Relative importance of phosphatidylinositol-3 kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK3/1) signaling during maturational steroid-induced meiotic G2–M1 transition in zebrafish oocytes

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Summary

Participation and relative importance of phosphatidylinositol-3 kinase (PI3K) and mitogen-activated protein kinase (MAPK) signalling, either alone or in combination, have been investigated during 17α,20β-dihydroxy-4-pregnen-3-one (DHP)-induced meiotic G2−M1 transition in denuded zebrafish oocyte. Results demonstrate that concomitant with rapid phosphorylation (activation) of Akt (Ser473) and MAPK (ERK1/2) at as early as 15 min of incubation, DHP stimulation promotes enhanced an GVBD response and histone H1 kinase activation between 1 and 5 h in full-grown oocytes *in vitro*. While p-Akt reaches its peak at 60 to 90 min and undergoes downregulation to the basal level by 240 min, ERK1/2 phosphorylation (activation) increases gradually until 120 min and remains high thereafter. Although, priming with MEK1/2 inhibitor U0126 is without effect, PI3K inhibitors, wortmannin or LY294002, delay the GVBD response significantly ($P < 0.001$) until 3 h but not at 5 h of incubation. Interestingly, blocking PI3K and MEK function together could abrogate steroid-induced oocyte maturation at all time points tested. While DHP stimulation promotes phospho-PKA catalytic (p-PKAc) dephosphorylation (inactivation) between 30–120 min of incubation, simultaneous inhibition of PI3K and MEK1/2 kinases abrogates DHP action. Conversely, elevated intra-oocyte cAMP, through priming with either adenylyl cyclase (AC) activator forskolin (FK) or dibutyryl cAMP (db-cAMP), abrogates steroid-induced Akt and ERK1/2 phosphorylation. Taken together, these results suggest that DHP-induced Akt and ERK activation precedes the onset of meiosis (GVBD response) in a cAMP-sensitive manner and PI3K/Akt and MEK/MAPK pathways together have a pivotal influence in the downregulation of PKA and resumption of meiotic maturation in zebrafish oocytes *in vitro*.

Keywords: GVBD, Oocyte, Meiosis, PKA, Zebrafish

Introduction

In teleosts, as in other lower vertebrates, before the onset of final oocyte maturation, a surge in pituitary gonadotropin (LH) leads to synthesis and secretion of maturation-inducing steroid (MIS) in the ovary. 17α,20β-Dihydroxy-4-pregnen-3-one (DHP) is the principal MIS in salmonids, cyprinids and many other teleost orders, but 17α,20β,21-trihydroxy-4-pregnen-3-one (20β-S) predominates in sciaenids and in marine perciform species (Nagahama, 1997). MIS action at the oocyte surface through membrane progestin receptor (mPR), a member of G-protein coupled receptor (GPCR), triggers the rapid activation of intra-oocyte signalling events as well as *de novo* synthesis of protein(s) from maternally stored mRNAs leading to the activation of maturation-promoting factor (MPF) (Zhu *et al.*, 2003). Active MPF promotes the progression from meiotic MI–MII through histone

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H1 kinase activation, chromosome condensation, spindle formation, dissolution of nuclear envelope (germinal vesicle breakdown; GVBD) and release of the first polar body that ultimately converge to produce functional female gametes (Nagahama & Yamashita, 2008). Although the nature of MIS, its cell surface-initiated action and its role in MPF activation is relatively ubiquitous, the molecular mechanisms underlying MIS action, more specifically recruitment of various intra-oocyte signalling events, varies considerably in fish and amphibian oocytes (Kajiura-Kobayashi *et al.*, 2000; Nagahama & Yamashita, 2008).

High intra-oocyte cAMP congruent with elevated cAMP-dependent protein kinase (PKA) activity is widely held responsible for the maintenance of prophase I arrest in teleosts (Das *et al.*, 2017 for review). Studies in Atlantic croaker, zebrafish and goldfish indicated that MIS action at the oocyte surface involved the participation of pertussis toxin-sensitive inhibitory G-protein ($G\alpha_i$), the downregulation of adenylyl cyclase (AC) activity and a decrease in intra-oocyte cAMP levels (Yoshikuni & Nagahama, 1994; Zhu *et al.*, 2003; Pace & Thomas, 2005a, Thomas, 2012; Das *et al.*, 2017). Insulin stimulation of meiotic maturation in zebrafish oocytes involves the downregulation of PKA activity, possibly through the PI3K/Akt/PDE3 pathway and high cAMP/PKA could successfully block meiosis resumption due to receptor tyrosine kinase (RTK) activation (Das *et al.*, 2013; Maitra *et al.*, 2014). Conversely, in Atlantic croaker, 20β-S (the natural MIS)-induced oocyte maturation involves a significant decrease in cAMP production, however PKA inhibitors failed to induce meiosis (Pace & Thomas, 2005b). Collectively, these data indicated that species-specific variations exist in upstream signalling events that participate in PKA inhibition leading to meiotic G2−M1 transition in teleost oocytes. Although the involvement of high AC activity in maintaining prophase arrest is ubiquitous in fish oocytes, it is not yet clearly understood if the high level of cAMP/PKA is maintained solely by the AC pathway or some other signalling is also involved in this process.

In addition to AC-mediated signalling cascades, MIS-induced oocyte maturation events in starfish (Okumura *et al.*, 2002), Atlantic croaker (Pace & Thomas, 2005b) and Indian shad (Pramanick *et al.*, 2014) have been shown to depend on cAMP-independent phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB, also known as Akt). Furthermore, the activation of the PI3K/Akt pathway is essential for insulin-/IGF1-stimulated oocyte maturation in *Xenopus* and zebrafish (Andersen *et al.*, 2003; Das *et al.*, 2013). While full activation of Akt requires dual phosphorylation on Thr308 and Ser473 by phosphatidylinositol-dependent kinase (PDK1) and the rictor−mTOR

complex respectively (for review see Manning & Cantley, 2007), membrane recruitment of Akt and PDK1, both containing pleckstrin homology domain, is mediated by phosphatidylinositol 3-phosphate (PIP3), a phospholipid second messenger produced by the action of PI3K. The family of PI3Ks is comprised of 14 enzymes that are separated into three classes, out of which class IB PI3Ks are activated by binding to free βγ subunits of heterotrimeric G-protein (Stephens *et al.*, 1997; Murga *et al.*, 1998; Manning & Cantley, 2007).

Mitogen-activated protein kinases (MAPK3/1, or extracellular signal-regulated kinases, ERK1/2) are rapidly activated in response to various growth factors and hormones including steroids. This signalling cascade family is regulated by upstream kinases including Mos and/or Raf and MEK (Pearson *et al.*, 2001). While Ras-dependent activation of Raf is non-genomic, several earlier studies have demonstrated that Mos/MAPK is activated almost universally during meiotic G2−M1 transition (Ferrell, 1999; Liang *et al.*, 2007). Further, GPCR-mediated ERK phosphorylation can be regulated by Gβγ via the recruitment of small G-protein Ras to the plasma membrane leading to ERK activation through the Raf/MEK/ERK pathway (Crespo *et al.*, 1994). Although its role as a component of cytostatic factor to suppress DNA replication between meiosis I and II is ubiquitous; the need for MAPK activation for GVBD is uncertain and species-specific in fish and amphibian models (Ferrell, 1999; Kajiura-Kobayashi *et al.*, 2000; Liang *et al.*, 2007, Khan & Maitra, 2013).

