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SHORT COMMUNICATION

Oxygen consumption rate and Na^+/K^+-ATPase activity in early developmental stages of the sea urchin *Paracentrotus lividus* Lam.

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Abstract Changes in oxygen consumption rate and Na^+/K^+-ATPase activity during early development were studied in the sea urchin *Paracentrotus lividus* Lam. The oxygen consumption rate increased from 0.12 μmol O_2 mg protein^{-1} h^{-1} in unfertilized eggs to 0.38 μmol O_2 mg protein^{-1} h^{-1} 25 min after fertilization. Specific activity of the Na^+/K^+-ATPase was significantly stimulated after fertilization, ranging up to 1.07 μmol P_i h^{-1} mg protein^{-1} in the late blastula stage and slightly lower values in the early and late pluteus stages.

Keywords Sea urchins · Early developmental stages · Oxygen consumption · Na^+/K^+-ATPase

Introduction

The common sea urchin *Paracentrotus lividus* is broadly distributed in the sublittoral zones of the northeast Atlantic, Mediterranean and Adriatic Seas where they are exposed to a wide range of temperature and light conditions. Populations in the Adriatic Sea show strong gonadal growth in winter followed by spawning in spring, and moderate gonadal growth in summer followed by spawning in fall (Tomšić et al. 2010). Low water temperatures and short-day photoperiods enhance reproductive development (Shpigel et al. 2004).

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It has been clearly demonstrated that the plasma membrane of unfertilized eggs of *Paracentrotus lividus* contains a Na/K transport system that is stimulated after fertilization (Ciapa et al. 1984). Na^+/K^+-ATPase activity accounts for a large portion of the energy demand in the early developmental stages of the purple sea urchin *Strongylocentrotus purpuratus* and the Antarctic sea urchin *Sterechinus neumayeri* (Leong and Manahan 1997; Marsh et al. 1999), and increases in enzyme activation and transcription of the α-subunit gene have been verified in the late stage blastula and the gastrula of a variety of sea urchin species (Mitsunaga-Nakatsubo et al. 1992, 1996).

In the present paper, we provide data on the energetics of early developmental stages of the sea urchin *Paracentrotus lividus* Lam. Since gonads are the marketable product of sea urchins, our study may be useful for cultivation and sustainable exploitation of the species (e.g. in the South Adriatic, Bistrina Bay).

Materials and methods

Fertilization and collection of early developmental stages

Adult sea urchins *Paracentrotus lividus* (Lamarck 1816) were collected in coastal non-polluted waters of the Bay of Gruž in the vicinity of the city of Dubrovnik (Croatia) during the summer of 2006 and autumn of 2007 and 2008. After induced spawning, individuals were returned to the sea. Freshly collected sea urchins were induced to spawn by injection of 1 ml 1.0 M KCl into the coelomic cavity. Sperm was collected into dry containers and stored at 4°C. Spawned eggs were cleaned from debris by washing through 500-μm mesh and suspended (1×10^6 eggs) in

500 ml filtered seawater (0.2 µm pore size Millipore filters). Before use, sperm was diluted 100-fold with filtered seawater. One ml of this sperm suspension was added to fertilize 1×10^6 eggs. Ten minutes after fertilization, eggs were gently washed through a 45-µm mesh screen and resuspended in 5–10 l seawater. Temperature was maintained at 20 ± 1°C during the experiment. Fertilized eggs were incubated in a culture container at a final concentration ranging from 30 to 250 embryos per ml. The container was put on a slowly moving shaker to keep the embryos in suspension. First, second and third cleavages appeared after 1.5, 2.0 and 3.0 h, respectively. About 12, 18, 24 and 48 h after fertilization, blastulae, gastrulae and early and late stage plutei were collected by filtration through 45-µm mesh, additionally centrifuged at 2,000×*g* for 15 min and then stored at −80°C until use.

Measurement of oxygen consumption

Oxygen consumption rates of unfertilized and fertilized eggs were measured in a closed Perspex chamber containing 50 ml filtered seawater (Millipore filter size 45 µm) at constant room temperature (20 ± 1°C). Measurements were recorded by a Digital Radiometer O_2 analyzer (Copenhagen). The tip of a pO_2 electrode (E-5046, Radiometer, Copenhagen) extended through the cover into the chamber. A magnetic stirrer gently rotated in the chamber to maintain a uniform O_2 tension. The oxygen electrode was calibrated by bubbling the seawater in the chamber with N_2 gas to provide a solution with zero O_2 saturation. Before O_2 was measured, the oxygen meter scale was adjusted to the actual barometric pressure in the oxygen-saturated medium. A suspension of eggs (470 eggs/ml) was uniformly suspended by magnetic stirring (50 revolutions/min). Unfertilized eggs were inseminated by 0.5 ml suspension of sperm diluted 10^4-fold immediately before injection into the chamber. An experiment was considered valid when more than 95% of the eggs had a regularly developed fertilization membrane within 2 min. The metabolic inhibitor rotenone (Fluka) was added into the chamber in the final concentration of 4.6 µmol/l. Methodological details of O_2 consumption measurement were described earlier (Lucu and Pavičić 1995).

