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Regulation of recombinant human insulin-induced maturational events in *Clarias batrachus* (L.) oocytes *in vitro*

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Summary

Regulation of insulin-mediated resumption of meiotic maturation in catfish oocytes was investigated. Insulin stimulation of post-vitellogenic oocytes promotes the synthesis of cyclin B, histone H1 kinase activation and a germinal vesicle breakdown (GVBD) response in a dose-dependent and duration-dependent manner. The PI3K inhibitor wortmannin abrogates recombinant human (rh)-insulin action on histone H1 kinase activation and meiotic G2–M1 transition in denuded and follicle-enclosed oocytes *in vitro.* While the translational inhibitor cycloheximide attenuates rh-insulin action, priming with transcriptional blocker actinomycin D prevents insulin-stimulated maturational response appreciably, albeit in low amounts. Compared with rh-insulin, human chorionic gonadotrophin (hCG) stimulation of follicle-enclosed oocytes *in vitro* triggers a sharp increase in 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DHP) secreted in the incubation medium at 12 h. Interestingly, the insulin, but not the hCG-induced, maturational response shows less susceptibility to steroidogenesis inhibitors, trilostane or DL-aminoglutethimide. In addition, priming with phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX) or cell-permeable dbcAMP or adenylyl cyclase activator forskolin reverses the action of insulin on meiotic G2–M1 transition. Conversely, the adenylyl cyclase inhibitor, SQ 22536, or PKA inhibitor H89 promotes the resumption of meiosis alone and further potentiates the GVBD response in the presence of rh-insulin. Furthermore, insulin-mediated meiotic maturation involves the down-regulation of endogenous protein kinase A (PKA) activity in a manner sensitive to PI3K activation, suggesting potential involvement of a cross-talk between cAMP/PKA and insulin-mediated signalling cascade in catfish oocytes *in vitro.* Taken together, these results suggest that rh-insulin regulation of the maturational response in *C. batrachus* oocytes involves down-regulation of PKA, synthesis of cyclin B, and histone H1 kinase activation and demonstrates reduced sensitivity to steroidogenesis and transcriptional inhibition.

Keywords: cAMP/PKA, Catfish, Insulin, Oocyte, Ovarian follicles, PI3K

Introduction

Follicular steroidogenesis and final oocyte maturation are considered to be under the guidance of pituitary gonadotropin [follicle-stimulating hormone/luteinizing hormone (FSH/LH)] and ovarian steroids, respectively (Negatu *et al.*, 1998). The actions of LH/human chorionic gonadotrophin (hCG) are mediated by binding to specific membrane-bound G-protein-coupled receptors and subsequent activation of signal transduction pathways, including adenylyl cyclase- and calcium-dependent signalling cascades (for review, Leung & Steele, 1992; Van Der Kraak & Wade, 1994). In teleost ovary, LH-dependent signals derived from the surrounding somatic cells induce the synthesis and secretion of maturation-inducing steroid (MIS) prior to meiotic G2–M1 transition (for review, Nagahama, 1997). While 17α,20β-dihdroxy-4-pregnen-3-one (17α,20β-DHP) is the principal MIS in salmonids, cyprinids and many other teleost orders (Nagahama, 1997; Nagahama & Yamashita, 2008), in sciaenid and other marine fishes 17α,20β,

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21-trihydroxy-4-pregnen-3-one (20β-S) has been identified as MIS (Trant & Thomas, 1989; Picha *et al.*, 2012).

Some metabolic cues including insulin and leptin are well known to regulate both metabolic and reproductive functions and their perturbation has been linked to many disorders in which gonadotropin release is at risk (Acosta-Martínez, 2012). Diabetic animals ovulate at a lower rate; exhibit altered ovarian steroidogenesis and increased incidence of follicular atresia (Poretsky *et al.*, 1999; Colton *et al.*, 2002). Ovaries of almost all vertebrates express receptors for binding of insulin and insulin-like growth factor (IGF)-1 (Hainaut *et al.*, 1991; Giudice, 1992; Maestro *et al.*, 1997; Poretsky *et al.*, 1999) and ovarian IGF system comprising of IGF1, IGF-II, IGF1-receptor, and IGF-binding protein-2 has been reported earlier in maturing and pre-ovulatory follicles in fish (Kagawa *et al.*, 1994; Nelson & Van Der Kraak, 2010). That insulin/IGF1 may have a significant role in the regulation of gonadal functions in teleosts has been hypothesized due to the presence of their receptors in the ovary and variation in ligand binding at different stages of reproductive cycle (Gutiérrez *et al.*, 1993).

In addition to its role in the regulation of glucose homeostasis, insulin has been shown to act as a potent regulator of meiotic maturation in fish and amphibia (El-Etr *et al.*, 1979; Hainaut *et al.*, 1991; Maestro *et al.*, 1997; Mukherjee *et al.*, 2006; Das *et al.*, 2013). In common carp and zebrafish insulin/IGFs could induce germinal vesicle breakdown (GVBD) in denuded oocytes divested of surrounding follicular cells (Mukherjee *et al.*, 2006; Das *et al.*, 2013), suggesting participation of growth factors in the regulation of final oocyte maturation through a pathway independent of steroid action. Conversely, insulin/IGFs potentially induce ovarian steroidogenesis and development of oocyte maturational competence (Srivastava & Van Der Kraak, 1994; Patiño & Kagawa, 1999; Weber & Sullivan, 2005; Weber *et al.*, 2007). More interestingly, in red seabream, mummichog and striped bass, IGF1 has been shown to induce oocyte maturation in intact follicles even in the presence of transcriptional and/or steroidogenesis inhibitors (Kagawa *et al.*, 1994; Negatu *et al.*, 1998; Weber & Sullivan, 2000). Despite the described actions of insulin/IGFs on oocyte maturation, the intracellular mechanisms by which they induce maturational competence in follicle-enclosed oocytes or alter follicular steroidogenesis to favour MIS production are not well characterized.

The importance of cAMP-independent signalling pathways that involves PI3K activation and participation of protein kinase B (PKB/Akt) in the regulation of oocyte maturation has been reported earlier (Schmitt & Nebreda, 2002; Pace & Thomas, 2005). Furthermore, oocyte-specific phosphodiesterase (PDE3) has been implicated as the potential downstream target to the insulin signalling cascade in Atlantic croaker, zebrafish, *Xenopus* and mouse oocytes (Andersen *et al.*, 1998, 2003; Pace & Thomas, 2005; Han *et al.*, 2006; Das *et al.*, 2013). Although exposure to MIS up-regulates PI3K activation in oocytes undergoing meiotic maturation in starfish (Sadler & Ruderman, 1998), Atlantic croaker (Pace & Thomas, 2005) and *Rana* (Ju *et al.*, 2002), PI3K inhibition fails to inhibit progesterone-stimulated GVBD in *Xenopus* oocytes (Liu *et al.*, 1995; Mood *et al.*, 2004).

The paradigm of cAMP regulation in somatic follicular cells and oocyte is considered to be diametrically opposite (Tsafriri *et al.*, 1996), while a high cAMP level is essential for gonadotropin (LH/hCG)-induced increase in follicular steroidogenesis (Leung & Steele, 1992; Van Der Kraak & Wade, 1994; Nagahama, 1997), forced elevation of intra-oocyte cAMP is sufficient to arrest oocytes at the G2–M1 boundary (Conti *et al.*, 2002). Moreover, activation of adenylyl cyclase, production of cAMP and increase in protein kinase A (PKA) activity by the LH–CG receptor (LHCGR) are attributed to steroid biosynthesis and MIS stimulation of oocyte maturation has been shown to involve down-regulation of cAMP/PKA activity (Yoshikuni & Nagahama, 1994; Nagahama, 1997; Zhu *et al.*, 2003; Khan & Maitra, 2013). Although species-specific variations exist, insulin/IGF regulation of follicular steroidogenesis and oocyte maturation has been reported earlier (Nagahama, 1997; Tyler *et al.*, 1999). However, potential involvement of the cAMP-dependent signalling cascade during insulin stimulation of follicular events is not yet well characterized and requires further investigation.