Zebrafish provides an excellent model for the study of ovarian growth, development, steroidogenesis, oocyte maturation and ovulation (Ge, 2005; Das *et al.*, 2016a, b). DHP has been shown previously to be the principal MIS in this species (Selman *et al.*, 1994; Kondo *et al.*, 1997). Significant progress has been made towards the identification and characterization of the membrane MIS receptors (mPRα and mPRβ) in zebrafish (Zhu *et al.*, 2003; Hanna *et al.*, 2006; Hanna & Zhu, 2011). We have previously demonstrated the involvement of the PI3K/Akt signalling pathway in insulin-induced MPF activation in this species (Das *et al.*, 2013). In addition, the role of elevated cAMP has been shown to inhibit insulin action on the oocyte GVBD response (Maitra *et al.*, 2014). However, relatively less information is available on the participation and potential interaction between rapid non-genomic signalling events during meiotic G2−M1 transition under the influence of MIS in this species. Accordingly, the primary objective of the present investigation was to study the potential involvement of PI3K-Akt and MEK-MAPK cascades, either alone or in combination, in the regulation of PKA activity and GVBD response in DHP-treated zebrafish oocytes.

Materials and Methods

Animal collection and maintenance

Adult zebrafish (*Danio rerio*), purchased from local pet stores in and around Santiniketan (Lat. 23°41′30″N, Long. 87°30′47″E), India, were maintained in 60 litre glass aquaria, under a 28 ± 1°C and 14 h light: 10 h darkness cycle with lights turned on at 0600 h. Fish were fed thrice daily with live blood worms and were acclimatized to laboratory conditions for at least 7 days before their use in experiments (Das *et al*., 2013). All animal experiments were carried out following the guidelines of the Institutional Animal Ethics Committee of Visva-Bharati University and approved by the committee according to Indian law.

Chemicals and antibody

Rabbit polyclonal anti-Akt:sc-8312, anti-p-Akt (Ser 473):sc-7985-R, anti-ERK1:sc-93, anti-p-ERK1/2 (Thr 202/Tyr204):sc-16982, anti-PKAα cat:sc-903 and anti-p-PKAα/β/γ cat:sc-32968 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies used in the present study have been reported previously to cross-react specifically with zebrafish proteins (Das *et al*., 2013; Maitra *et al*., 2014). Unless otherwise specified, hormones, inhibitors, anti-Rabbit IgG and other reagents were from Sigma-Aldrich, India.

Oocyte preparation and *in vitro* culture

For all *in vitro* experiments, zebrafish were autopsied in the evening (2000 h) when a lower percentage of oocytes matures spontaneously in the absence of exogenous stimuli (Hanna & Zhu, 2011). Gravid females (*n* ~ 30–40) were anesthetized by brief cold shock, decapitated, ovaries excised aseptically and placed immediately in oxygenated zebrafish Ringer (116 mM NaCl, 2.9 mM KCl, 1.8 mM CaCl$_2$, 5 mM HEPES; pH 7.2) supplemented with penicillin (100 IU/ml) and streptomycin (100 mg/ml) (Kondo *et al*., 1997). This preparation was also used as incubation medium in subsequent *in vitro* experiments. The protocol for harvesting post-vitellogenic intact follicles, enzymatic removal of the follicular layer, selection of oocytes divested of surrounding follicular cells and with centrally placed germinal vesicle (GV) were according to our standard laboratory protocols and have been described earlier (Das *et al*., 2013, 2016a). In brief, ovarian follicles were incubated in Ca^{2+}-free zebrafish Ringer's solution containing 0.001% collagenase type-IA (1.25 U/ml) for 1 h at 20 ± 1°C with mild agitation. Follicular cells were removed by repeated (30×) gentle pipetting through a narrow pipette (1 mm diameter) at 10 min intervals during incubation. Oocytes that showed signs of damage or the presence of fibrous amorphous follicle cells (incomplete denudation) were discarded manually. Complete removal of the follicular cells was ascertained by the absence of DAPI-positive nuclei surrounding the denuded oocytes (Das *et al*., 2013), and were unresponsive to meiotic maturation due to hCG stimulation (Das *et al*., 2013). Healthy, denuded oocytes of desired sizes (mean diameter ~550–600 μm) were washed thoroughly with fresh zebrafish Ringer solution and were cultured in a 24-well tissue culture plate (50–60 oocytes/well) for the indicated time in hours and at 25 ± 1°C under gentle agitation. AC activator forskolin (FK, 5 μM) that specifically raised the intra-cellular cAMP level, PI3K inhibitor wortmannin (Wrt, 10 μM), LY294002 (LY, 25 μM), and MEK1/2 inhibitor U0126 (10 μM) were dissolved in DMSO. DHP was dissolved in ethanol. Cell-permeable, chemically stable cAMP analogue, dibutyryl (db) cAMP (1 mM) that increases intracellular cAMP level were dissolved in nuclease free water (Fermentas) just before the experiment. Inhibitors were added to the culture medium 2 h prior to DHP (5 nM) addition. The doses of DHP, LY294002, forskolin and db-cAMP used were as described earlier in this fish species (Das *et al*., 2013, 2016a; Maitra *et al*., 2014). Selection of the most effective dose of wortmannin and U0126 to block Akt and ERK1/2 phosphorylations as well as cross-specificity of each inhibitor were checked through immunoblot analysis. All chemicals were prepared at 1000-fold stock and oocytes in control wells received equivalent amount of solvent (1 μl/well) only. GVBD was monitored at indicated time intervals following immersion in clearing solution (ethanol: formaldehyde: acetic acid, 3: 6: 1 v/v) under an inverted microscope (Victory FL, Dewinter Optical Inc.) and processed for preparation of oocyte extract and immunoblot analysis.

Preparation of oocyte extract

Samples were harvested at appropriate time intervals, washed (3×) and homogenised in chilled oocyte extraction buffer containing 100 mM sodium β-glycerophosphate, 20 mM HEPES, 15 mM MgCl$_2$, 5 mM EGTA, 100 μM p-PMSF, 3 μg/ml leupeptin, 1 mM DTT and 1 μg/ml aprotinin; pH 7.5. Supernatant was separated by spinning the samples at 17,500 *g* for 20 min at 4°C and was either used immediately or stored at −86°C until further use. As activation of MAPK cascade potentially involves interaction with large scaffolding complexes (Raman *et al*., 2007), whole homogenates, supernatants and pellet fractions were prepared and tested for relative protein content (Methods S1).

