General and Comparative Endocrinology 181 (2013) 88–97

Contents lists available at SciVerse ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Participation of cAMP-dependent protein kinase and MAP kinase pathways during *Anabas testudineus* oocyte maturation

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ARTICLE INFO

Article history:
Available online 19 November 2012

Keywords:
cAMP dependent protein kinase
H89
PKI
Oocyte maturation
MAPK
Anabas testudineus

ABSTRACT

Possible involvement of cyclic nucleotide dependent protein kinase (PKA) and MAP kinase (MAPK) pathways during oocyte maturation in *Anabas testudineus* was investigated. Pre-incubation with phosphodiesterase (PDE) inhibitor, 3-isobutyl-1-methylxanthine (IBMX), inhibited 17α, 20β-DHP-induced GVBD dose dependently. PKA inhibitor, H89 could induce resumption of meiosis independent of 17α, 20β-DHP, in dose and duration dependent manner. The maximum response was obtained with the dose of 10 μM of H89 and 95% of cells underwent GVBD within 18 h. Moreover, stimulation with 17α, 20β-DHP inhibited endogenous PKA activity significantly within first hour and this effect was attenuated by PDE inhibitor IBMX at all time points. The pattern of PKA inhibition corresponded well with kinetics of histone H1 kinase activation and p34cdc2 phosphorylation. These results suggest physiological relevance of cAMP/PKA signaling in perch oocytes undergoing G2/M transition. MAPK was demonstrated as two distinct isoforms (ERK1 and ERK2) which resolved in the range of 42–44 kDa in immunoblot. Though total protein content did not show significant variation, H89 stimulation was able to stimulate phosphorylation of ERK1/2 from 5 h onwards and the strongest response was observed between 10 and 18 h. MEK inhibitor, U0126 completely blocked PKA inhibition induced MAPK activation and GVBD. In addition, inhibition of endogenous PKA by a more selective peptide inhibitor [PKI-(6–22)-amide] was sufficient to resume GVBD and MAPK activation in intact perch oocytes. Also, significant ERK1/2 phosphorylation could be stimulated in cell-free extracts of perch oocytes supplemented with PKI-(6–22)-amide. The results suggest an interaction between cAMP/PKA and MAPK pathways in mediating meiosis resumption in perch oocyte.

1. Introduction

Release from meiotic arrest induces oocytes to undergo two consecutive meiotic division cycles without an intervening S phase. Full grown *Anabas* oocytes remain arrested in prophase of meiosis I and are induced to mature upon exposure to 17α, 20β-dihydroxy 4-pregnen-3-one (17α, 20β-DHP), the natural maturation inducing steroid (MIS) in this species [2]. Available information indicates that, binding of MIS to novel G-protein coupled receptors on oocyte plasma membrane (mPRα), activates a cascade of intracellular second messengers leading to formation and activation of maturation promoting factor (MPF), a heterodimeric protein kinase of cyclin B and cdc2 [54,60]. MPF activation leads to completion of oocyte maturation, an event characterized by nuclear membrane dissolution (GVBD), chromosome condensation, and formation of metaphase spindles and extrusion of first polar body to produce fertilizable gamete, a prerequisite for successful fertilization [54].

There is abundant evidence showing the important role played by the second messenger cAMP in maintaining prophase arrest in vertebrate oocyte [6,11]. Hormonal stimulation of meiotic maturation is associated with a rapid fall in intra-oocyte cAMP level [20] and is mediated through activation of a pertussis toxin-sensitive inhibitory G protein (Gi) and subsequent downregulation of adenylyl cyclase activity [37,54]. The link between decreasing cAMP concentration and the downstream regulatory component is presumably through inhibition of cAMP-dependent protein kinase (PKA) activity. Signal transduction cascades involving the cAMP-PKA are highly conserved among a wide variety of organisms [8]. PKA is a serine/threonine kinase, composed of two catalytic subunits held in an inactive state in association with a regulatory subunit dimer. The binding of two cAMP molecules to each regulatory subunit allows the catalytic subunits to dissociate and results in phosphorylation of substrate proteins [51]. Although PKA involvement in the maintenance of meiotic arrest is widely accepted in fish, amphibian and mammalian oocytes [7,11,18], the biochemical steps linking PKA inhibition and MPF activation and the putative PKA substrates that are phosphorylated or dephosphorylated during re-entry into cell cycle, are largely unknown.

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http://dx.doi.org/10.1016/j.ygcen.2012.10.016

Mitogen-activated protein kinases (MAPK), also termed extracellular signal-regulated kinases (ERKs) are rapidly activated in response to hormones, growth factors, cytokines and stress controlling diverse biological functions [41,46]. The most widely studied MAPKs are 44- and 42-kDa isoforms, designated as ERK1 and ERK2, play important role in the process of oocyte maturation [22,33]. Although several earlier studies have established the participation of Mos/MAPK in metaphase II arrest [21,30,49], involvement of MAPK in progesterone induced MPF activation and GVBD is contradictory in fish, amphibian and mammalian models [22,28].

In somatic cells the cAMP/PKA and MAPK pathways do not operate independently, rather interact with each other during the regulation of cell proliferation [15,52]. Available information indicates that though cAMP-PKA participates in non-MAPK stimulated mitogenic pathway in thyroid cells [26], it has been reported to upregulate p44 MAPK cascade in PC12 cells [14]. Though, considerable evidence has been generated from PKA regulation of oocyte maturation in amphibian and mammalian oocytes, data implicating cAMP/PKA in MAPK regulation in fish oocytes are missing.