Clarias batrachus (Order: Siluriformes; Family: Clariidae), a species of high table value and economic importance in the Indian sub-continent, are group-synchronous single-clutch seasonal spawners and the annual breeding cycle has been divided into the preparatory (February–April), the pre-spawning (May–June), the spawning (July–August) and the post-spawning (September–January) phases (Nath & Maitra, 2001). MIS stimulation of *C. batrachus* follicle-enclosed oocytes promotes GVBD response and maturation promoting factor (MPF) activation through a pathway that involves down-regulation of cAMP/PKA activity (Haider & Chaube, 1995, 1996; Chaube & Haider, 1997; Haider & Baqri, 2000a, b, 2002). The action of any member of the insulin family of peptides, however, has not been investigated so far. Earlier insulin has been shown to act as an amplifier of the gonadotropin action modulating follicular steroidogenesis (Poretsky & Kalin, 1987; Srivastava & Van Der Kraak, 1994); however, data implicating insulin regulation of endogenous PKA activity in teleost oocytes are missing. The major

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objective of the present study was to investigate the regulation of maturational events during recombinant human insulin (rh-insulin)-stimulated meiotic G2–M1 transition in *Clarias batrachus* oocytes *in vitro*. Specifically, our results demonstrate that, compared with hCG, rh-insulin stimulation of maturational response is less susceptible to transcriptional and steroidogenesis inhibitors (trilostane or DL-aminoglutethimide) and requires down-regulation of cAMP-dependent protein kinase (PKA) activity.

Materials and methods

Animals

Adult female *C. batrachus* (body weight range 100–150 g) were obtained from local fish farms in and around Santiniketan (latitude 23°41′30″N, longitude 87°30′47″E), West Bengal, India during the late pre-spawning and spawning phases (June–August) and were maintained in 200 l glass aquaria under an ambient photoperiod (13 h light:11 h dark) and temperature (28.5 ± 2°C) for at least 7 days prior to their use in experiments. Fish were fed *ad libitum* twice daily with tropical dry fish palate and live blood worm. Water in the aquaria was circulated using motor pumps and replenished on every alternate day. All animal experiments were carried out following the guidelines of the Institutional Animal Ethics Committee of Visva-Bharati University.

Chemicals and antibody

Leibovitz's L-15 tissue culture medium was purchased from Gibco(R), Invitrogen; pharmacological adenylyl cyclase inhibitor, SQ22536 was purchased from Tocris Bioscience (R&D Systems, India). Insulin, recombinant human (cat. no. 91077C) and recombinant human IGF1 (cat. no. 13769) were purchased from Sigma-Aldrich, India. Mouse monoclonal anti-cyclinB1: sc-245; and rabbit polyclonal anti-cdc2p34 (PSTAIRE): sc-53; anti-p-PKAα/β/γ cat (Thr 198): sc-32968; and anti-PKAα cat: sc-903 antibodies were obtained from Santa Cruz Biotech (Santa Cruz, CA, USA). Maturation-inducing steroid (MIS) enzyme immunoassay (EIA) antiserum (cat. no. 498502), acetylcholinesterase (AChE) tracer (cat. no. 498500), EIA standard (cat. no. 498504), pre-coated (mouse anti-rabbit IgG) 96-well plates (cat. no. 400006) and Ellman's reagent (cat. no. 400050) were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Reagents unless otherwise specified, were from Sigma-Aldrich, India.

Oocyte preparation and *in vitro* culture

Gravid females were sacrificed by decapitation, ovaries were removed aseptically and placed immediately in L-15 medium, (pH 7.4) supplemented with penicillin (100 IU/ml) and streptomycin (100 μg/ml). Follicle-enclosed oocytes (average diameter 1.35 ± 0.112 mm) were separated manually using fine tip forceps, seeded into sterile tissue culture plates (40 follicles/2 ml medium) and cultured *in vitro* at 25 ± 1°C for 24 h under gentle agitation (40 rpm). Denuded oocytes were prepared by treating the post-vitellogenic follicles with 0.001% collagenase type IA (Sigma–Aldrich, cat. no. C9891, 1.25 U/ml) and repeated pipetting as described earlier (Das *et al.*, 2013). Complete removal of the surrounding follicular cells was ascertained microscopically by staining with 4′6-diamidino-2-phenylindole (DAPI) and GVBD bioassay with hCG treatment.

Recombinant human insulin (molecular weight ~5808 Da), identical in structure and function to full length human insulin and produced by recombinant DNA technology in *Saccharomyces cerevisiae*, was reconstituted aseptically in cell culture grade water containing 0.01 N hydrochloric acid (pH 2) at 4.4 mg/ml (≥27.5 IU/mg) as per manufacturer's instructions. Recombinant human IGF1 was dissolved in sterile water. Various doses of rh-insulin, and IGF1 were diluted with medium (final volume 10 μl) just before use and added to the culture; control wells received an equal volume of vehicle only. Maturational steroid 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DHP) and DL-aminoglutethimide (DL-A, 100 μM), an inhibitor of cholesterol side-chain cleavage cytochrome P450 (P450scc), were dissolved in ethanol. Pharmacological phosphoinositide 3 kinase (PI3K) inhibitor wortmannin (Wrt, 1 and 10 μM), phosphodiesterase (PDE) inhibitor 3-isobutyl-1-methylxanthine (IBMX; 1 mM), adenylyl cyclase activator, forskolin (FK; 5 μM), transcription inhibitor, actinomycin D (ActD; 100 nM), translational blocker, cycloheximide (Chx; 100 μg/ml), and 3β-hydroxy steroid dehydrogenase (3β-HSD) inhibitor trilostane (Trilo, 1 μg/ml) were dissolved in dimethyl sulphoxide (DMSO) and added to the culture 2 h prior to rh-insulin stimulation. Adenylyl cyclase inhibitor, SQ22536 (SQ; 10 μM) and PKA inhibitor H89 (10 μM) were also dissolved in DMSO and were added to the culture either alone or in combination with rh-insulin. Follicles in control wells received an equivalent amount of DMSO (10 μl/ml of medium) only. Cell-permeable di-butyryl (db)cAMP (1 mM) was dissolved in nuclease-free water (Fermentas) just before use and added to the culture medium 2 h prior to hormonal stimulation. Doses of IBMX, FK, dbcAMP, H89, Wrt, ActD, Chx were as described earlier (Haider & Chaube, 1996; Das

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et al., 2013; Maitra *et al.*, 2014). GVBD was determined microscopically by immersing the oocytes in clearing solution (ethanol:formaldehyde:acetic acid, 3:6:1 v/v). At least three different replicates were taken for each time points and each experiment was done using follicles collected from different donor fish. Viability was checked by the 0.1% trypan blue dye exclusion method and was found to be ≥90% after 24 h of incubation.

Preparation of oocyte extract

Samples were harvested at appropriate time intervals, washed (3×) and homogenized in oocyte extraction buffer containing 100 mM sodium β-glycerophosphate, 20 mM HEPES, 15 mM $MgCl_2$, 5 mM ethyleneglycoltetraacetic acid (EGTA), 100 μM *p*-phenylmethylsulphonyl fluoride (*p*-PMSF), 3 μg/ml leupeptin, 1 mM dithiothreitol (DTT) and 1 μg/ml aprotinin; pH 7.5 (Hirai *et al.*, 1992). Supernatant was separated by spinning at 17,500 *g* for 20 min at 4°C and was either used immediately or stored at –86°C until further use.

