Comparative Biochemistry and Physiology, Part A 187 (2015) 168–176

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

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

Transcript levels of the soluble sperm factor protein phospholipase C zeta 1 (PLCζ1) increase through induced spermatogenesis in European eel

Marina Morini [a], David S. Peñaranda [a], María C. Vílchez [a], Víctor Gallego [a], Rasoul Nourizadeh-Lillabadi [b], Juan F. Asturiano [a], Finn-Arne Weltzien [b], Luz Pérez [a,*]

[a] Grupo de Acuicultura y Biodiversidad, Instituto de Ciencia y Tecnología Animal, Universitat Politècnica de València, Camino de Vera s/n, 46022 Valencia, Spain
[b] Department of Basic Sciences and Aquatic Medicine, Norwegian University of Life Sciences—Campus Adamstuen, P.O. Box 8146 Dep, 0033 Oslo, Norway

ARTICLE INFO

Article history:
Received 14 April 2015
Received in revised form 22 May 2015
Accepted 31 May 2015
Available online 4 June 2015

Keywords:
Teleost
Reproduction
Fertilization
Spermatozoa
Anguilla anguilla

ABSTRACT

Activation at fertilization of the vertebrate egg is triggered by Ca^{2+} waves. Recent studies suggest the phospholipase C zeta (PLCζ), a sperm-specific protein, triggers egg activation by an IP3-mediated Ca^{2+} release and allow Ca^{2+} waves at fertilization. In the present study we cloned, characterized, and phylogenetically positioned the European eel PLCζ (PLCζ1). It is 1521bp long, with 10 exons encoding an open reading frame of 506 amino acids. The amino acid sequence contains an EF-hand domain, X and Y catalytic domains, and a carboxy-terminal C2 domain, all typical of other PLCζ orthologous. The tissue distribution was studied, and the gene expression was determined in testis during induced sexual maturation at three different thermal regimes. Also, brain and pituitary expression was studied through sex maturation at constant temperature. *plcζ1* was expressed in brain of male and female, in testis but not in ovaries. By first time in vertebrates, it is reported *plcζ1* expression in the pituitary gland. Testis *plcζ1* expression increased through spermatogenesis under all the thermal regimes, but being significantly elevated at lower temperatures. It was very low when testis contained only spermatogonia or spermatocytes, while maximum expression was found during spermiogenesis. These results support the hypothesis for an eel sperm-specific PLCζ1 inducing egg activation, similarly to mammals and some teleosts, but different from some other teleost species, which express this protein in ovaries, but not in testes.

1. Introduction

Sperm fusion with the egg induces egg activation in all animals studied so far through a rise in intracellular Ca^{2+} (Stricker, 1999; Tarin, 2000; Horner and Wolfner, 2008; Kashir et al., 2010). Three models have been proposed for mechanisms by which fertilization-induced Ca^{2+} waves are initiated: a) Ca^{2+} bolus/conduit (Jaffe, 1983, 1991), where the sperm trigger the entering of extracellular Ca^{2+} into the oocyte; b) membrane receptor (Jaffe, 1990; Evans and Kopf, 1998), with an intracellular Ca^{2+} release provoked by the binding of an oocyte surface receptor with a sperm ligand; or c) a soluble sperm factor (Swann et al., 2006; Parrington et al., 2007; Saunders et al., 2007) released into the oocyte after gamete fusion, triggering egg activation. This sperm factor corresponds to a sperm-specific phospholipase C (PLC) called PLCζ (Ito et al., 2011; Swann and Lai, 2013). After fertilization, PLCζ induces a reaction chain by cleaving phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (Igarashi et al., 2007; Miao and Williams, 2012). These two metabolites, in turn, cause IP3-mediated Ca^{2+} release from the endoplasmic reticulum, and the activation of such targets as DAG-sensitive protein kinase Cs (PKCs) (Miyazaki et al., 1993; Saunders et al., 2002; Swann and Yu, 2008; Yu et al., 2008).