Electrophoresis and immunoblot analysis

Oocyte lysates (µg protein; Lowry *et al.*, 1951) from indicated treatment groups were subjected to SDS-PAGE and immunoblot analysis according to our standard laboratory protocols as described earlier (Das *et al.*, 2013; Maitra *et al.*, 2014). In brief, 40 µg of oocyte lysates were mixed with 2× SDS sample buffer (125 mM Tris–HCl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.002% bromophenol blue) at a ratio of 1:1, boiled for 5 min, cooled at room temperature and then resolved in 15% SDS-PAGE. After the transfer of proteins, the Hybond-P PVDF membrane (GE Healthcare Biosciences, UK) were blocked in 5% BSA in TBST (50 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.6), overnight at 4°C followed by primary antibody (1:500 in blocking buffer) and alkaline phosphatase tagged anti-rabbit IgG (1:1000) for 4 and 2 h respectively at room temperature. Bands were developed by adding BCIP-NBT, recorded in Gel Doc apparatus (Bio-Rad) and imported into Adobe Photoshop. Band intensities were quantified by ImageJ software and expressed as arbitrary units (AU).

Histone H1 kinase assay

Histone H1 kinase activation, a reliable marker for p34cdc2 activation, was assayed as described earlier (Das *et al.*, 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, Govt. of India); 1 mM EGTA, 10 mM MgCl2; 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 phosphocellulose paper (Whatman, Brentford, UK), washed three times with 1% phosphoric acid, dried, and radioactivity was measured in a liquid scintillation counter (Perkin Elmer). For determination of histone H1 phosphorylation by autoradiography, the reaction was stopped by adding 40 µl of 2× SDS sample buffer. Samples were heated at 95°C for 5 min and then resolved on 15% SDS-PAGE, along with pre-stained molecular weight marker proteins (Fermentas). The band corresponding to histone H1 was gel excised and transferred to PVDF membrane and recorded in a Storm 860 phosphoimager. Data were analyzed using Quantity One image software (GE Healthcare Biosciences).

Data analysis

All values were mean ± standard error of the mean (SEM) of at least three independent observations using oocytes from different donor fish ($n \geq 5$). Data were analyzed by one-way analysis of variance (ANOVA), followed by Duncan's multiple range test for multiple group comparisons, a *P*-value < 0.05 was considered to be statistically significant.

Results

DHP stimulation of Akt and MAPK (ERK1/2) phosphorylation in maturing zebrafish oocyte

Kinetics of Akt and ERK1/2 phosphorylation was determined by immunoblot analysis using anti-p-Akt (Ser473) and anti-p-ERK1/2 (Thr202/Tyr204) antibody respectively that specifically recognize the phosphorylated (active) form of these proteins. Although total protein did not vary significantly, DHP stimulation of denuded zebrafish oocytes could promote rapid phosphorylation of Akt (Fig. 1*A*) and ERK1/2 (Fig. 1*B*) as early as 15 min of incubation *in vitro*. While p-Akt (Ser473) reached its peak between 60 to 90 min and came to the basal level by 120 min (Fig. 1*A*); p-ERK1/2 increased significantly ($P < 0.05$) at 2 h of DHP stimulation and remained high afterwards (until 4 h) (Fig. 1*B*). Moreover, the kinetics of histone H1 kinase activation, a reliable marker for p34cdc2 kinase activity (MPF activation), underwent a sharp increase between 3 to 5 h of steroid stimulation (Fig. 1*C*) and revealed a tight temporal relationship with elevated GVBD response (Fig. 1*C*).

Selection of doses and testing cross-reactivity for PI3K and MEK1/2 inhibitors and their effect on the GVBD response

As both Akt and ERK phosphorylation were detected as early as after 15 min of incubation, i.e. much earlier than the highest GVBD response at 5 h, the need for these pathways during meiosis resumption was investigated. Doses for wortmannin and U0126 were selected using western blot analysis for their ability to block Akt and ERK1/2 phosphorylations respectively. As shown in Fig. 2, although the total protein did not vary significantly, maximum inhibition of Akt and ERK1/2 phosphorylation was found with a 10 µM dose for both the inhibitors at the respective time points tested (Fig. 2*A*, *B*). Furthermore, the cross-specificity of the selected doses of the inhibitors was also checked. As shown in Fig. 2(*C*, *D*), no changes in band intensities were observed due to the selected doses of wortmannin (10 µM) and U0126 (10 µM) on DHP-induced p-ERK1/2 and p-Akt immunoblot respectively. Moreover, denuded oocytes were pre-incubated with two different PI3K inhibitors, either wortmannin (Wrt, 10 µM) or LY294002 (LY, 25 µM) as
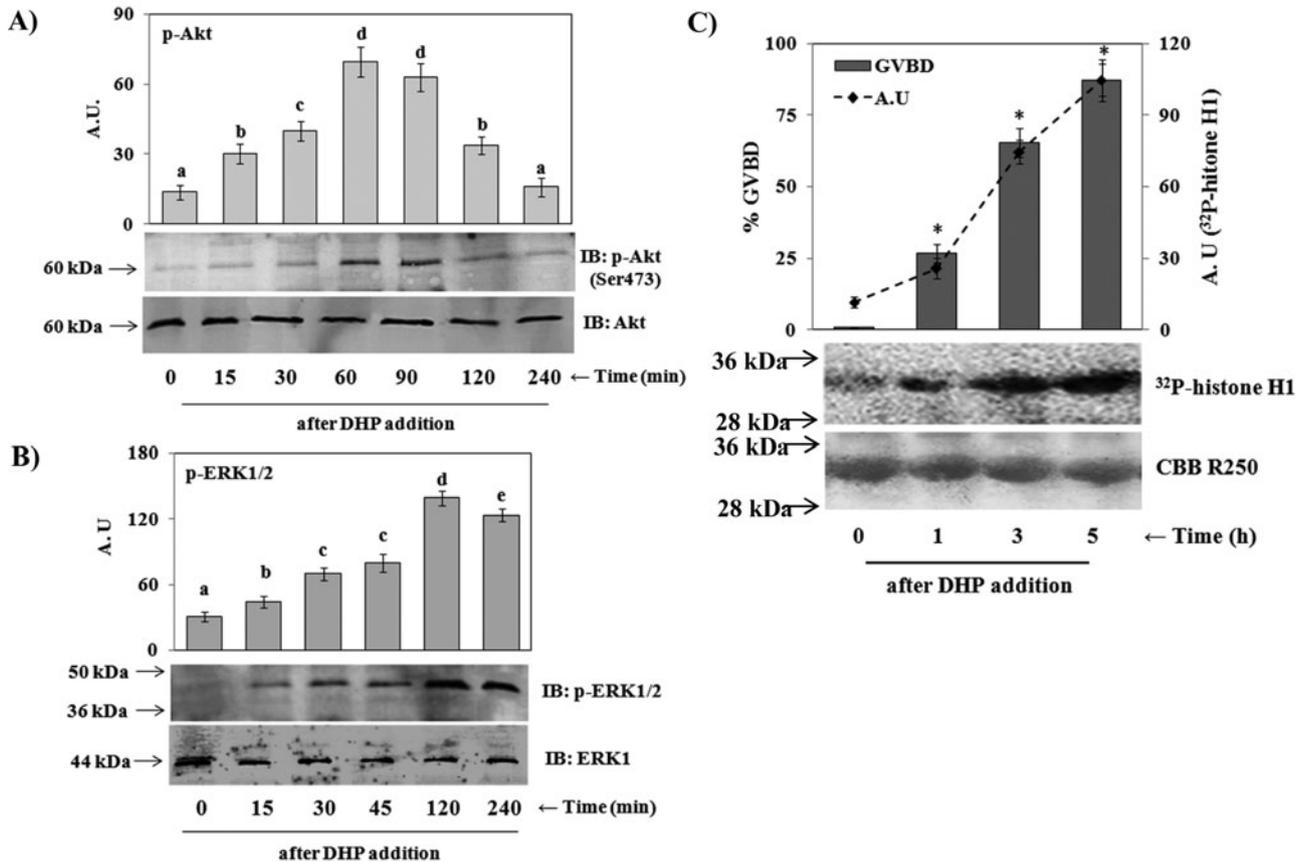
Figure 1 MIS stimulation of Akt and ERK1/2 phosphorylation for germinal vesicle breakdown (GVBD) and histone-H1 kinase activation in zebrafish oocyte *in vitro*. Denuded oocytes were treated with DHP (5 nM) and oocyte lysates from the indicated time intervals were analyzed by SDS-PAGE followed by immunoblot analysis (lower panels) using either anti-p-Akt (Ser473) (*A*) or anti-p-ERK1/2 antibody (*B*) that specifically recognizes the activated form of the protein or assayed for histone-H1 kinase activation, a reliable marker of MPF activation by autoradiography along with GVBD response (*C*). Anti-Akt or anti-ERK1 immunoblot, or Coomassie brilliant blue R-250 (CBB R250) staining served as the internal loading control. The corresponding densitometric analysis were also given in arbitrary units (AU, upper panels). The percentage of GVBD was scored microscopically. Values are mean ± standard error of the mean (SEM) of three independent experiments. Data were analyzed by one-way ANOVA ($P < 0.001$) and Duncan's multiple range test ($P < 0.05$). [a–e]Groups with the same lowercase letters above the bars are not significantly different and those with different letters differ significantly ($P < 0.05$). Immunoblot data are representative of at least three independent experiments that showed identical results.