Electrophoresis and immunoblot analysis

Protein concentration was determined by Lowry *et al.*, (1951) using bovine serum albumin (BSA) as standard. Oocyte lysates (60 μg protein/well) from control and treated groups were resolved in 15% SDS–PAGE, transferred to Hybond-P PVDF membrane (GE Healthcare Biosciences, Buckinghamshire, UK) using transfer buffer (25 mM Tris, 193 mM glycine, 20% methanol; pH 8.5) for 45 min. Membranes were kept in blocking solution containing 5% non-fat milk in TBST (50 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.6) overnight at 4°C and incubated in primary antibody diluted 1:500 in blocking buffer for 4 h at room temperature, washed with TBST and incubated in alkaline phosphatase tagged anti-mouse or anti-rabbit IgG (1:1000). Bands were developed by adding 5-bromo-4-chloro-3′-indolyphosphate/nitro-blue tetrazolium (BCIP/NBT), recorded in a Gel Doc apparatus (Bio-Rad) and imported into Adobe Photoshop software.

Histone H1 kinase assay

Histone H1 phosphorylation, a reliable marker for p34cdc2 kinase activation, was assayed as described earlier (Khan & Maitra, 2013). In brief, 20 μl of oocyte lysate (100 μg protein) from each treatment group were incubated at 30°C for 2 min in the presence of 100 μM histone H1 (Type-III-S); 500 μM ATP; 1.5 μCi [γ^{32}P]-ATP (3500 Ci/mmol; Board of Radiation and Isotope Technology, Department of Atomic Energy, Government of India); 1 mM EGTA; 10 mM $MgCl_2$; 4.5 mM 2-mercaptoethanol and 20 mM Tris–HCl (pH 7.4). The kinase reaction was stopped by adding 20 μl of 300 mM phosphoric acid and 80 μl of reaction mixture was spotted on Whatman P81 phospho cellulose paper (Whatman, Brentford, UK), washed three times with 1% phosphoric acid, dried and radioactivity was measured in a liquid scintillation counter (Perkin Elmer).

Measurement of 17α,20β-DHP in the incubation medium

Media from catfish follicle incubations were collected and stored at -80°C prior to measurement of steroid content. The level of 17α,20β-DHP was measured by competitive enzyme immunoassay (EIA) kit following the manufacturer's protocol (Cayman Chemical, Ann Arbor, MI, USA) and as reported earlier (Nelson & Van Der Kraak, 2010). The antiserum of MIS was highly specific and cross-reacted with 17α,20β-DHP, 17α,20α-dihydroxy progesterone, 20β-hydroxy progesterone and allopregnanolone at 100, 0.04, 0.1 and <0.01% respectively. The minimum detection limit (76% B/B_0) was 3.9 pg/ml. The intra-assay [% coefficient of variation (CV), $n = 6$] and inter-assay % CV, ($n = 8$) at 19.12–75.86% binding were less than 5.6 and 9% respectively.

Assay for endogenous PKA activity in follicular lysates

The PKA activity in follicular lysates was measured in a cell-free reaction system using kemptide as a substrate (Khan & Maitra, 2013). Briefly, 10 μl of the lysate (100 μg protein) was incubated at 30°C for 30 min in assay buffer [20 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS); pH 7.2, 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 4.05 mM $MgCl_2$, 27 mM ATP, kemptide (80 μM), 5 μl of cAMP and 10 μl of CaMK/PKC inhibitor cocktail and 10 μCi [γ^{32}P]-ATP (3500 Ci/mmol; Board of Radiation and Isotope Technology, Department of Atomic Energy, Govt. of India) in a final volume of 60 μl. The reaction was terminated by spotting 25 μl of the reaction mixture onto P81 phospho-cellulose paper, washed three times with 0.75% phosphoric acid and once in acetone and the radiolabel incorporation was determined by scintillation counting of the dried paper. For each set of data at least three replicates were taken and the assay was further conducted from five different fish. Phosphorylation of kemptide by the catalytic subunit of PKA (PKAc), purified from bovine heart muscle (1.7 μg/ml), served as a positive control. Specificity of the assay was determined by addition of protein kinase inhibitor peptide (PKI, 1 μM) in parallel incubations.

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Data analysis

Data [mean ± standard error of the mean (SEM)] were analyzed by one-way analysis of variance (ANOVA), followed by Duncan's Multiple Range Test for multiple group comparison and significance level were checked, a P-value < 0.05 was considered statistically significant. Steroid concentrations were normalized to pg/oocyte and below the minimum detection limit were shown as 'nd.'

Results

Insulin induction of meiotic maturation in catfish follicle-enclosed oocytes

Priming of follicle-enclosed oocytes with rh-insulin promoted GVBD response in a dose- and duration-dependent manner; 5 μM (0.8 IU/ml) eliciting best result as 51.84 ± 4.21% of oocytes underwent GVBD at 24 h of incubation (Fig. 1*a*) and up-regulated histone H1 kinase activation significantly ($P < 0.001$) over the unstimulated or vehicle-treated group (Control) (Fig. 1*b*). Priming with PI3K inhibitor, wortmannin (1 and 10 μM) attenuated insulin action on ^{32}P incorporation in histone H1 protein significantly ($P < 0.001$) (Fig. 1*b*). Furthermore, immunoblot analysis of the oocyte extract revealed that stimulation with either 17α,20β-DHP or rh-insulin lead to the synthesis of an anti-cyclin B immuno-reactive protein at 48 kDa (Fig. 1*c*, lower panel) and probing oocyte lysates with anti-p34cdc2 (PSTAIRE) antibody revealed a distinct protein band at 34 kDa (Fig. 1*c*, upper panel). Interestingly, priming with 1, 10 and 50 nM of IGF1, but not rh-insulin, could promote a dose-dependent increase in the GVBD response (Fig. 2).

Effect of protein synthesis and steroidogenic enzyme inhibitors on insulin-induced maturational response

To examine the importance of *de novo* synthesis of proteins and follicular steroidogenesis per se on rh-insulin-induced GVBD response, follicle-enclosed oocytes were pre-incubated with either inhibitors of transcription, translation or steroidogenic enzymes for 2 h followed by hormonal stimulation. As shown in Fig. 3*a*, in Chx pre-incubated cells, stimulation with either rh-insulin or hCG failed to induce GVBD response. Although it was less effective in blocking resumption of meiosis in insulin-treated oocytes, pre-incubation with ActD could reduce hCG-induced GVBD to the level comparable with vehicle-treated control (Fig. 3*a*). Furthermore, priming with either 3β-HSD inhibitor, trilostane (Trilo, 1 μg/ml) or P450scc blocker, DL-aminoglutethimide (DL-A, 100 μM) could attenuate the hCG-induced GVBD response significantly ($P < 0.001$) (Fig. 3*b*). Conversely, even in the presence of either trilostane or DL-aminoglutethimide, rh-insulin could trigger a GVBD response significantly ($P < 0.001$) (Fig. 3*b*). Enzyme immune assay (EIA) for maturation-inducing steroid (17α,20β-DHP) in the follicle incubation medium using highly specific antiserum revealed that while rh-insulin (5 μM) could elicit a modest increase (38.7 ± 3.4 pg/oocyte), incubation with hCG (10 IU/ml) stimulated a sharp increase in 17α,20β-DHP (98.4 ± 4.5 pg/oocyte) at 12 h (Fig. 3*c*). Moreover, priming with either DL-A or trilostane either alone or in the presence of hCG/rh-insulin abrogated the MIS response (Fig. 3*c*). However, 17α,20β-DHP was not detected (nd) in incubation medium from either 1, 10 or 50 nM of either the IGF1 or rh-insulin-treated groups (data not shown).