Anabas testudineus (Order Perciformes, Family Anabantidae) is a fresh water air-breathing teleost widely distributed in swamps, canals, ponds, lakes and estuaries of Asia. The species can tolerate extremely unfavorable water conditions and considered as economic food fish in South-East Asia. Unlike the previous reports in various other fish species, immature *Anabas* oocytes arrested in prophase I contain an inactive form of MPF (pre-MPF) and conversion of pre-MPF to active MPF occurs several hours after stimulation by 17α, 20β-DHP [1], a situation more close to *Xenopus laevis* [37]. In the present study, attempts have been made to understand the effect of PKA inhibition on MPF activation through the participation of MEK-MAPK pathway. It has been observed PKA inhibition up regulates MPF activation and GVBD and this effect was attenuated by forced increase of intra-oocyte cAMP level by phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine. In PKA inhibited cells, inhibition of MEK by its pharmacological inhibitor U0126 blocks phosphorylation of ERK1/2 and meiotic resumption.

2. Materials and methods

2.1. Animals

A. testudineus adult females (body weight range 100–120 g) were obtained from local fish farms in and around Santiniketan [Lat. 23°41′30″N, Long. 87°30′47″E], West Bengal, India during pre-spawning and spawning stages (May–August) and were maintained in 200 L glass aquaria under ambient photoperiod and temperature. During this time ovaries contained mostly fully grown follicles measuring 0.5–0.65 mm in diameter with centrally placed intact germinal vesicle (GV). Reagents unless otherwise specified, were from Sigma–Aldrich, India.

2.2. Incubation of perch oocytes

Ovaries were surgically removed from gravid females and immediately placed in ice-cold M199 medium (pH7.6, salinity 0.6%) supplemented with streptomycin (100 μg/ml) and penicillin (100 IU/ml). These oocytes were denuded, defolliculated and then washed several times with the medium according to the method described earlier [9]. Viability of denuded oocytes was checked by trypan blue dye exclusion test. Denuded oocytes were seeded into 24 well culture plate (100 oocytes/well) containing fresh medium (1 ml/well) and cultured *in vitro* at 25 ± 1 °C under gentle agitation. Healthy oocytes with centrally placed GV were selected microscopically after 1 h of incubation. Pharmacologic phosphodiesterase (PDE) inhibitor, IBMX was diluted in DMSO just before the experiment and added to the culture medium at different concentrations (0.1–1 mM) 1 h prior to 17α, 20β-DHP (1 μg/ml) addition. Oocytes in control wells received equivalent amount of DMSO (10 μl/1 ml of medium) only. To determine the effects of PKA inhibition, H89 was added to the appropriate wells at different concentration (0.1–10 μM) and incubated for 24 h. Stock solutions of MEK inhibitor U0126 was prepared with DMSO on the day of experiment and kept at 0 °C. Just prior to the incubation they were diluted with the incubation medium to give different working concentration (5–20 μM). Maturational processes were assessed by immersing the oocytes in a clearing solution containing 1% acetic acid, 6% ethanol and 3% formalin [27], facilitating easy microscopic examination of the occurrence of GVBD. At least three different replicates (100 denuded oocytes/replicate) were taken for each time points and each experiment was done using oocytes collected from five fish.

2.3. Preparation of oocyte extracts

Oocytes were washed thoroughly in oocyte extraction buffer (OEB) containing 100 mM sodium β-glycerophosphate, 20 mM HEPES, 15 mM $MgCl_2$, 5 mM EGTA, 100 mM p-PMSF, 3 mg/ml leupeptin, 1 mM DTT and 1 mg/ml aprotinin; pH 7.5 [19] and homogenized in the same buffer (1 μl/oocyte) using polytron homogenizer. The homogenate was centrifuged at 17,500*g* for 30 min at 4 °C. The supernatant were either used immediately or stored at −20 °C until use.

2.4. Histone H1 kinase assay

Kinase activity of p34cdc2 was measured as described earlier [1,57]. Briefly, 20 μl of oocyte extract was incubated at 30 °C for 2 min in presence of 100 mM histone H1 (Type-III-S); 500 mM ATP; 1.5 mCi 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 $MgCl_2$; 4 mM 2-mercaptoethanol and 20 mM Tris–HCl; pH 7.4. For determination of histone H1 phosphorylation by autoradiography, the reaction was stopped by adding 40 μl of 2× SDS sample buffer [25]. Samples were heated at 95 °C for 5 min and then resolved on 12.5% SDS–PAGE, along with pre-stained molecular weight marker proteins (Fermentas, Cat. No. SM#0441, Lot No. 00033594). The band corresponding to histone H1 was gel excised and transferred to PVDF membrane and recorded in Storm 860 phosphoimager. Data was analyzed using Quantity One image software (GE Healthcare Biosciences).

2.5. Determination of PKAc enzyme activity

The PKA activity was measured in a cell-free reaction system using kemptide as a substrate. Denuded perch oocytes exposed to various reagents as mentioned earlier, were collected at different time intervals up to 9 h, washed briefly in OEB and the PKA kinase activity was determined in oocyte lysates using PKA assay kit (Upstate, Millipore; Cat. No. #17-134) as per manufacturer's protocol. Briefly, 10 μl of the lysate (100 μg protein) was incubated at 30 °C for 30 min in assay buffer (20 mM MOPS; pH 7.2, 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 4.05 mM magnesium chloride, 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-phosphocellulose paper (Millipore), washed thrice in 0.75% phosphoric acid and once in acetone and the radiolabel incorporation was determined by scintillation counting of the dried paper (Tricrab2800,