Determination of Na^+/K^+-ATPase activity

Na^+/K^+-ATPase activity was determined by a modified method described in detail by Lucu and Flik (1999). Defrozen sea urchin embryos were homogenized (1 g fresh weight/5 ml medium) in ice-chilled hyposmotic medium (hypo) that consisted of 12.5 mM NaCl, 1 mM HEPES, 1 mM DTT and 0.5 mM EDTA. The pH was adjusted by TRIS base at 8.1. Immediately before use, the protease inhibitor aprotinin was added (0.1 ml/100 ml hypo). Embryos were homogenized in a Dounce homogenization device by 80 strokes and then centrifuged at 1,000×*g* for 15 min. The supernatant was separated, and Na^+/K^+-ATPase activity was assayed in triplicate by incubating 150 µl of supernatant with 500 µl assay solutions A and E. Assay solution A contained 100 mM NaCl, 12.5 mM KCl, 5.0 mM $MgCl_2$, 0.1 mM EDTA, 3 mM Na_2ATP and 15 mM imidazole. The pH was adjusted by HEPES at 7.6. Solution E was composed as A, but KCl was omitted and ouabain (2.0 mM/l) was added. The difference in phosphate released from ATP in solutions A and E reflects the ouabain-sensitive Na^+/K^+-ATPase activity. After incubation in a thermostated bath at 37°C for 30 min, the reaction was stopped by the addition of 1 ml ice-cold 8.6% trichloracetic acid. Liberated phosphate was quantified spectrophotometrically by the addition of 1 ml of 1.14% (wt/vol) ammonium heptamolybdate dissolved in sulphuric acid, supplemented immediately before use with $FeSO_4 \times 7H_2O$ (4.6 g/50 ml). After 30-min incubation, the change in absorbance at 700 nm was recorded. Total ATPases were quantified by triplicates of 100 µl 1.62 mM P_i Sigma standards in assay solution.

The protein content of homogenates was estimated by the Coomassie Brilliant Blue reagent procedure (Bradford 1976; Bio-Rad Perth, Scotland). The reference standard for protein determination was bovine serum albumin (BSA; 2 mg/ml). Protein concentration was measured spectrophotometrically at 595 nm wavelength.

Calculated values were expressed as means ± SD of two small sample groups to determine whether differences between them are significant (Quattro Pro Computer test). Statistical analyses consisted of paired two-tailed Student *t*-test. *P* values of <0.05 were considered significant. Significant differences between treatments were accessed by ANOVA in combination with Turkey test. Normality and homogeneity of variances assumptions were checked (Zar 1999).

Results and discussion

The rate of oxygen consumption of unfertilized eggs was 0.12 µmol O_2 mg protein^{-1} h^{-1}. Immediately after fertilization, the oxygen consumption rate increased by a factor of 3.2 (0.38 µmol O_2 mg protein^{-1} h^{-1} 10 min after fertilization; Fig. 1). The increased respiration following fertilization of eggs is due to the elevation of the latent capacity of the mitochondrial electron transport chain coupled with oxidative phosphorylation. This change most probably results from a transient rise in calcium and extracellular pH, leading to structural and enzymatic

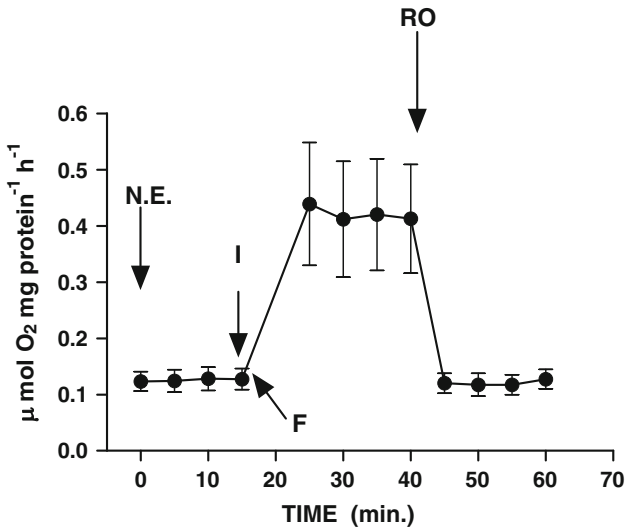

Fig. 1 Time course of oxygen consumption rate of unfertilized and fertilized eggs of the sea urchin *Paracentrotus lividus* Lam. During a 15-min exposure in an oxygen electrode chamber, unfertilized eggs showed a low and stable oxygen consumption rate (*N.E.*; 0–15 min). The times of insemination (*I*), formation of the fertilization membrane (*F*) and addition of rotenone (*RO*) are indicated by *arrows*. Mean values ± SD of six samples each