Effect of cAMP/PKA modulators on insulin-induced GVBD in follicle-enclosed oocytes

As IGF1/rh-insulin could promote oocyte maturation in the absence of appreciable MIS synthesis and, compared with the hCG-stimulated GVBD response, rh-insulin at high dose (5 μM) showed less susceptibility to pre-incubation with steroidogenesis inhibitors, participation of cAMP-dependent signalling cascade was hypothesized. To explore this possibility, next we examined the effect of various cAMP/PKA modulators on insulin-stimulated GVBD response. As shown in Fig. 4*a*, priming with either IBMX (1 mM) or cell-permeable dbcAMP (1 mM) or FK (5 μM) attenuated rh-insulin action on oocyte GVBD significantly ($P < 0.001$).

To examine further the participation of cAMP/PKA on insulin action, intact post-vitellogenic ovarian follicles were incubated with adenylyl cyclase inhibitor, SQ22536 or PKA inhibitor, H89 either alone or in combination with rh-insulin. While priming with SQ22536 (10 μM) or H89 (10 μM) alone was sufficient to trigger GVBD response in 51.2 ± 3.8 and 47.5 ± 3.1% of follicle-enclosed oocytes respectively (Fig. 4*b*), could further potentiate insulin-induced maturation response significantly ($P < 0.001$) at all the time points tested (Fig. 4*c*).

Effect of rh-insulin on endogenous PKA activity

In order to explore the involvement of active PKA in negative regulation of insulin action, endogenous PKA activity was determined indirectly by immunoblot analysis using phospho-PKAc (p-PKAc) antibody and directly by kinase assay in cell-free reaction system using kemptide as a substrate. Immunoblot analysis revealed that regardless of the treatments total protein

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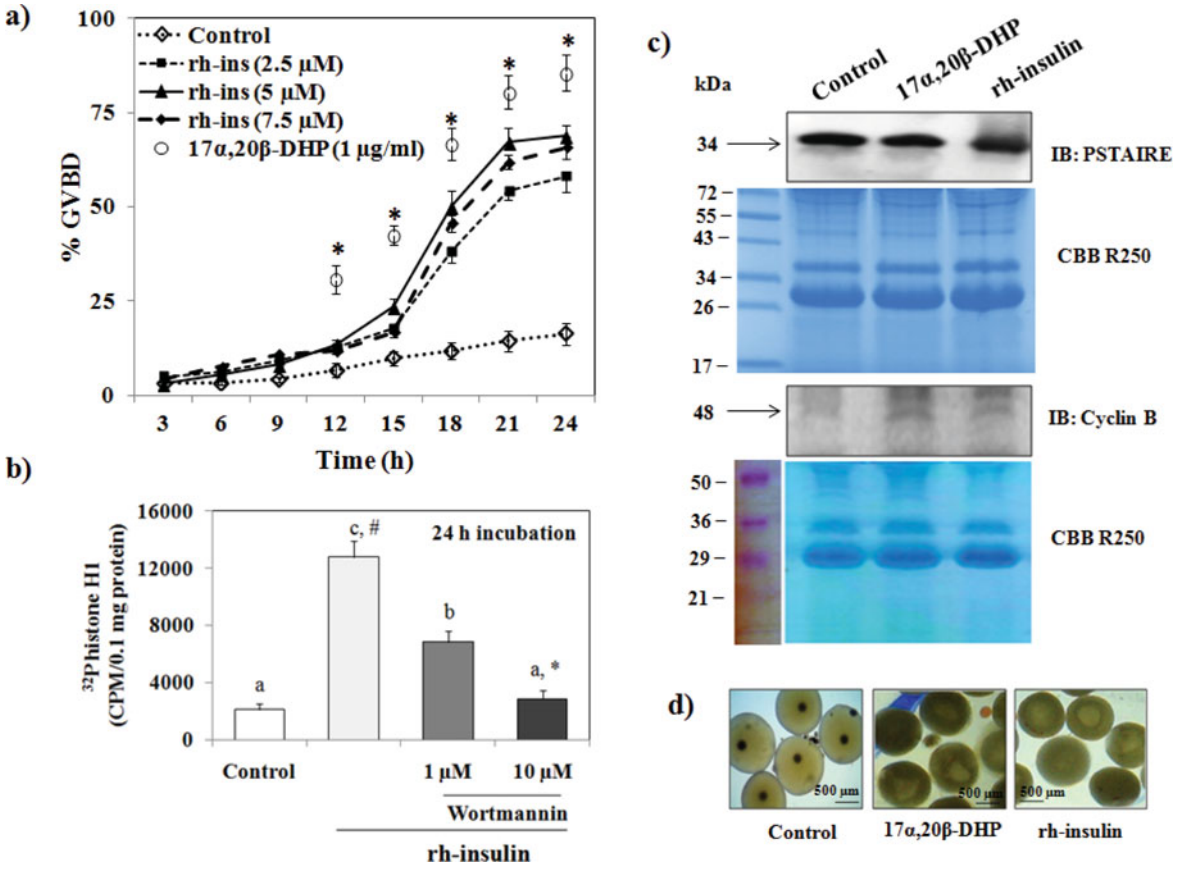

Figure 1 Recombinant human (rh)-insulin stimulation of meiotic G2–M1 transition in catfish follicle-enclosed oocytes *in vitro.* (a) Fully grown catfish follicles (diameter ~1350 μm) were primed with either 17α,20β-DHP (1 μg/ml) or different doses of rh-insulin (2.5, 5 or 7.5 μM equivalent to 0.4, 0.8 or 1.2 IU/ml respectively) or medium only (Control) and scored for GVBD at indicated time intervals. (b) Oocyte lysates from various treatment groups were assayed for histone H1 kinase activation by scintillation counting. (c) Immunoblot analysis of follicular lysate using anti-p34cdc2 (PSTAIRE) (upper panel) and anti-cyclin B antibodies (lower panel). Gels stained with Coomassie brilliant blue R250 (CBB R250) reveal equal protein loading and migration of size markers. (d) Representative photomicrographs of follicle-enclosed oocytes stimulated either without (control) or with 17α,20β-DHP (1 μg/ml) or rh-insulin (5 μM) for 24 h and recorded under stereo-zoom microscope. Data are representative of at least three independent experiments from separate fish showing identical results. Values are mean ± standard error of the mean (SEM) of three independent experiments and data were analyzed by one-way analysis of variance (ANOVA) ($P < 0.001$) and Duncan's Multiple Range Test ($P < 0.05$). $^{a-c}$Groups with same lowercase letter above the bars are not significantly different and those with different letters differ significantly. #$P < 0.001$ compared with the vehicle-treated group and $^{*}P < 0.001$ compared with the rh-insulin-stimulated positive control group.

(PKA catalytic subunit, PKAc) remained unaltered (Fig. 5, lower panel). In contrast, compared with the unstimulated control, p-PKAc (active) decreased significantly in response to stimulation with either rh-insulin or 17α,20β-DHP, providing indirect evidence for PKA inactivation (Fig. 5, upper panel). Moreover, insulin stimulation abrogated endogenous PKA activity significantly ($P < 0.001$) in a manner sensitive to PI3K activation.