well as pharmacological MEK inhibitor, U0126 (10 μM) for 2 h followed by DHP stimulation. While prior inhibition of PI3K/Akt signalling delayed the GVBD response markedly up to 3 h of incubation, MEK1/2 inhibitor could neither block nor delay MIS-induced oocyte maturation significantly (Fig. 2*E*). Conversely, the simultaneous inhibition of PI3K/Akt and MEK-MAPK pathways could successfully reverse DHP-stimulated meiotic maturation at all the time points tested (Fig. 2*E*).

Inhibition of PI3K and MEK1/2 reverse DHP-mediated inactivation of PKA

MIS stimulation of resumption of meiotic maturation involves the activation of inhibitory G-protein (Gα$_i$),

allowing a transient decrease in intra-oocyte cAMP levels in zebrafish and other fish models (Zhu *et al.*, 2003; Pace & Thomas, 2005a). Accordingly, in the present study, PKA involvement was examined during the DHP-induced GVBD response by immunoblot analysis using p-PKAα/β/γ cat (Thr198) antibody. Phosphorylation of the PKA catalytic subunit (PKAc) has been shown to correlate well with its catalytic activity, as PKAc is always phosphorylated on the activation loop allowing proper substrate recognition and catalysis (Moore *et al.*, 2002; Khan & Maitra, 2013; Maitra *et al.*, 2014). Time kinetics data revealed that although total protein (PKAc) did not vary significantly, DHP stimulation could attenuate p-PKAc significantly ($P < 0.05$) as early as after 30 min of incubation (lane 2, Fig. 3*A*) that progressed further to
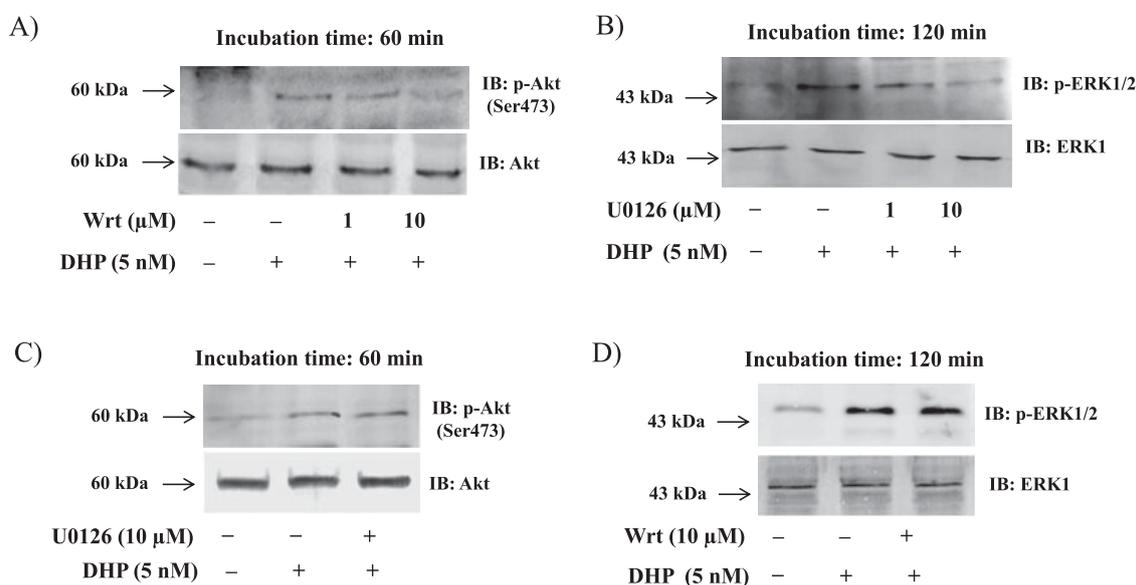
Figure 2 Effect of PI3K and MEK1/2 inhibition, either alone or in combination, on meiotic G2–M1 transition *in vitro*. To determine the highest effective doses of PI3K and MEK1/2 inhibitors and their cross-specificity, fully grown immature defolliculated oocytes were primed without (Con) or with increasing concentration of wortmannin (Wrt; 1, 10 μM) and U0126 (1, 10 μM), followed by DHP stimulation (5 nM). Oocyte lysates from indicated time intervals were subjected to immunoblot analysis using anti-p-Akt (Ser473) and anti-p-ERK1/2 antibodies. Akt and ERK1 immunoblots served as the endogenous loading control. Furthermore, denuded oocytes were incubated without (Con) or with either wortmannin (Wrt, 10 μM) or LY294002 (LY, 25 μM) or U0126 (10 μM) or Wrt/LY + U0126 for 2 h followed by DHP (5 nM) stimulation; GVBD was scored microscopically. Data are representative of at least five independent experiments from separate fish showing identical results. Values are the 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 ($P < 0.05$). Mean values with different superscripts, small letters, capital letters and numbers ([a,b, A–C, 1,2]) indicate significant differences among different time groups (i.e. 1 h, 3 h and 5 h respectively).

reach the basal level at 120 min of incubation *in vitro* (lane 4, Fig. 3A). Next, the potential involvement of PI3K/Akt and MEK/MAPK pathways, either alone or in combination, on PKA phosphorylation (activation) was examined. While high levels of p-PKAc, signifying elevated PKA activity in G2-arrested oocytes, underwent a sharp decrease in the DHP-treated group (lanes 1 and 2, Fig. 3B), priming with wortmannin could partially reverse DHP action on p-PKAc dephosphorylation (lane 3, Fig. 3B). In contrast, although pre-incubation with U0126 was largely without effect (lane 4, Fig. 3B), the simultaneous inhibition of MEK and PI3K function could abrogate DHP-mediated p-PKAc dephosphorylation (lane 5, Fig. 3B), indicating PI3K/Akt and MEK/MAPK signalling cascades, together but not alone, might have a pivotal influence in PKA deactivation in DHP-induced zebrafish oocytes allowing the resumption of meiotic maturation *in vitro*.