During the last ten years, several studies have demonstrated the importance of the soluble sperm factor to allow Ca^{2+} waves at fertilization. Injection of recombinant PLCζ cRNA (Saunders et al., 2002) or protein (Kouchi et al., 2004) into mouse eggs leads to Ca^{2+} oscillations at fertilization. Saunders et al. (2002) showed that when endogenous PLCζ was removed by immunodepletion, mouse sperm protein extracts lost their ability to release Ca^{2+}. Moreover, in vitro fertilization of mouse eggs with sperm from transgenic mice expressing lower amounts of PLCζ (due to a short hairpin RNAs targeting PLCζ) induced Ca^{2+} oscillations that ended prematurely, negatively affecting egg activation and embryonic development (Knott et al., 2005). Furthermore, infertile men whose sperm failed in egg activation showed abnormal expression and localization of PLCζ in the sperm

* Corresponding author at: Grupo de Acuicultura y Biodiversidad, Instituto de Ciencia y Tecnología Animal, Edificio 7G, Universitat Politècnica de València, Camino de Vera, s/n, 46022 Valencia, Spain.
E-mail address: mlpereig@dca.upv.es (L. Pérez).

http://dx.doi.org/10.1016/j.cbpa.2015.05.028
1095-6433/

(Yoon et al., 2008; Heytens et al., 2009). Until now, mammalian PLCζ orthologues have been reported in mice, monkeys, humans, boars, hamsters, and bulls (Cox et al., 2002; Saunders et al., 2002; Yoneda et al., 2006; Young et al., 2009; Cooney et al., 2010). In non-mammals, PLCζ orthologues were reported in the chicken (Coward et al., 2005), medaka (Ito et al., 2008), quail (Mizushima et al., 2009) and in two pufferfish species *Takifugu rubripes* (Fugu) and *Tetraodon nigroviridis* (Tetraodon) (Coward et al., 2011). In these non-mammalian species, like chicken or medaka, PLCζ mRNA is expressed in the testis, in line with the situation in mammals. In contrast, in two pufferfish species, *plcζ1* is expressed in the ovary, but not in the testis (Coward et al., 2011).

Due to its unique life cycle and its phylogenetical position, the European eel (*Anguilla anguilla*) is a particularly interesting model to investigate the regulatory mechanisms of reproductive physiology and for providing insights into ancestral regulatory functions in teleosts. Prepubertal silver eels migrate across the Atlantic Ocean to reach their probable spawning area in the Sargasso Sea (Tesch, 1977). Gonadal development and maturation probably take place during the supposedly 6–7 month migration period, at low temperature, whereas the spawning takes place at high temperatures, considered to be around 20 °C (Boetius and Boetius, 1967, 1980). However, as detailed information from the field is still lacking, it is difficult to simulate the variable environmental factors which would occur during the migration (temperature, photoperiod, pressure, etc.). That is why, in captivity, silver eels are blocked in a pre-pubertal stage (Dufour et al., 2003; Pasqualini et al., 2004; Vidal et al., 2004) and must receive a long-term hormonal treatment to induce sexual maturation and spermiation (Boetius and Boetius, 1967; Ohta et al., 1996, 1997; Asturiano et al., 2005; Huang et al., 2009; Pérez et al., 2000; Gallego et al., 2012).

In this study, we characterized and cloned the *A. anguilla plcζ1* mRNA, analysed the structure and investigated the position of this protein among vertebrates by phylogenetic analyses, studied the tissue distribution of this gene and finally, for the first time in teleost, we studied the expression profile of *plcζ1* in the brain and gonad through spermatogenesis. The impact of water temperature on the maturation process of European eel has been highlighted in females (Pérez et al., 2011; Mazzeo et al., 2014) and males (Gallego et al., 2012, 2014; Baeza et al., 2014), and in order to simulate the natural conditions during the reproductive migration and testing its potential effect on *plcζ1* expression, three different thermal regimes were tested for the gene expression profile experiments, two variable regimes (changing gradually from 10 to 20 °C or from 15 to 20 °C), and one constant regime (20 °C).

2. Material and methods

2.1. Fish maintenance, hormonal and thermic treatments, and sampling

Three hundred and seventeen male European eels (mean body weight 100 ± 2 g) from the fish farm Valenciana de Acuicultura, S.A. (Puzol, Valencia; East coast of Spain) were hormonally matured at the Aquaculture Laboratory at the Polytechnic University of Valencia. They were randomly distributed and kept in six 200-L fibreglass tanks (approximately 50 males per aquaria, 2 aquaria per treatment) equipped with separate recirculation systems, thermostats/coolers, and covered to maintain constant shadow.

