Available online at www.sciencedirect.com

SCIENCE DIRECT

Developmental Biology 285 (2005) 70 – 79

DEVELOPMENTAL BIOLOGY

www.elsevier.com/locate/ydbio

Activation of a pertussis toxin-sensitive, inhibitory G-protein is necessary for steroid-mediated oocyte maturation in spotted seatrout

Margaret C. Pace*, Peter Thomas

The University of Texas at Austin Marine Science Institute, 750 Channel View Drive, Port Aransas, TX 78373, USA

Received for publication 29 December 2004, revised 11 May 2005, accepted 9 June 2005

Abstract

Oocyte maturation (OM) is initiated in lower vertebrates and echinoderms when maturation-inducing substances (MIS) bind oocyte membrane receptors. This study tested the hypothesis that activation of a G_i protein is necessary for MIS-mediated OM in spotted seatrout. Addition of MIS significantly decreased adenylyl cyclase activity in a steroid specific, pertussis toxin (PTX)-sensitive manner in oocyte membranes and microinjection of PTX into oocytes inhibited MIS-induced OM, suggesting the steroid activates a G_i protein. MIS significantly increased [^{35}S]GTPγS binding to ovarian membranes, confirming that MIS receptor binding activates a G-protein, and immunoprecipitation studies showed the increased [^{35}S]GTPγS binding was associated with $G\alpha_{i1\text{-}3}$ proteins. Radioligand binding studies in ovarian membranes using GTPγS and PTX demonstrated that the MIS binds a receptor coupled to a PTX-sensitive G-protein. This study provides the first direct evidence in a vertebrate model that MIS-induced activation of a G_i protein is necessary for OM. These results support a mechanism of MIS action involving binding to a novel, G-protein coupled receptor and activation of an inhibitory G-protein, the most comprehensive and plausible model of MIS initiation of OM proposed to date.

Keywords: Oocyte maturation; G-protein; Germinal vesicle breakdown; Membrane progestin receptor; Spotted seatrout

Introduction

Postvitellogenic, fully grown oocytes are arrested at the first meiotic prophase prior to oocyte maturation. This arrest is released in lower vertebrates and echinoderms upon binding of species-specific maturation inducing substances (MISs) to receptors located on the oocyte plasma membrane. The resumption of meiosis is characterized by germinal vesicle breakdown (GVBD), chromosome condensation, spindle formation and extrusion of the first polar body (Masui and Clark, 1979). Once oocyte maturation is complete, meiosis is again arrested at the second meiotic metaphase and the mature oocyte (egg) is capable of ovulation and fertilization (Masui, 1985, 2001).

Many of the signaling molecules involved in the later stages of oocyte maturation have been well characterized and are conserved across species (reviewed in Masui, 1985; Schmitt and Nebreda, 2002; Hammes, 2003). The early signaling events initiated by MIS binding, however, are less well described and do not appear to be as conserved. Evidence suggests that oocyte meiotic arrest in several species is maintained via constitutive activity of a stimulatory G-protein (G_s) which stimulates adenylyl cyclase activity to elevate cAMP levels in the oocyte (Eppig, 1991; Kalinowski et al., 2004). It is proposed that induction of oocyte maturation in these vertebrates is through the inactivation of this constitutively active G_s and a subsequent reduction in cAMP concentrations and PKA activity (Gallo et al., 1995). Inhibition of G_s activity in *Xenopus* and zebrafish oocytes was sufficient to induce GVBD in the absence of a MIS signal (Gallo et al., 1995; Kalinowski et al., 2003). Whether a reduction in cAMP alone is sufficient to promote oocyte maturation, however, is not clear. Also

* Corresponding author. Fax: +1 361 749 6777.
E-mail address: mpace@utmsi.utexas.edu (M.C. Pace).

0012-1606/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2005.06.003

not clear is what type of receptor the MIS is binding, whether the MIS receptor is signaling through a G-protein and which receptor, if any, is regulating the constitutive G_s activity in the oocytes.

Evidence from other models suggests that activation of a G-protein is necessary for oocyte maturation. Experiments in rainbow trout suggested that activation of a pertussis toxin (PTX)-sensitive G_i protein is involved in the initiation of oocyte maturation, although microinjection of these oocytes with PTX, which inactivates $G_{i/o}$ G-proteins, did not inhibit GVBD (Yoshikuni and Nagahama, 1994). Studies in starfish and Atlantic croaker oocytes also found that activation of a G_i protein was necessary for maturation and in these species microinjection of oocytes with PTX significantly inhibited MIS-induced GVBD (Shilling et al., 1989; Thomas et al., 2002).