Effect of high intra-oocyte cAMP on DHP-induced Akt and ERK1/2 phosphorylations

To determine the possibility of any cross-talk between major signalling cascades, next we examined the effect of high cAMP on steroid-stimulated Akt and MAPK phosphorylation. Denuded oocytes were pre-incubated with AC activator forskolin and cell-permeable db-cAMP, both of which are known to elevate intra-cellular cAMP levels and have been used earlier in this fish species (Maitra *et al*., 2014). While total protein (Akt and ERK1) remained unchanged, elevated intra-oocyte cAMP levels could successfully attenuate DHP-stimulated Akt (Ser473) and ERK1/2 phosphorylation (Fig. 4A) as well as histone H1-kinase activity (Fig. 4B) suggesting, congruent with elevated p34cdc2 activity, that elevated Akt and MAPK phosphorylation (activation) levels are sensitive to high levels of cAMP.

Discussion

Data from the present study demonstrated that DHP stimulation of zebrafish oocytes *in vitro* induced phosphorylation of Akt (Ser473) as early as after 15 min incubation. While the kinetics of histone H1 kinase activation showed a tight temporal relationship with the GVBD response between 1–5 h of MIS
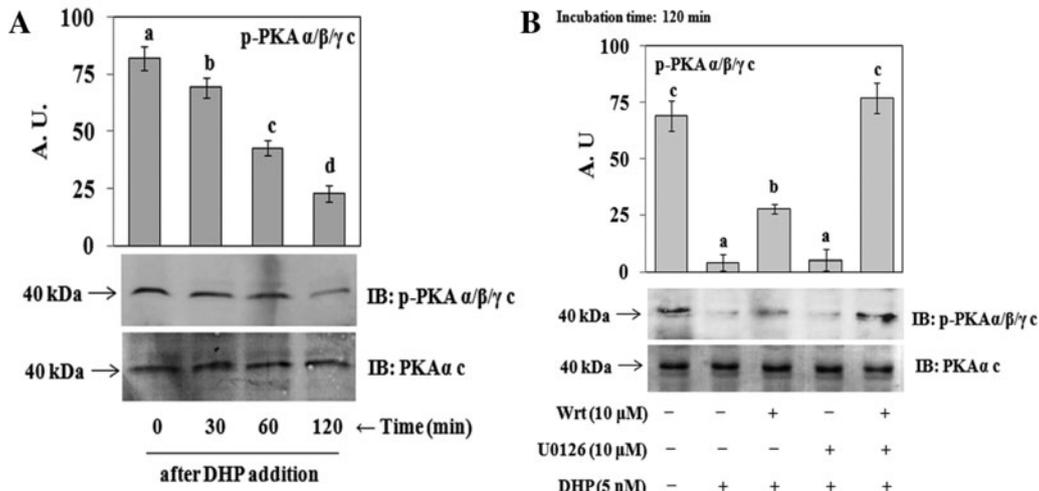
Figure 3 Kinetics of steroid-induced p-PKAc dephosphorylation (inhibition) (*A*) and effect of priming with PI3K and/or MEK1/2 inhibitors on DHP-induced p-PKAc dephosphorylation (*B*). Defolliculated oocytes were either stimulated with DHP (5 nM) alone or were primed (2 h) with wortmannin (Wrt, 10 μM) and U0126 (10 μM) followed by DHP (5 nM) stimulation for indicated time intervals. Oocyte lysates were analysed by SDS-PAGE and subjected to immunoblot analysis using anti-p-PKAα/β/γ cat antibody. Anti-PKAα c immunoblot served as internal loading control. Corresponding densitometric analysis were also given. Values are mean ± standard error of the mean (SEM) of three independent experiments. Data are analyzed by one-way ANOVA followed by Duncan's multiple range test. [a–d]Groups with same lowercase letters above the bars are not significantly different and those with different letters differ significantly ($P < 0.05$). Immunoblot data are representative of at least three independent experiments from separate fish showing identical results.

stimulation, p-Akt (Ser473) reached its peak much earlier, i.e. during 60 to 90 min of DHP stimulation *in vitro*. Previously, stimulation with MIS (20β-S) has been shown to promote phosphorylation of Akt in Atlantic croaker oocyte (Pace & Thomas, 2005b). In starfish oocyte, Akt is phosphorylated within a few minutes of 1-methyladenine addition; microinjection of constitutively active Akt mRNA could induce meiosis in the absence of 1-methyladenine, the natural MIS in this species (Okumura *et al.*, 2002). Moreover, in mouse cumulus-free oocytes, p-Akt has been detected at 20 min and Akt phosphorylation precedes onset of GVBD both *in vivo* and *in vitro* (Kalous *et al.*, 2006). The available information indicates that insulin/IGF1 stimulation of meiotic G2–M1 transition in zebrafish and *Xenopus* oocytes involves Akt activation (Andersen *et al.*, 2003; Das *et al.*, 2013). Although growth-factor-mediated Akt activation requires RTK activation, in oocytes MIS regulation of Akt phosphorylation (activation) may primarily involve the activation of GPCR (Sadler & Ruderman, 1998; Pace & Thomas, 2005b). Earlier evidence has established that rapid, non-genomic activation of Akt could be triggered through Gβγ subunits of heterotrimeric G-protein that might dissociate upon ligand binding to membrane GPCR followed by the activation of class 1B PI3K (Stephens *et al.*, 1997; Murga *et al.*, 1998).

Data from the present study demonstrated that DHP-induced meiotic maturation in zebrafish oocytes

involves the early activation of MAPK3/1 (ERK1/2), an event initiated within 15 min (i.e. much earlier than MPF activation), progresses up to 120 min and remains high thereafter. Rapid activation of ERK1/2 through a non-genomic pathway has been shown earlier in zebrafish mPRα/mPRβ transfected cells (Hanna *et al.*, 2006). Although the need for ERK activation prior to meiosis resumption in teleost is highly debated, available information indicates that ERK activation is almost universal during oocyte maturation (Ferrell, 1999). While it is activated before GVBD in equine, porcine and bovine oocytes, in rat and mouse ERK is activated after MPF activation and GVBD (Liang *et al.*, 2007) suggesting species-specific differences exist for the time kinetics of ERK activation during G2–M1 transition. In addition, progesterone stimulation induces the synthesis of maternally stored *mos* mRNA that activate MEK/ERK pathway and the overexpression of Mos or constitutively active ERK2 is enough to resume meiosis even in the absence of steroid stimulation in *Xenopus* (Sagata *et al.*, 1989; Posada *et al.*, 1993).