Effect of rh-insulin on denuded catfish oocytes

Although forced elevation of cAMP attenuates and PKA down-regulation potentiates rh-insulin action on meiotic G2–M1 transition in follicle-enclosed oocytes, possibility of insulin action directly on the oocyte membrane cannot be ruled out. In order to explore this possibility, denuded oocytes were incubated with 17α,20β-DHP and rh-insulin *in vitro.* Complete removal of surrounding follicular cells was confirmed by the absence of DAPI staining (right panel, Fig. 6*a*). Moreover, the effectiveness of the denudation procedure was also determined functionally as the denuded oocytes failed to undergo GVBD due to hCG stimulation (data not shown). As shown in Fig. 6*b*, compared with either vehicle-treated (Control) or in oocytes pre-incubated with wortmannin (10 μM), insulin (5 μM) stimulation promoted GVBD response

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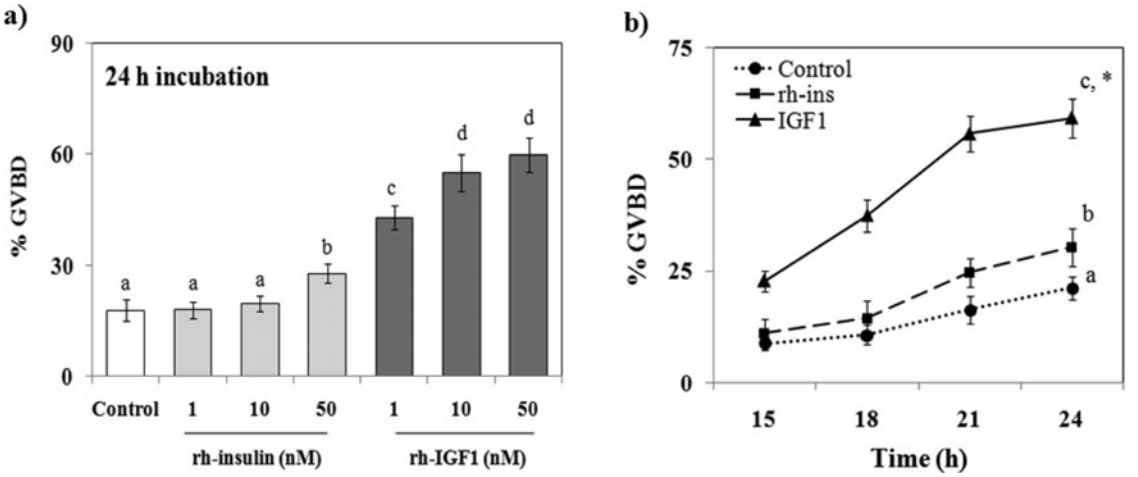

Figure 2 Effect of three different doses (1, 10 or 50 nM) of IGF1 and rh-insulin on maturational response at 24 h in follicle-enclosed oocytes (a); and time kinetics of insulin and IGF1 (50 nM for each)-induced GVBD (b). Values are mean ± standard error of the mean (SEM) of three independent experiments and data were analyzed by one-way analysis of variance (ANOVA) ($P < 0.001$) and Duncan's Multiple Range Test ($P < 0.05$). [a–d]Groups with same lowercase letter above the bars are not significantly different and those with different letters differ significantly.

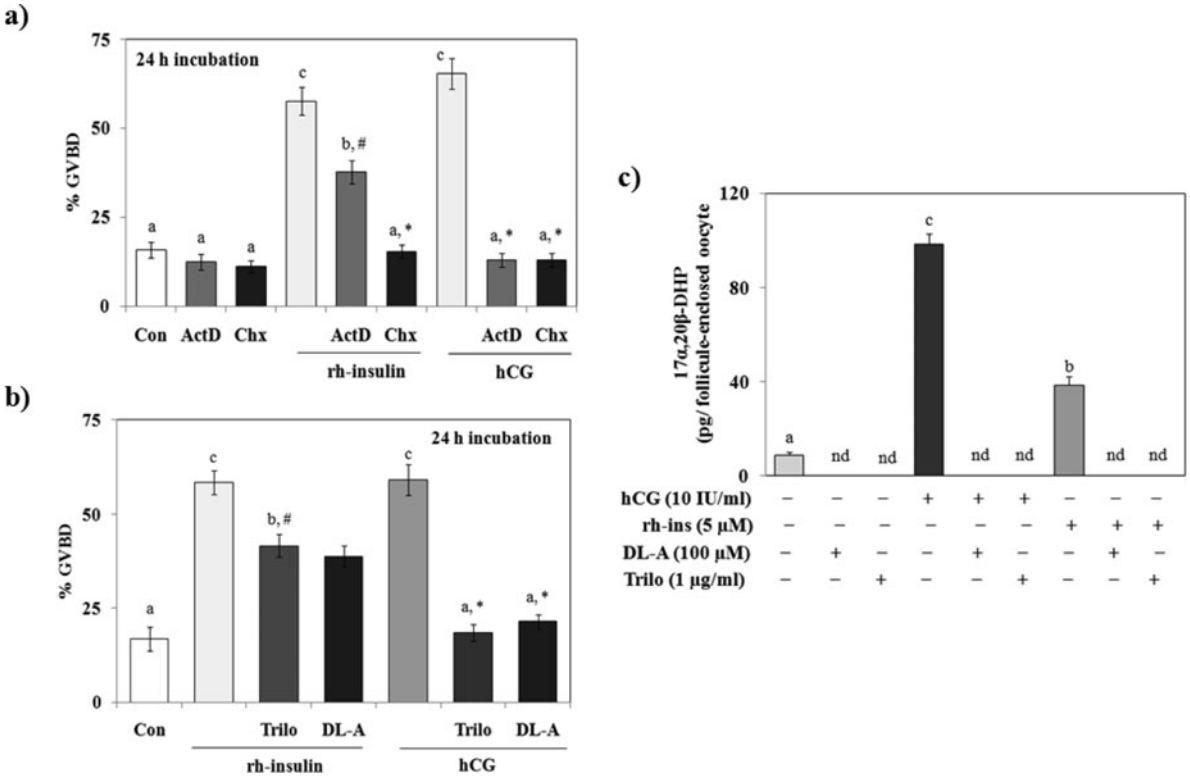

Figure 3 Relative importance of *de novo* protein synthesis (a), and follicular steroidogenesis (b) during rh-insulin (5 μM)- and hCG (10 IU/ml)-induced GVBD response in follicle-enclosed oocytes *in vitro.* Level of 17α,20β-DHP in the culture media due to stimulation with hCG/rh-insulin either pre-incubated or not with steroidogenesis inhibitors at 12 h of incubation (c). Values are mean ± standard error of the mean (SEM) of three independent experiments and data were analyzed by one-way analysis of variance (ANOVA) ($P < 0.001$) and Duncan's Multiple Range Test ($P < 0.05$). [a–c]Means with different lowercase letter above the bars differ significantly. #$P < 0.001$ compared with vehicle-treated (Control) and *$P < 0.001$ compared with hCG-stimulated positive control. 17α,20β-DHP level below the minimum detection limit were shown as 'nd.'

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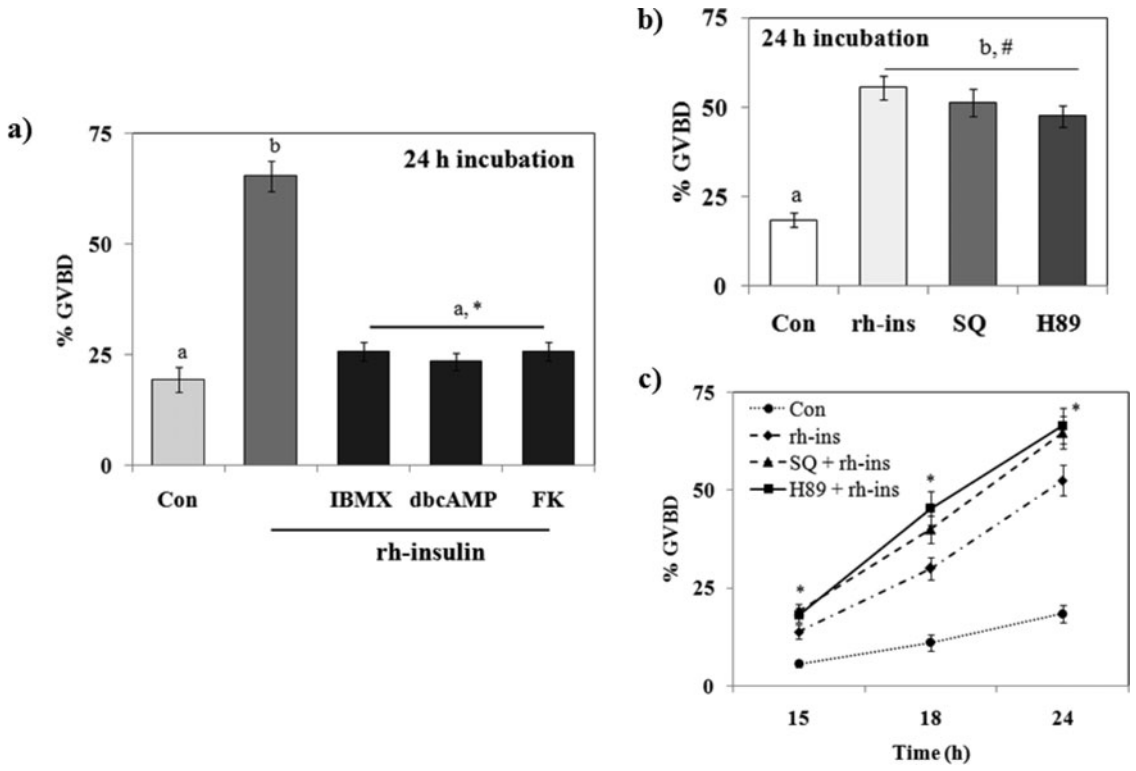