The spotted seatrout (*Cynoscion nebulosus*) is a well-characterized fish model of oocyte maturation. Unlike many oocyte maturation models whose MISs are ambiguous or unknown (Le Goascogne et al., 1985; Lutz et al., 2001; Hammes, 2003), the naturally occurring seatrout MIS has been identified as the progestin 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S) and its binding to oocyte plasma membranes characterized in detail (Trant and Thomas, 1989; Thomas and Trant, 1989; Patiño and Thomas, 1990; Thomas and Das, 1997; Thomas et al., 2001). This makes seatrout an ideal model in which to study whether steroid-mediated G-protein activation is necessary for oocyte maturation.

The purpose of the current study was to test the hypothesis that direct, steroid-induced activation of a G_i protein is necessary for oocyte maturation in spotted seatrout. We present evidence that steroid-mediated G-protein activation significantly reduces oocyte adenylyl cyclase activity in a steroid specific, PTX-sensitive manner. Data also show that 20β-S is binding a receptor in oocyte membranes that is coupled to a PTX-sensitive, inhibitory G-protein and PTX microinjection studies indicate that activation of this G-protein is necessary for GVBD in spotted seatrout.

Materials and methods

Chemicals

All nonradioactive steroids were obtained from Steraloids (Newport, RI). Dulbecco modified Eagle Medium with phenol red (DMEM, nutrient mixture F-12), actinomycin D, penicillin G, human chorionic gonadotropin (hCG), polyclonal $G\alpha_o$, $G\alpha_s$ and $G\alpha_{i1,2}$ antibodies, guanosine 5′-diphosphate (GDP), guanosine 5′-triphosphate (GTP), guanosine 5′-*O*-3-thiotriphosphate (GTPγS), phosphoenol pyruvate, pyruvate kinase and basic laboratory chemicals were purchased from Sigma (St. Louis, MO). Protease inhibitor cocktail, Protein A/G Plus-agarose beads and Supersignal West Pico Chemiluminescent Substrate System were purchased from Pierce Biotechnology (Westford, IL). The $G\alpha_{i3}$ antibody was purchased from Biomol (Plymouth Meeting, PA). 1,2-[^{3}H]-11-Deoxycortisol (42 Ci/mmol) was purchased from New England Nuclear (Boston, MA) and enzymatically converted to 20β-S as described by Scott et al. (1982). Nicotinamide adenine[adenylate-^{32}P] dinucleotide ([^{32}P]NAD, 1000 Ci/mmol), guanosine 5′-[γ-^{35}S]triphosphate, triethylammonium salt ([^{35}S]GTPγS, 1000 Ci/mmol) and [1,2,6,7-^{3}H]testosterone (78.5 Ci/mmol) were purchased from Amersham Pharmacia (Piscataway, NJ). All radioactivity was counted in a liquid scintillation counter (LS 6000, Beckman Instruments, Fullerton, CA).

Animal collection

Adult seatrout undergoing gonadal development were collected by gill net in Redfish Bay, Aransas Pass, Texas, during April and May. Fish were quickly removed from the net and returned to the laboratory. Female seatrout were anesthetized and an ovarian biopsy sample was obtained by inserting a catheter attached to a syringe via the cloaca into the oviduct. The average diameter of the oocytes was determined under a binocular microscope. Oocytes with an average diameter of 430 to 450 μm were considered fully grown and suitable for experimentation. Fish were humanely sacrificed by severing the spinal cord following procedures approved by the University of Texas at Austin Animal Care and Use Committee. The ovaries were immediately removed, weighed and either transferred to DMEM, pH 7.6, containing 100 mg/l streptomycin and 60 mg/l penicillin, for in vitro incubations, or frozen on dry ice and stored at −80°C.

Oocyte membrane preparation

Ovarian tissues were placed in ice-cold homogenization buffer (25 mM HEPES, pH 7.6, 10 mM NaCl, 10 mM $MgCl_2$, 1 mM dithioerythritol, 1 mM EDTA) and finely minced. Tissue fragments were repeatedly expelled through an 18- and then a 22-guage needle and vigorously washed through 400 μm Nitex mesh to remove follicle cells and immature oocytes. Removal of >90% of the follicle cells from the final oocyte preparation was confirmed by microscopy. Next, oocytes were homogenized in 2 ml of homogenization buffer containing 10 μl protease inhibitor cocktail with five passes through a glass Tenbroeck tissue grinder. The homogenate was centrifuged twice at 500 × *g* for 5 min and the pellet discarded. The supernatant was centrifuged at 20,000 × *g* for 20 min and the resulting supernatant discarded. The pellet was resuspended in 1.5 ml of homogenization buffer and further separated over a 1.2 M sucrose pad (1 volume pellet resuspension: 1 volume sucrose buffer) by centrifugation at 6500 × *g* for 45 min. After centrifugation, the middle layer membrane fraction