Acta Histochemica 115 (2013) 257–263

Contents lists available at SciVerse ScienceDirect

Acta Histochemica

journal homepage: www.elsevier.de/acthis

Identification of aquaporins in eggs and early embryogenesis of the sea urchin *Paracentrotus lividus*

Andrea Amaroli[a,*], Sara Ferrando[a], Maria Cristina Gagliani[b], Lorenzo Gallus[a], Maria Angela Masini[a]

[a] *Dipartimento di Scienze della Terra, dell'Ambiente e della Vita (DISTAV), University of Genoa, Genoa, Italy*
[b] *Dipartimento di Medicina Sperimentale, University of Genoa, Genoa, Italy*

ARTICLE INFO

Article history:
Received 28 June 2012
Received in revised form 24 July 2012
Accepted 25 July 2012

Keywords:
Echinodermata
Sea urchin
Water channels
Aquaporins
Aquaporin inhibitors
Water homeostasis
Egg fertilization

ABSTRACT

Sea urchins are echinoderms, marine invertebrates found at the base of the deutorostome lineage, which show separate sexes and are external spawners. In the sea urchin, efficient regulation of water homeostasis is essential for many biological processes such as cellular respiration, normal fertilization and correct embryo growth. In order to clarify some of these processes, the present study reports on the identification and function of aquaporin proteins in the sea urchin. Our results show, by immunoblot, immunoelectron microscopy and immunofluorescence analysis, the presence of aquaporin1- and aquaporin3-like proteins in virgin eggs and in early embryogenesis of *Paracentrotus lividus* and, by using known inhibitors of aquaporin functions, the functional and relevant role of aquaporin-3 in the fertilization process. AQP3 in particular seems to play a crucial role in high velocity water flux formations involved in the detachment of the vitelline layer during the slow block of polyspermy, while the presence of AQP1 and the increase of AQP3 in the first phase of the *P. lividus* developmental cycle, suggest their involvement in the appropriate homeostasis for embryo development.

Introduction

For about a century, sea urchins have been recognized as an excellent model for the study of fertilization and embryo development, since large quantities of gametes are readily available and the whole life cycle is easily obtainable (Neil and Vacquier, 2004). Sea urchins are echinoderms, marine invertebrates found at the base of the deutorostome lineage, which show separate sexes and are external spawners. When released into sea water, the spermatozoa start to swim and interact with the eggs by a species-specific recognition owing to the presence of sperm-receptors in the vitelline layer, a glycoprotein meshwork of filamentous layer that surrounds the egg plasma membrane (Hirohashi and Lennarz, 1998). In a normal fertilization process, or monospermy, only one sperm enters the egg (Gilbert and Singer, 2006). The entrance of multiple spermatozoa could lead to anomalies in the zygote owing to inappropriate chromosome and centriole numbers. For this reason, to avoid polyspermy, the sea urchin egg has evolved two mechanisms: a fast reaction, achieved by an electrical change in the egg plasma membrane (from −70 mV to +20 mV), and a slower reaction, involving the exocytosis of the cortical granules that starts about 20 s after sperm attachment and is complete by the end of the first minute of fertilization (Just, 1919; Jaffe, 1980). The cortical granules release their mucopolysaccharide contents, which produce an osmotic gradient that causes water to rush into the space between the plasma membrane and the vitelline layer, causing the layer to expand and become the fertilization envelope (Glabe and Vacquier, 1978). Following zygote formation in the sea urchin, the first three cleavages are synchronous in all blastomeres. On segregation of the fourth cleavage, a vegetal-animal gradient of cell division begins and at the seventh division a 128-cell blastula is formed. The sea urchin developmental cycle continues up to the formation of a larva known as a *pluteus* (for more details see Gilbert and Singer, 2006). This developmental process is synchronized with steps in which water exchange plays a crucial role. Water is the major component of all living cells, and efficient regulation of water homeostasis, also in the sea urchin, is essential for many biological processes such as cellular respiration (Gomes et al., 2009), normal fertilization and correct embryo growth (Kruse et al., 2006; Damiano, 2011). Until now, although the life cycle of the sea urchin and the relevant role of water exchange appear clear, there is very limited information about the mechanisms that permit water exchange in a very short time, and which are not explicable as simple diffusion across the envelopes and membrane of the egg.