The present results show that pharmacological inhibition of PI3K/Akt pathway delays the DHP-induced (5 nM) GVBD response in zebrafish oocytes for the first 4 h of incubation and are in agreement with our earlier observation with low level of DHP (3 nM) stimulation in this species (Das *et al.*, 2016a). Conversely, PI3K/Akt activation is apparently
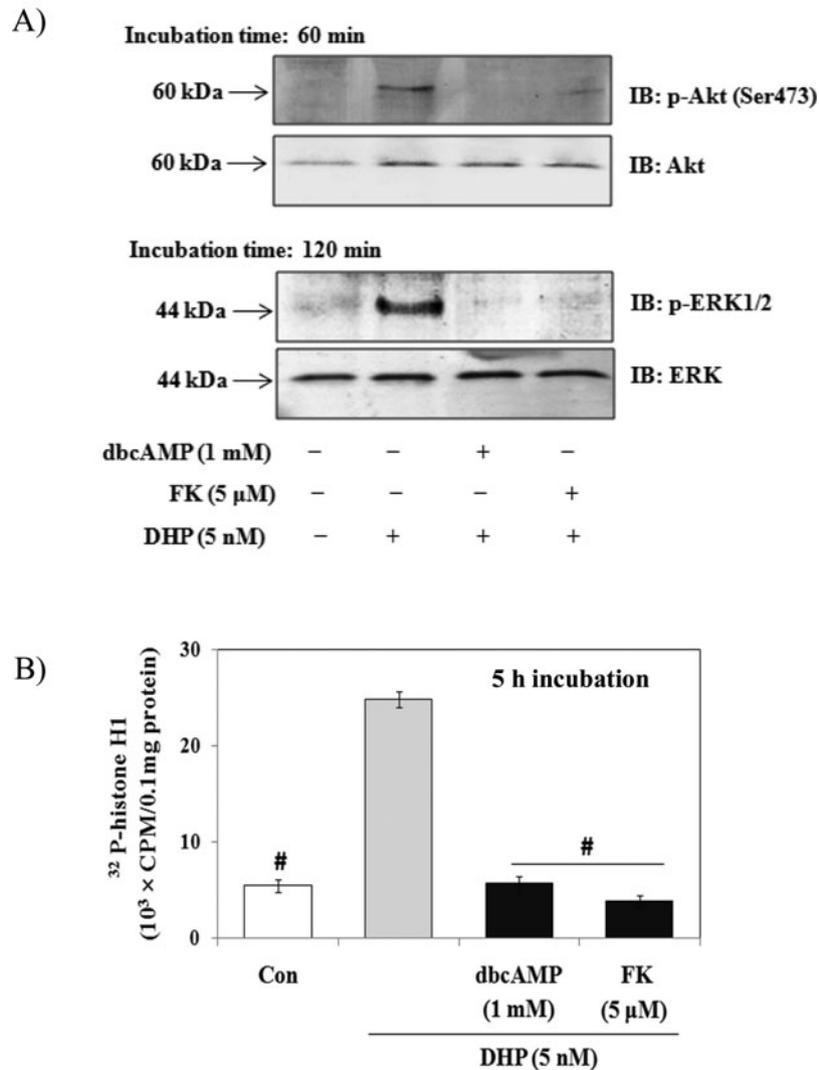
Figure 4 Effect of high cAMP on Akt and ERK1/2 phosphorylation (*A*) and histone H1-kinase activation (*B*). Defolliculated oocytes were either stimulated with DHP (5 nM) alone or were pre-incubated (2 h) with forskolin or db-cAMP followed by steroid stimulation. Oocyte lysates from various treatment groups were subjected to immunoblot analysis using anti-p-Akt (Ser473) or anti-p-ERK1/2 antibodies and assayed for histone H1 kinase activation by scintillation counting. Anti-Akt and anti-ERK1 (total protein) immunoblot served as internal loading control. Values are mean ± standard error of the mean (SEM) of three independent experiments. Data are analyzed by one-way ANOVA followed by Duncan's multiple range test. # $P <$ 0.001, compared with DHP-stimulated group. Immunoblot data are representative of at least three independent experiments from separate fish showing identical results.

indispensable for insulin-induced meiotic maturation in zebrafish, in which PDE3 might be a downstream target (Das *et al.*, 2013). These observations suggest that although it is functionally important, activation of PI3K/Akt might have a differential role during growth factor-induced and steroid-induced meiosis resumption (Das *et al.*, 2016a). However, the expression of a dominant negative PI3K (p85deltaSH2N) or overexpression of PI3K to inhibit or promote zebrafish oocyte maturation either in the presence or absence of MIS respectively, could provide strong evidence for an essential role for PI3K in the regulation of meiosis resumption in this species in the future. Interestingly, the present data conform with earlier reports in *Xenopus*, in which PI3K inhibition either delays (Bagowski *et al.*, 2001) or is without effect on progesterone but not insulin/IGF1-stimulated meiotic maturation, suggesting that PI3K-mediated signalling acts as an auxiliary pathway for progesterone action (Andersen *et al.*, 2003, Mood *et al.*, 2004).

Furthermore, present data demonstrate that priming with MEK1/2 inhibitor, U0126 alone could neither block nor delay DHP-induced GVBD response in zebrafish oocytes. Earlier evidence has established

that, in zebrafish oocytes, MEK inhibition *in vitro* could suppress insulin-stimulated meiotic maturation significantly, but not completely, and ERK1/2 activation by okadaic acid promotes the GVBD response suboptimally (Maitra *et al.*, 2014). Although ERK activation is neither necessary nor sufficient for initiating meiosis in goldfish and Atlantic croaker oocytes (Kajiura-Kobayashi *et al.*, 2000; Pace & Thomas, 2005b), recent studies in perch oocyte have suggested that there is an involvement of ERK1/2 activation in PKA-inhibition-induced oocyte maturation (Khan & Maitra, 2013). Furthermore, MEK inhibitor PD098059 inhibits follicular fluid-meiosis-activating-sterol (FF-MAS)-induced GVBD but not spontaneous oocyte maturation in mouse (Faerge *et al.*, 2001).