Figure 4 cAMP/PKA regulation of insulin-stimulated GVBD response in follicle-enclosed oocytes *in vitro.* Follicle-enclosed oocytes were pre-incubated (2 h) either without (Con) or with IBMX (1 mM), dbcAMP (1 mM), FK (5 μM) followed by rh-insulin (5 μM) stimulation for 24 h and GVBD was scored (a). Effect of priming with SQ22536 (10 μM), H89 (10 μM), rh-insulin (5 μM), either alone (b) or in combination (c), on GVBD response. Values are mean ± standard error of the mean (SEM) of at least three independent experiments from different donor fish and data were analyzed by one-way analysis of variance (ANOVA) ($P < 0.001$) and Duncan's Multiple Range Test ($P < 0.05$). [a,b]Groups with different lowercase letters above the bars differ significantly. #$P < 0.001$ compared with vehicle-treated group and *$P < 0.001$ compared with insulin-stimulated positive control.

and histone H1 kinase activation significantly ($P < 0.05$) within 14 h of incubation and the GVBD% was comparable with that in the 17α,20β-DHP incubated group.

Discussion

Insulin-mediated resumption of meiotic maturation in catfish oocytes has been investigated. Based on the primary structure of insulin from at least 100 vertebrate species, Conlon (2001) stated that in addition to invariant cysteine residues, precisely 10 amino acids at specific locations do participate either in receptor binding or in maintaining the ligand-receptor conformation and are fully conserved during vertebrate evolution. Available information indicates that porcine, bovine and human insulin could promote meiotic maturation in fish and amphibian oocytes indicating heterologous insulin are functionally competent (Maller & Koontz, 1981; Diss & Greenstein, 1991; Hainaut *et al.*, 1991; Kagawa *et al.*, 1994; Maestro *et al.*, 1997; Dasgupta *et al.*, 2001; Mukherjee *et al.*, 2006; Chourasia & Joy, 2008; Das *et al.*, 2013). Moreover, binding of human and porcine insulin to insulin/IGF receptors has been shown previously in *Xenopus* and carp ovary (Diss & Greenstein, 1991; Maestro *et al.*, 1997).

Data of the present study demonstrate that rh-insulin stimulation alone is sufficient to promote meiotic G2–M1 transition in *C. batrachus* post-vitellogenic ovarian oocytes in a dose- and duration-dependent manner. The highest effective dose of rh-insulin (5 μM) triggering meiotic maturation is comparable with similar earlier studies in other teleosts (Mukherjee *et al.*, 2006; Chourasia & Joy, 2008; Das *et al.*, 2013). Furthermore, immunoblot analysis reveal that insulin induction promotes *de novo* synthesis of cyclin B and histone H1 kinase activation in catfish follicle-enclosed oocytes *in vitro* and the data are comparable with stimulation with 17α,20β-DHP, the natural MIS in this species. The present data are in agreement with similar earlier observations on maturational events in *Xenopus* (El-Etr *et al.*, 1979; Maller & Koontz, 1981; Hainaut *et al.*,

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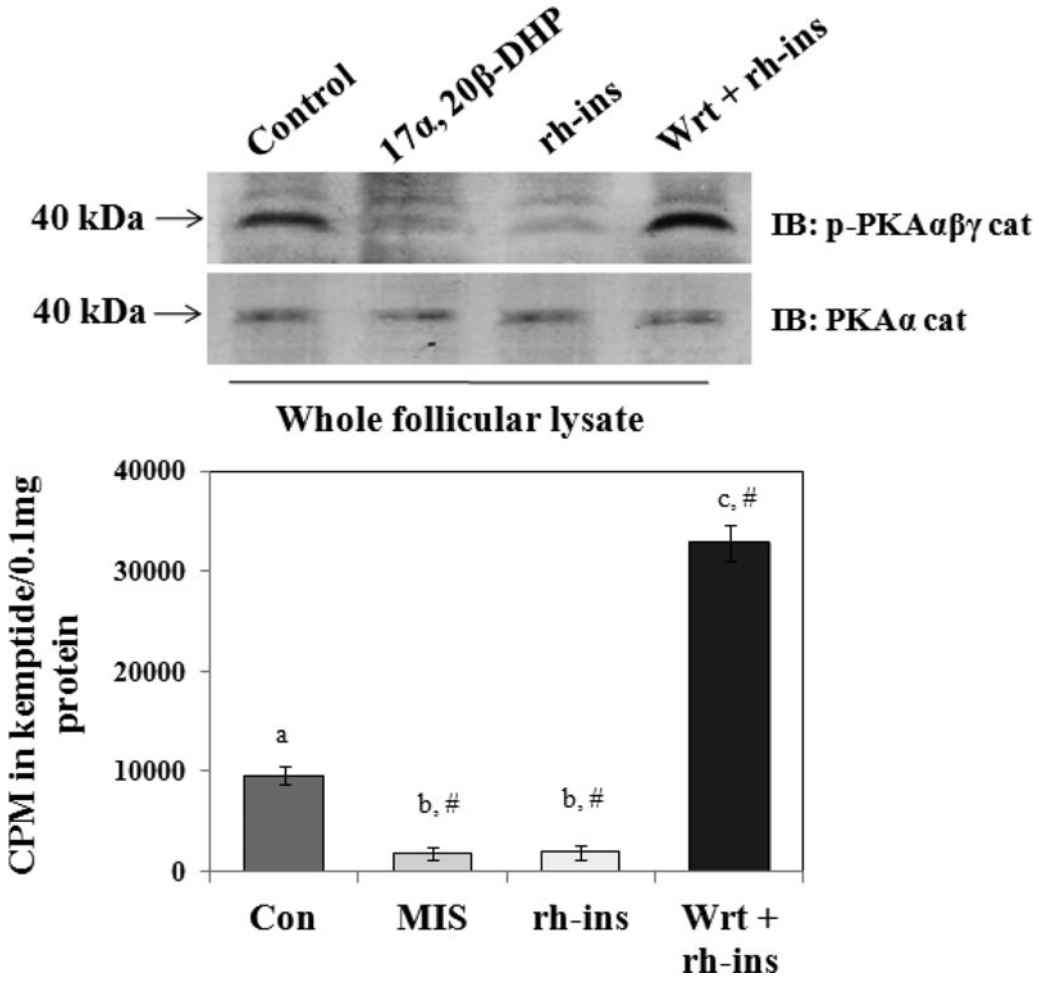

Figure 5 Effect of rh-insulin stimulation on endogenous PKA activity in follicle-enclosed oocytes *in vitro.* Follicular lysate of indicated treatment groups were analysed by SDS-PAGE and subjected to immunoblot analysis using anti-p-PKAα/β/γ cat antibody that specifically recognize the phosphorylated (active) form of the protein. PKAα c immunoblot (total protein) served as internal loading control (upper panel). Data are representative of at least three observations with identical results. Follicles from indicated treatment groups were harvested and PKA activity was measured in follicular lysates, MIS-treated sample served as the positive control (lower panel). Values are mean ± standard error of the mean (SEM) of three independent experiments and data were analyzed by one-way analysis of variance (ANOVA) ($P < 0.001$) and Duncan's Multiple Range Test ($P < 0.05$). [a–c]Groups with same lowercase letter above the bars are not significantly different and those with different letters differ significantly. #$P < 0.001$ compared with control group.

