steroid-induced GVBD might be due to differential response of the PKA isoforms or due to the action of the drug on other kinases (Chijiwa et al., 1990; Leemhuis et al., 2002). These kinases may interact with cAMP-PKA pathway (Liu and Simon, 1996). Further detailed studies are required to define the differential responses of H89 on the GVBD response in the catfish.

The role of PKA activity in oocyte maturation needs to be re-examined in the context of recent studies. In *Xenopus,* catalytic activity of PKA is not necessary to block meiotic maturation induced by progesterone or Mos, although it is important for the inhibition of Cdc25C-induced maturation (Schmitt and Nebreda, 2002). In Atlantic croaker, inhibition of the cAMP-independent signal pathway by PI 3-kinase/Akt blocked 20β-S-induced GVBD. The involvement of the PI 3-kinase/Akt pathway in inhibition of GVBD has also been reported in striped bass (Weber and Sullivan, 2001). These studies are to be extended to the catfish; however, we have shown a positive involvement of other signaling pathways such as PKC and MAPK in oocyte maturation (Mishra and Joy, 2006d; Mishra and Joy, 2006e). It appears that the relative importance of various signal transduction pathways and their interactions during oocyte maturation may show species differences.

Okadaic acid-sensitive PP (PP1 and PP2A) has been demonstrated to play an important role in oocyte maturation (Huchon et al., 1981; Schwartz and Schultz, 1991; Sun et al., 2002). OA stimulates oocyte maturation in starfish, *Xenopus* and mammals (Goris et al., 1989; Rime and Ozon, 1990; Gavin et al., 1991; Schwartz and Schultz, 1991; Sun et al., 2002) and has been attributed to MPF (Goris et al., 1989) or MAPK (Zernika-Goetz et al., 1997; Sun et al., 2002) activation. In the catfish, the incubation of follicles with various concentrations of OA alone did not induce GVBD but together with 2-OHE$_2$ it did (Mishra and Joy, 2006d). Thus, the effect of OA on meiotic resumption is dependent on the presence of the MIS in the catfish unlike in *Xenopus*, starfish or mammals in which the action is MIS (hormone) independent (Goris et al., 1989; Pondaven et al., 1989; Rime and Ozon, 1990). OA has stimulated MAPK activation by suppressing PP1 and PP2A activity, leading to a higher percentage of GVBD.

In the present study, OA reversed fully the inhibitory effect of IBMX on the 2-OHE$_2$-induced GVBD. Similar observations were also reported in mouse (Rime and Ozon, 1990; Gavin et al., 1991; Schwartz and Schultz, 1991) and rat (Lu et al., 2001). In *Xenopus*, *in vivo* activation of the MPF by OA does not involve PKA (Rime et al., 1992). Rime et al. have suggested that the OA-induced inhibition of PP1 and/or PP2A acts at a step distal to the site of action of cAMP-PKA, activating both MPF and MAPK (see also Sun et al., 2002; Fan et al., 2002). In the catfish, a similar mechanism may operate to overcome the inhibitory effect of IBMX on the 2-OHE$_2$-induced GVBD. Further studies are required to identify the specific site(s) in the cascade of MPF and/or MAPK activation.

In conclusion, the results of the present study show that cAMP-PKA inhibits the 2-OHE$_2$-induced GVBD in the catfish. Okadaic acid *per se* did not support oocyte maturation, as it does in other species, but it potentiated the effect of 2-OHE$_2$ on GVBD. OA could overcome the inhibitory effect of IBMX on GVBD, implying an action distal to the cAMP–PKA cascade.

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Abbreviations

2-OHE$_2$	2-hydroxyestradiol-17β
GVBD	germinal vesicle breakdown
H89	*N*-(2-[p-bromocinnamylamino]ethyl)-5-isoquinoline sulfonamide hydrochloride
IBMX	3-isobutyl-1-methyl-xanthine
LH	luteinizing hormone
MIS	maturation-inducing substance
MPF	maturation-promoting factor
OA	okadaic acid
PDE	phosphodiesterase
PP	protein phosphatases

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