The present results show that pharmacological inhibition of MEK1/2 or PI3K/Akt together, but not alone, could successfully abrogate DHP-induced oocyte maturation in zebrafish oocytes *in vitro*. These data are in agreement with the earlier observation in *Xenopus* oocytes, in which attenuation of Mos/MEK function in addition to abrogating PI3K activity could effectively block the GVBD response induced by either high or low concentrations of progesterone. However, inhibition of any one pathway alone fails (Mood *et al.*, 2004). Recently, we have reported that the PI3K/Akt pathway is indispensable for insulin-induced meiotic maturation in zebrafish, in which PDE3 might be a downstream target (Das *et al.*, 2013). Inhibition of the PI3K/Akt pathway has been shown to abrogate 1-methyladenine-induced resumption of meiosis in starfish (Sadler & Ruderman, 1998), 20β-S-induced GVBD response in Atlantic croaker and striped bass (Weber & Sullivan, 2001; Pace & Thomas, 2005b), and DHP-induced oocyte maturation in Indian shad (Pramanick *et al.*, 2014). Based on the above data it is reasonable to conclude that, while activation of either PI3K/Akt and MEK/MAPK signalling pathway may act in parallel to rescue MIS-induced GVBD response, simultaneous inhibition of these pathways might have a pivotal influence and may restrict the withdrawal of G2 (prophase I) arrest in zebrafish oocytes.

The available information indicates that high AMP level congruent with elevated PKA activity helps to maintain meiotic prophase I arrest in vertebrate oocytes and release from meiotic arrest accompanies AC inhibition, lowering of cAMP levels, and PKA inhibition (Conti *et al.*, 2002; Nagahama & Yamashita, 2008; Das *et al.*, 2017). In *Xenopus* oocytes, during progesterone-stimulated oocyte maturation, the exact timing and extent of cAMP decrease and the necessity of PKA downregulation are still unclear and are characterised by conflicting reports (Daar *et al.*, 1993; Schmitt & Nebreda, 2002; Eyers *et al.*, 2005; Nader *et al.*, 2016). However, in zebrafish oocytes and

also in zebrafish mPRα/β-transfected somatic cells, DHP stimulation promotes inhibitory G-protein (Gαi) allowing the downregulation of AC activity and a sharp fall in cAMP levels (Zhu *et al.*, 2003; Hanna *et al.*, 2006; Thomas, 2012). Data from the present study demonstrate that stimulation of zebrafish oocytes with DHP downregulates PKA activity (p-PKAc dephosphorylation) within 30 min to 120 min. Previously, we have shown that inhibition of either AC or PKA by pharmacological inhibitors alone is sufficient to promote a GVBD response even without hormonal stimulation in this species (Maitra *et al.*, 2014; Das *et al.*, 2016a). Furthermore, PKA inhibition alone has been found to remove meiotic arrest independent of MIS stimulation in various other teleosts (Haider & Baqri, 2002; Khan & Maitra, 2013; Hajra *et al.*, 2016). Taken together these data indicate that downregulation of cAMP/PKA signalling in teleost oocytes plays a critical role in the process of MPF activation and the GVBD response.

In a recent study in *Xenopus*, it has been demonstrated that while lowering endogenous cAMP levels failed to induce spontaneous maturation, meiotic arrest could be released even in the presence of high levels of cAMP and resumption of meiosis is apparently insensitive to PKA inhibition (Nader *et al.*, 2016). Earlier we have demonstrated that forced elevation of intra-oocyte cAMP prevents ERK1/2 phosphorylation as well as a GVBD response in zebrafish oocytes (Maitra *et al.*, 2014; Das *et al.*, 2016a). The present study shows that DHP downregulates PKA activation and pharmacological inhibition of PI3K and MEK function attenuates DHP-induced oocyte maturation. Therefore, it was important to check whether PI3K and MEK inhibitors could potentially interfere with MIS-induced PKA inhibition (dephosphorylation). The present results revealed that although inhibition of MEK1/2 had been apparently without effect, blocking of PI3K could significantly reverse MIS action on p-PKAc dephosphorylation. Moreover, simultaneous inhibition of MEK-dependent and PI3K-dependent signalling could attenuate DHP-mediated p-PKAc dephosphorylation, suggesting that, unlike *Xenopus*, the downregulation of PKA activity is possibly an obligatory function during DHP-induced meiosis resumption in zebrafish oocytes. The involvement of the PI3K/Akt/PDE3 cascade in the downregulation of intra-oocyte cAMP level concomitant with PKA inactivation has been reported previously in fish and amphibian oocytes (Andersen *et al.*, 2003; Pace and Thomas, 2005b; Das *et al.*, 2013; Maitra *et al.*, 2014). Moreover, the inhibition of PI3K has been shown to promote PKA activation that in turn successfully attenuates insulin-induced meiotic G2-M1 transition in catfish follicle enclosed oocytes *in vitro* (Hajra *et al.*, 2016). Furthermore, studies in somatic cancer cells
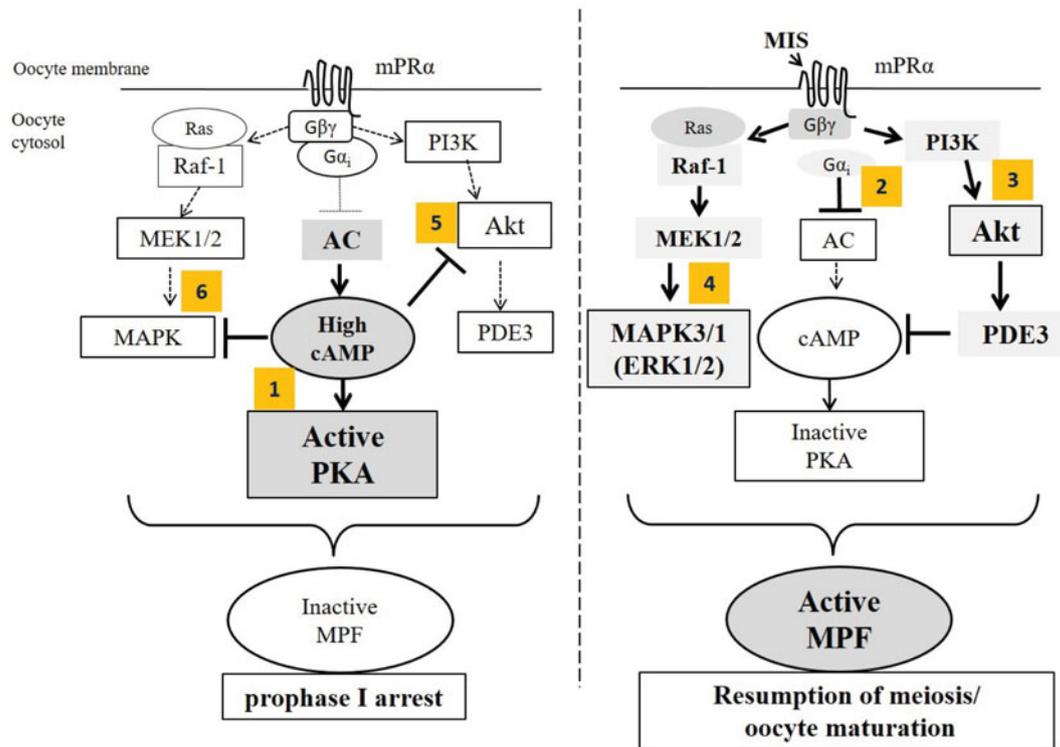
Figure 5 Proposed model for rapid activation of intra-oocyte signalling cascades in maturational steroid (DHP)-stimulated zebrafish oocytes and cross-talk between cAMP-mediated and PI3K/Akt- and MEK/MAPK-dependent signalling events. It is now well accepted that in the absence of MIS (left panel), high intra-oocyte cAMP/PKA inhibits MPF activation and resumption of meiosis (1). Conversely, it is documented that MIS action at the oocyte surface via its cognate G-protein coupled receptor (mPR) promotes activation of inhibitory G-protein (Gαi), down-regulation of AC activity and cAMP level to promote meiosis (2). Interestingly, present data demonstrate that DHP stimulation triggers rapid phosphorylation of both Akt and ERK1/2 (3, 4) and forced elevation of intra-oocyte cAMP (through priming with Ac activator FK or non-degradable db-cAMP) attenuates both Akt and ERK1/2 phosphorylation/activation (5, 6). Rapid phosphorylation of Akt and MEK/MAPK in MIS treated cells, possibly involves release of Gβγ and rapid activation of PI3Kγ and Ras/Raf/MEK cascades respectively. Specifically, present results demonstrate that attenuation of MEK in combination with PI3K inhibition, but not alone, could abrogate DHP action on cAMP-dependent protein kinase (PKA) dephosphorylation (inhibition), hitherto considered as the major upstream regulator of MPF activation and meiotic G2-M1 transition.