1991), common carp (Mukherjee *et al.*, 2006) and rohu (*Labeo rohita*) (Dasgupta *et al.*, 2001) oocytes stimulated either by IGF1, insulin or both. Although insulin has either little or no activity on oocyte maturation in goldfish and striped bass (Lessman, 1985; Weber & Sullivan, 2000), earlier evidence suggests that it can either act synergistically or can potentiate the effect of maturational steroid(s) in *Xenopus* (Hirai *et al.*, 1983) and fish (Kagawa *et al.*, 1994) oocytes indicating that species-specific differences may exist in insulin's action on resumption of meiotic maturation.

Although insulin receptors are widely distributed throughout all ovarian compartments including granulosa, theca and stromal tissues in human and in other mammalian models (Poretsky *et al.*, 1999) and high-affinity insulin binding sites have been reported earlier in amphibian oocytes (Maller & Koontz, 1981; Hainaut *et al.*, 1991) and in carp ovaries (Gutiérrez *et al.*, 1993; Maestro *et al.*, 1997), the possibility that at higher concentration insulin induction of oocyte maturation *in vitro* via IGF receptors, at least in part (the specificity spill over phenomenon), cannot be ruled out (Reinecke, 2010; Das *et al.*, 2013). The present data demonstrate that stimulation with IGF1 could induce GVBD response in a dose-dependent manner; however, rh-insulin at equivalent concentration are either without effect (1 and 10 nM) or could marginally promote (50 nM) G2–M1 transition suggesting rh-insulin at a lower dose is less effective than IGF1. However, presently no data are available on relative

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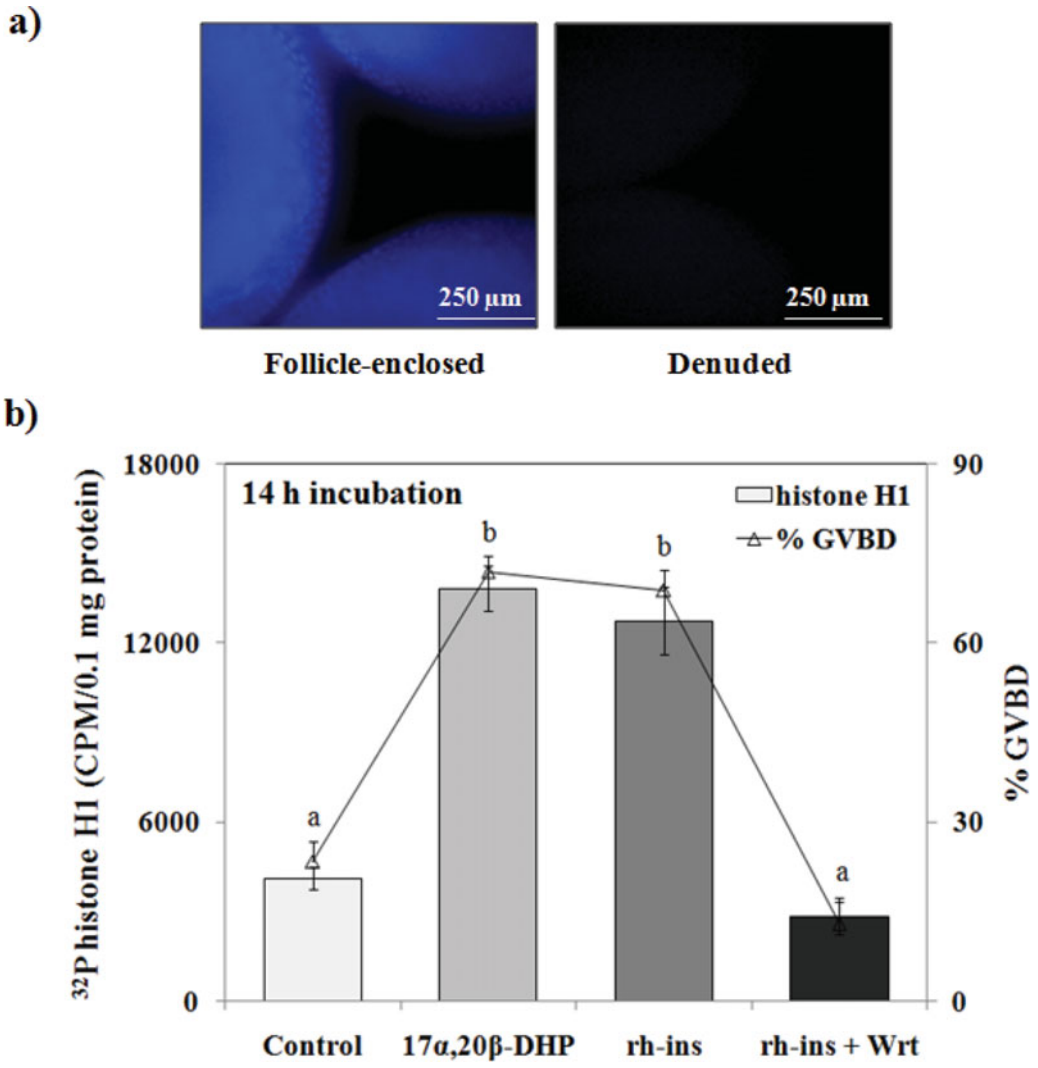

Figure 6 Comparison of surface images of fully grown ovarian follicles (left) and denuded oocytes (right) stained with DAPI under an upright-fluorescent microscope (a). Denuded oocytes were incubated *in vitro* either without (Control) or with rh-insulin (5 µM) in presence of PI3K inhibitor, wortmannin (10 µM) or not for 14 h and scored for GVBD (b). 17α,20β-DHP (1 µg/ml)-stimulated group served as positive control. Oocyte lysates were also assayed for histone H1 kinase activation by scintillation counting. Values are mean ± standard error of the mean (SEM) of three independent experiments and data were analyzed by one-way analysis of variance (ANOVA) ($P < 0.001$) and Duncan's Multiple Range Test ($P < 0.05$). [a,b]Groups with same lowercase letter above the bars are not significantly different and those with different letters differ significantly.

abundance of either insulin or IGF receptors in catfish ovary or how much ligand is required to saturate their respective cognate receptors.

Activation of the PI3K/Akt signalling cascade is implicated as a key intermediate downstream to receptor tyrosine kinase activation. Present data demonstrate that incubation with specific PI3K inhibitor, wortmannin, could effectively reverse rh-insulin stimulation of histone H1 kinase activation, the marker for MPF activation suggesting the specificity of insulin action in catfish follicles. Previously, priming with specific PI3K inhibitors could abrogate insulin-/IGF-induced meiotic maturation in *Xenopus* and zebrafish oocytes (Andersen *et al.*, 1998; Das *et al.*, 2013). Moreover, involvement of PI3K has been shown in the MIS-induced GVBD response in starfish (Sadler & Ruderman, 1998), striped bass (Weber & Sullivan, 2001) and Atlantic croaker (Pace & Thomas, 2005) oocytes. Furthermore, in striped bass and in common carp, inhibition of PI3K has been shown to block MIS biosynthesis and could attenuate gonadotropin (hCG) as well as an insulin-/IGF1-induced GVBD response (Weber & Sullivan, 2001, Mukherjee *et al.*, 2006).