The fish were gradually acclimatized for one week to seawater (37 ± 0.3‰ of salinity) and the water temperature was kept at 20 °C or changed to 15 °C in one week or to 10 °C in two weeks, depending on thermal groups. Starting three weeks after arrival to the Aquaculture Laboratory, the eels were treated with weekly intraperitoneal injections of human chorionic gonadotropin (hCG, Profasi®, Serono, Italy); 1.5 IU g^{-1} fish; during 13 weeks to induce maturation and spermiation, as previously described by Pérez et al. (2000).

During the experiment, the animals were maintained in three thermal regimes (2 aquaria per treatment): T10–T20: 10 °C (first 5 weeks, with one week of temperature acclimation), 15 °C (next 3 weeks) and 20 °C (last 6 weeks); T15–T20: 15 °C (first 6 weeks, with two weeks of temperature acclimation) and 20 °C (last 9 weeks); and T20: 20 °C during the whole experimental period. These thermal regimes were previously described by Gallego et al. (2012).

Groups of 5–8 eels per treatment were anaesthetized with benzocaine (60 ppm) and sacrificed by decapitation each week along the hormonal treatment. Morphometric parameters such as total body and gonad weights were recorded to calculate the gonadosomatic index (GSI = (gonad weight / total body weight) × 100) for each fish (Pankhurst, 1982). Furthermore, testicular tissue samples were fixed in 10% formalin buffered at pH 7.4 for histological processing and subsequent determination of maturational status. Samples of pituitary, testis, liver, heart, gill, muscle, spleen, fins, and kidney were collected for analyses of gene expression levels by qPCR. Brains were dissected into five parts: olfactory bulbs, telencephalon, mes-/di-encephalon, cerebellum, and medulla oblongata. All the samples were stored in 0.5 ml of RNAlater (Ambion Inc., Huntingdon, UK) at −20 °C until extraction of total RNA (Peñaranda et al., 2010).

Because eels stop feeding at the silver stage and throughout sexual maturation, the fish were not fed during the experiment and were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC).

2.2. Gonadal histology

Fixed testis samples were dehydrated in ethanol and embedded in paraffin. Sections of 5–10 μm thickness were cut with a Shandom Hypercut manual microtome and stained with haematoxylin and eosin. Slides were observed with a Nikon Eclipse E-400 microscope, and pictures were taken with a Nikon DS-5M camera attached to the microscope. Stages of spermatogenesis were determined according to the most advanced germ cell type present and their relative abundance, degree of development of the seminal tubules, GSI and sperm production by the male in the same week of the sacrifice. Stage 1 Spermatogonia (SPG): dominance of spermatogonia, in some cases, a few spermatocytes were present in low number, mean GSI = 0.08 (0.0–0.36); Stage 2 Spermatocytes (SPC): spermatocytes were present in proportion ≥50% with spermatogonia, in some cases appeared low number of spermatids, mean GSI = 0.72 (0.27–1.54); Stage 3 spermatids (SD): spermatids were the dominant germ cell, some sperm cells can appear, mean GSI = 3.28; and Stage 4 spermatozoa (SZ): spermatozoa was the dominant germ cell, mean GSI = 7.35 (3.41–12.8) (Fig. 1).

2.3. Isolation of PLCζ sequence

2.3.1. European eel genome database analysis

The TBLASTN algorithm of the CLC DNA Workbench software (CLC bio, Aarhus, Denmark) was used to retrieve the genomic sequence of the PLCζ from the European and Japanese eel genomes (Henkel et al., 2012a,b).

Exons and splice junctions were predicted using the empirical nucleotidic splicing signatures, i.e.: introns begin with “GT” and end with “AG”. The peptidic sequences of *T. nigroviridis* PLCζ1 sequence (Accession: HQ185299. GI: 322510422. 1889 bp mRNA) were used as query.

Percentage of European eel PLCζ1 identity with other osteichtian PLCζ sequences was calculated with Sequences Identities And Similarities (SIAS) server (imed.med.ucm.es/Tools/sias.html).

2.3.2. Partial cloning of the PLCZ1 gene

cDNA was generated using 1 μg of total RNA. A mixture of cDNA from different tissues of female silver eels was used as template for