Since the first description of aquaporin (AQP) by Agre and colleagues (Preston and Agre, 1991; Preston et al., 1992), and the 2003 Nobel Prize award in Chemistry, much information on the physiological significance of these channel proteins in

* Corresponding author at: DISTAV, Corso Europa 26, 16128 Genoa, Italy.
E-mail address: amaroli@dipteris.unige.it (A. Amaroli).

0065-1281/$ – see front matter © 2012 Elsevier GmbH. All rights reserved.
http://dx.doi.org/10.1016/j.acthis.2012.07.006

transmembrane water transport has been amassed. The aquaporins (AQPs) are a family of small pore-forming integral membrane proteins that have representatives in all kingdoms of living things (Kruse et al., 2006). Thirteen mammalian AQPs have been identified to date, and although they all share structural similarities, their expression is tissue specific (Carbrey and Agre, 2009; Ishibashi, 2009; Herrera and Garvin, 2011). According to their structural and functional properties, AQPs are divided into three subgroups. The classical AQP, comprising AQP0, 1, 2, 4, 5, which are selective only for water, and AQP6 and AQP8 which are also permeable, respectively, to anions and urea (Yasui et al., 1999; Damiano, 2011). The aquaglyceroporins, comprising AQP3, 7, 9, and 10 are permeable to water, urea and glycerol and in the case of AQP9 also to monocarboxylates, purines and pyrimidines (Ishibashi et al., 1998; Tsukaguchi et al., 1998; Damiano, 2011). The super-AQPs constitute a subgroup recently proposed for AQP11 and 12, whose permeability has not yet been fully determined (Ishibashi, 2009). Nevertheless, this classification is only a simplification, and has been well reviewed by Carbrey and Agre (2009). Different molecules, such as ammonia and hydrogen peroxide (Jahn et al., 2004; Bienert et al., 2007), arsenite and antimonite (Liu et al., 2002), carbon dioxide and nitric oxide gases are able to cross the AQPs (Nakhoul et al., 1998; Herrera and Garvin, 2011).

Starting from this basis, in order to clarify the mechanisms that permit the high velocity water fluxes which occur during the sea urchin developmental cycle, the present study reports the identification and function of AQP proteins in the eggs and early embryogenesis of the sea urchin. Our results, by immunoblot, immunoelectron microscopy and immunofluorescence analysis, show the presence of aquaporin1- and aquaporin3-like proteins in virgin eggs and in early embryogenesis of *Paracentrotus lividus* and, by the use of known inhibitors of aquaporin functions, the functional and relevant role of aquaporin-3 in the fertilization process.

Materials and methods

Reagents

All common chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA), and were of superior analytical grade. The antibodies: rabbit anti-water channel aquaporin 1 (anti-AQP1), aquaporin 2 (anti-AQP2), aquaporin 3 (anti-AQP3) or anti-mouse α-tubulin were purchased from Sigma–Aldrich, USA; Cat. No. A5560, Cat. No. A7310, Cat. No. A0303, respectively. The anti-rabbit secondary antibody conjugated with Alexa Fluor 488 was purchased from Invitrogen, Life Technologies, Paisley, UK. The anti-rabbit and the anti-mouse secondary antibody conjugated with alkaline phosphatase were bought from Santa Cruz Biotechnology, Santa Cruz, CA, USA; Cat. No. sc-2034. The Bio-Rad protein assay kit was purchased from Bio-Rad, Milan, Italy; Cat. No. 500-0006. The 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) solution, premixed, was bought from Sigma–Aldrich, USA; Cat. No. B6404-100ML. The Paraplast® wax for embedding was purchased from McCormick Scientific, Leica Microsystems, Richmond, IL, USA.