Available information indicates that transcriptional inhibition fails to block insulin-/IGF1-induced meiotic maturation in follicle-enclosed oocytes (Negatu *et al.*, 1998; Weber & Sullivan, 2000; Mukherjee *et al.*,

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2006). Results of the present study reveal that while priming with ActD attenuates, pre-incubation with Chx abrogates insulin action on meiotic G2–M1 transition – suggesting that species-specific differences may exist. Furthermore, hCG stimulation fails to promote a GVBD response in either Chx or ActD pre-incubated follicles, indicating that rh-insulin and hCG may adopt disparate signalling strategies during resumption of meiosis in catfish follicle-enclosed oocytes. Transcriptional activation as well as *de novo* synthesis of new proteins during gonadotropin-induced re-initiation of meiotic maturation have been reported earlier (Nagahama *et al.*, 1985; Kagawa *et al.*, 1994; Negatu *et al.*, 1998; Picha *et al.*, 2012). Furthermore, priming with IGF-II has been shown to up-regulate expression and synthesis of the membrane progestin receptor (mPRα) and resumption of meiosis in southern flounder (Picha *et al.*, 2012).

Previously, priming with the 3β-HSD antagonist cyanoketone has been shown to attenuate either the salmon gonadotropin, SG-G100- or FK-induced GVBD response in *C. batrachus* intact follicles, indicating biosynthesis of steroid is a pre-requisite for lifting prophase I arrest and resumption of meiosis in this species (Haider & Chaube, 1996; Haider & Baqri, 2002). The present data demonstrate that, compared with rh-insulin, priming with hCG promotes a sharp increase in 17α,20β-DHP secreted in the culture medium and pre-incubation with either trilostane or DL-aminoglutethimide could abrogate MIS synthesis. While hCG stimulation fails to induce GVBD response in trilostane/DL-aminoglutethimide pre-incubated oocytes, rh-insulin could significantly enhance GVBD response over vehicle-treated control under similar conditions. Earlier evidence suggests that mechanisms underlying insulin/IGFs regulation of ovarian function in teleosts involve ovarian steroid production (Srivastava & Van Der Kraak, 1994; Patiño & Kagawa, 1999; Weber & Sullivan, 2005; Weber *et al.*, 2007; Chourasia & Joy, 2008). Although priming with DL-aminoglutethimide/cyanoketone fails to attenuate IGF1-induced GVBD response (Kagawa *et al.*, 1994; Negatu *et al.*, 1998; Weber & Sullivan, 2000; Mukherjee *et al.*, 2006), trilostane and ActD priming could successfully inhibit hCG-induced GVBD and follicular steroidogenesis in striped bass (King *et al.*, 1994).

Furthermore, IGF1 at the dose level tested here could promote meiosis resumption when the level of MIS secreted in the culture medium remains below the detection limit (data not shown), suggesting that, compared with hCG, the rh-insulin-/IGF1-induced GVBD response may have less dependence on follicular steroidogenesis to promote meiotic G2–M1 transition. Clearly the possibility of functional variation as well as differences in mode of action between insulin and gonadotropin (hCG) in the catfish ovary require further investigation in future.

In teleosts, as in other vertebrates studied, down-regulation of cAMP/PKA is considered a pre-requisite to induce final oocyte maturation (Haider & Baqri, 2000a, 2002; Conti *et al.*, 2002; Mishra & Joy, 2006; Khan & Maitra, 2013). While high cAMP/PKA levels act as a positive regulator to promote follicular steroidogenesis under the influence of pituitary gonadotropins (Leung & Steele, 1992; Nagahama, 1997), high intra-oocyte cAMP levels (using gap-junction complexes that increases oocyte cAMP pool or membrane estrogen receptor mediated production of cAMP) maintain prophase I arrest in fish oocytes (Nagahama & Yamashita, 2008; Peyton & Thomas, 2011). The present data demonstrate that priming with either FK or PDE inhibitor IBMX or cell-permeable dbcAMP could effectively reverse the insulin action on meiotic G2–M1 transition, indicating that forced elevation of cAMP (considered as pro-steroidogenesis signal) may have a negative influence on insulin-induced GVBD. Recently we have shown that high cAMP levels could successfully abrogate the insulin-induced GVBD response as well as the mitogen-activated protein kinase kinase/mitogen-activated protein kinase (MEK/MAPK) signalling axis in zebrafish oocytes divested of the surrounding theca–granulosa layer (Maitra *et al.*, 2014). Moreover, data from the present study demonstrate that either adenylyl cyclase inhibitor, SQ22536 or PKA inhibitor H89, sufficient to trigger GVBD response, could further potentiate rh-insulin action on meiotic G2–M1 transition *in vitro*, indicating that down-regulation of cAMP/PKA may have a positive influence on insulin action in folliculated oocytes.

Considering the response of insulin-treated follicles to cAMP/PKA modulators, we assumed the involvement of PKA in insulin-stimulated GVBD response. Earlier evidence suggests that a high cAMP level maintains PKA in an active dissociated state, allowing phosphorylation of PKAc on its activation loop (Moore *et al.*, 2002) and down-regulation of PKA activity corresponds well to the PKAc dephosphorylation in perch oocytes (Khan & Maitra, 2013). Results of the present study reveal that the high level of p-PKAc (active) in control oocytes undergoes significant dephosphorylation due to stimulation with rh-insulin *in vitro* and the data corroborates well with the MIS-treated group, providing indirect evidence for PKA inactivation. Moreover, insulin induction attenuates the endogenous PKA activity significantly in maturing oocytes and, most interestingly, PI3K inhibition reverses insulin action on PKA inhibition. This finding suggests that insulin possibly works by down-regulation of PKA activity in a manner sensitive to PI3K-mediated signalling pathway. Available

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information from *Xenopus* and mammals reveal that insulin/IGFs stimulation of PI3K activation triggers phosphorylation of Akt which potentially activates oocyte-specific PDE3 (Andersen *et al.*, 1998, 2003; Conti *et al.*, 2002; Han *et al.*, 2006). On the basis of the above findings it may reasonably be concluded that PI3K inhibition possibly leads to the accumulation of cAMP promoting PKA activation and inhibition of insulin-induced meiotic maturation. Although, hCG-induction of elevated cAMP and PKA activation has been reported earlier in goldfish prematurational fully grown follicles (Srivastava & Van Der Kraak, 1994), evidence in favour of insulin-induced down-regulation of PKA activity favouring maturational response is being reported for the first time in a teleost species.

Moreover, the present data demonstrate that denuded oocytes undergo meiotic G2–M1 transition due to stimulation with either 17α,20β-DHP or rh-insulin and priming with wortmannin could successfully attenuate rh-insulin action. Previous studies in zebrafish and common carp have shown that insulin/IGF possibly act on the oocyte membrane and trigger a GVBD response (Mukherjee *et al.*, 2006; Das *et al.*, 2013). Although the mechanism of insulin action on the oocyte membrane is not fully understood, recently we have reported that PDE3 may act as a downstream target of the PI3K/Akt signalling cascade during rh-insulin stimulation of the GVBD response in zebrafish oocytes (Das *et al.*, 2013), indicating a proximal interaction between the RTK-mediated and cAMP-dependent signalling cascades. Taken together, the results of the present study demonstrate that insulin induction of the GVBD response requires PKA inhibition, synthesis of cyclin B and histone H1 kinase activation in a manner sensitive to PI3K activation. Most interestingly, high cAMP levels attenuate, while PKA inhibition potentiates the insulin-mediated maturational response in catfish oocytes *in vitro*.

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Conflict of interest

The authors declare no conflicts of interests.

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