have revealed that endogenous ribosomal S6-kinase (p-RSK), the substrate of ERK1/2, when remaining inactive, interacts with the PKA regulatory subunit (RI) thereby decreasing the interaction between RI and PKAc and leading to PKA activation (Chaturvedi *et al.*, 2006; Gao & Patel, 2009). Conversely, upon activation by ERK1/2, phosphorylated RSK1 blocks PKAc and decreases the ability of cAMP to activate the PKA holoenzyme (Chaturvedi *et al.*, 2006; Gao & Patel, 2009). Interestingly, it has been reported previously that RSK2, the major isoform in *Xenopus* oocytes, phosphorylates and inhibits Myt1. Myt1, in turn, phosphorylates and inactivates cdc2 (Palmer *et al.*, 1998), while constitutively active RSK1 triggers meiotic G2–M1 transition (Gross *et al.*, 2001). Clearly, in the future it would be interesting to examine whether potential synergism between PI3K/Akt and MEK1/2 in regulation of PDE3 activity along with inactive

RSK-mediated activation of PKA may ensure prophase arrest in zebrafish oocytes.

Next we examined the effect of high cAMP on the regulation of p-Akt and p-ERK in zebrafish oocytes undergoing meiotic G2–M1 transition due to MIS stimulation. Data from the present study demonstrated that prior elevation of intra-oocyte cAMP could successfully attenuate phosphorylation of Akt and MAPK, suggesting the possibility of a proximal interaction between these signalling pathways. In cultured human primary T lymphocytes, an increase in cAMP levels has been shown to inhibit PI3K/Akt and Ras-dependent activation of ERK1/2 that correlates well with the blockade of cell cycle progression (Grader-Beck *et al.*, 2003). Furthermore, cAMP inhibits the synthesis of p27Kip1 and cyclin D2 in rat C6 glioma cells and prevents S phase entry by blocking Akt and ERK activities (Wang *et al.*, 2001). Moreover, the

ability of brain-derived neurotrophic factor (BDNF) to rescue cortical neurons from apoptosis after serum deprivation that required PI3K signal transduction cascade is attenuated by cAMP (Poser *et al.*, 2003). Although further studies are required in the oocyte model, experimental evidence in cultured somatic cells suggests that high cAMP blocks the membrane localization of PDK1 and inhibits the lipid kinase activity of PI3K (Kim *et al.*, 2001). Furthermore, thyroid stimulating hormone (TSH) inhibition of Akt activity via cAMP in PCCL3 cells suggests that a novel Epac-Rap1b-PP2A signalling module controls cAMP-dependent Akt regulation (Lou *et al.*, 2002; Hong *et al.*, 2008). The attenuation of MIS-induced Akt activation by cAMP is reported here, however, for the first time in an oocyte model.

Moreover, cAMP inhibition of MAPK activation has been reported earlier in both normal and transformed cell types (Cook & McCormick, 1993; Filardo *et al.*, 2002; Funaki *et al.*, 2010) as well as in oocytes (Sun *et al.*, 1999; Lu *et al.*, 2001; Maitra *et al.*, 2014). Cross-talk between the cAMP and MAPK signalling pathways has been shown in COS and NIH3T3 cells, in which cAMP-dependent inhibition of the MAPK is mediated by Raf-1 (Dumaz & Marais, 2003). PKA blocks Raf-1 activation by triggering inhibitory phosphorylation at Ser43, Ser233 and Ser259 residues, which allows binding of the 14-3-3 protein to Raf-1 and prevents its binding to Ras–GTP (Gerits *et al.*, 2008). Although high cAMP attenuation of c-Mos translation and MAPK activation cannot be ruled out during late stages of MIS stimulation (Sun *et al.*, 1999; Lu *et al.*, 2001), predominance of the phosphorylation cascade (through Gβγ–Ras–Raf–MEK) is highly probable during rapid (within 15 min) activation of MAPK (ERK1/2 phosphorylation) in zebrafish oocytes *in vitro*. Potential cross-talk between cAMP/PKA and membrane-initiated signalling axes in zebrafish oocytes has been reported previously and high intra-oocyte cAMP levels prevent insulin-induced (RTK-mediated), as well as okadaic acid-induced, MAPK phosphorylation and oocyte GVBD (Maitra *et al.*, 2014).

Although a pharmacological approach has its own level of limitations, the use of specific kinase inhibitors forms the main stay behind targeted therapy in cell-cycle regulation in the clinical set up and is widely prevalent in the treatment of cancer patients in recent times. As far as we know, the present study shows for the first time the involvement and potential cross-talk between PI3K/Akt or MEK/MAPK pathways to promote MIS-induced PKA dephosphorylation and oocyte maturation through the use of specific pharmacological inhibitors. Regardless, further in-depth studies involving the expression of a dominant negative PI3K (p85δSH2N) to inhibit MIS action

and/or overexpression of PI3K to promote oocyte maturation independent of steroid stimulation would provide strong evidence to reach a robust conclusion in future. Collectively our results indicate that, in the absence of maturational steroid, high intra-oocyte cAMP/PKA potentially blocks MPF activation (Fig. 5, left panel). Conversely, DHP stimulation inhibits the cAMP/PKA-mediated signalling pathway, and triggers PI3K/Akt and MEK/MAPK signalling pathways to induce maturation (Fig. 5, right panel). Therefore, the present data strengthen the view that PI3K/Akt, MEK/MAPK and cAMP/PKA-mediated signalling pathways may not be mutually exclusive and potentially forming a triad, components of which are selectively accessible to diverse stimuli (endocrine and/or autocrine/paracrine) and influence meiotic cell cycle progression.

Statement of interest

None

Supporting information

Additional Supporting Information is available online at the publisher's website.

Methods S1
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Table S1

Supplementary material

To view supplementary material for this article, please visit https://doi.org/10.1017/S0967199417000545

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