P. lividus fertilization and differentiation

Gametes from the purple sea urchin *P. lividus*, Lamarck 1816, were obtained in the laboratory according to the usual methods: eggs were obtained by intracelomic injection of 0.5 M potassium chloride and were collected in ultrafiltered pelagic seawater (SW); sperm were collected "dry" and used immediately. For fertilization, 5 μl of the sea urchin sperm was added to 1 ml of SW, and 10 μl of this diluted sperm was added to the bowls, each containing about 2000 eggs in 20 ml of SW. Fertilization and development were carried out at 20 °C in a temperature-controlled room until the 16-cell stage. The developmental cycle was examined using a Leica MS5 stereomicroscope (Leica Microsystems, Wetzlar, Germany), the experimental egg samples collected by centrifugation at the stage of virgin eggs (T0); eggs fertilized from 1 min that show the complete detachment of vitelline layer (T1); eggs fertilized and developed at the 4-cell stage (T2); and eggs fertilized and developed at the 16-cell stage (T3). The experimental sperm samples (SP) were collected by centrifugation 10 min after 5 μl of the sea urchin sperm was added to 1 ml of SW. In all the experiments the SW was collected from the water column in the Portofino Marine Park and maintained for at least one month in a glass tank of 100 l at 20 °C. The salinity of this water was 3.7%, and the pH 8.00 (Aluigi et al., 2009).

Immunoblot analysis

Pellets from the experimental samples (T0, T1, T2, T3, SP) were homogenized in 0.5% Triton X-100 in phosphate-buffered saline, pH 7.4 (Angelini et al., 2003). A 30 μg total protein extract from the homogenate was run in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS) and then transferred to a nitrocellulose membrane. Non-specific binding sites were blocked with a blocking buffer containing Tris-buffered saline pH 7.4 and 0.1% Tween-20 with 3% non-fat milk powder for 45 min at 25 °C. The blot was incubated overnight at 4 °C with a blocking buffer containing the anti-AQP1, AQP2 or AQP3 primary antibodies, diluted 1:500. After washing, the blot was incubated with anti-rabbit immunoglobulin secondary antibodies conjugated with alkaline phosphatase, diluted 1:500, for 3 h at 25 °C. After rinsing thoroughly, detection was performed directly on the membrane using the BCIP/NBT solution.

The loading controls were performed as suggested by Salaun et al. (2005) by incubation with anti-mouse tubulin antibodies. The total protein estimation in cell homogenates were assayed according to the standard method as indicated in Bio-Rad protein assay kit. The immunoblot was acquired with Adobe Photoshop Elements 8.0 (Adobe Systems, San Jose, CA, USA) using a Canoscan Lide35 scanner (Canon, Tokyo, Japan). We used ImageJ 1.33j software (National Institutes of Health, USA) to evaluate the optical density (O.D.) and the apparent molecular mass of the bands of the experimental samples. The experiments were performed at least in triplicate.

Immunofluorescence

The experimental samples (T0, T1, T2, T3) were fixed in 4% paraformaldehyde in phosphate-buffered saline, pH 7.4 (PBS). Subsequently, samples were rinsed with PBS, dehydrated and embedded in Paraplast®. Dewaxed sections were preincubated with normal serum (1:50) and immunohistochemically treated overnight at room temperature with the appropriate primary antibody: anti-AQP1, dilution 1:200; anti-AQP2, dilution 1:200; anti-AQP3, dilution 1:200. Treated sections were rinsed in PBS and incubated for 2 h at room temperature with the appropriate secondary antibody conjugated with Alexa Fluor 488 (dilution 1:400). A control experiment was performed, omitting the primary antibodies and applying only the secondary antibody. No staining was detected in the control experiment. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, 1:10,000 in PBS for 5 min). Sections were observed with a Leica DMRB light and epi-fluorescence microscope, equipped with four Leica objectives: N Plan 2.5×/0.07, PL Fluotar 10×/0.30, PL Fluotar 20×/0.50, PL Fluotar 40×/0.70, PL Fluotar 100×/1.3 Oil. Images were obtained with a Leica CCD camera DFC420C and with the software Leica