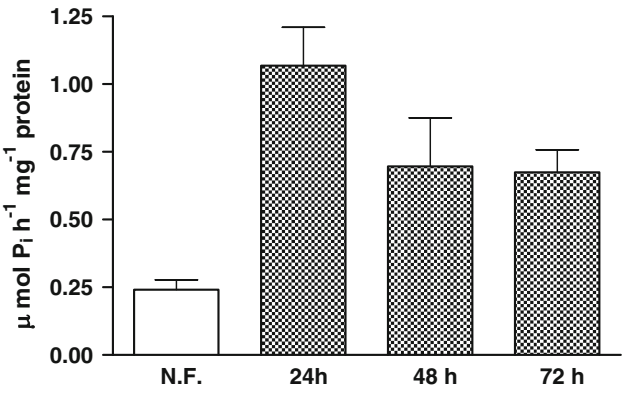

Fig. 2 Specific activity of the Na^+/K^+-ATPase in homogenates of unfertilized eggs (*N.F.*) and of early developmental stages (blastulae/gastrulae, early and late plutei) at different times (24, 48, 72 h) after fertilization. Mean values ± SD of six samples each. Level of significance between differences in enzyme activities was tested by Turkey test; $^{**}P < 0.001$; $^{*}P < 0.05$

changes that ultimately initiate DNA synthesis and rapid cell divisions (Epel 1990; Runft et al. 2002).

When eggs 25 min after insemination were exposed to 4.6 µmol/l rotenone, oxygen consumption rate returned to the level of unfertilized eggs (Fig. 1). In the presence of rotenone, the perivitelline space and the fertilization membrane were not morphologically malformed, but cleavage was completely blocked and further development of the embryo was inhibited. A marked increase in the oxygen consumption rate upon fertilization was also found for other sea urchin species by Fujiwara et al. (1986) and Selak and Scandella (1987). Rotenone is a highly specific metabolic poison that affects cellular aerobic respiration, blocking mitochondrial electron transport by inhibiting NADH-ubiquinone reductase (Singer and Ramsay 1994). Cellular uptake of oxygen is blocked, and production of cellular energy in the form of nucleoside triphosphates is greatly reduced. The inhibitory effect of rotenone is attributed to irreversible binding and inactivation of complex I of the mitochondrial electron transport chain, thereby inhibiting oxidative phosphorylation. In sea urchin eggs, intracellular Ca^{2+} of mitochondria is a multisite activator of oxidative phosphorylation. Ca^{2+} signals and mitochondrial ATP production stimulate embryonic development (Eisen and Reynolds 1985; Rizutto et al. 2000). Mitochondria isolated from unfertilized and fertilized eggs of sea urchins *Strongylocentrotus purpuratus* exhibit coupled 5′-diphosphate (ADP)-dependent oxidation of flavine- and pyridine-linked substrates. In unfertilized eggs, cytochrome was extracted more efficiently. The toxicity of rotenone has been attributed to the inhibition of cell respiration by blocking the oxidation of nicotinamide adenine dinucleotide (NAD) at complex I of the electron transport chain, thus maintaining a high $NADH/NAH^+$ ratio (Pastorino et al. 1995). This may result in a collapse of mitochondrial proton motive force necessary for ATP production (Lindahl and Oberg 1961).

Characterization of the Na^+/K^+-ATPase specific activity in early developmental stages of the sea urchin *Paracentrotus lividus* Lam. strongly suggests that the Na^+/K^+-ATPase contained within unfertilized eggs was stimulated after fertilization. In unfertilized eggs, ouabain slightly inhibits total ATPase activity. Activity of the enzyme increased after fertilization, reaching a maximum of 1.07 µmol P_i h^{-1} mg $protein^{-1}$ 24 h after fertilization in late stage blastulae and early gastrulae, while the values slightly decreased in early and late stage plutei 48 and 72 h after fertilization (Fig. 2).

Early developmental stages have a higher specific rate of energy utilization than adult stages. In the embryonal stages, a substantial fraction of the ATP energy was attributed to Na^+/K^+-ATPase activity. For the blastula and gastrula stages, 23 and 19% of respective metabolic energy could be accounted to sodium pump activity (Leong and Manahan 1997). About 75% of the metabolism of blastula and gastrula stages can be attributed to a combination of Na^+/K^+-ATPase activity and protein synthesis. In spite of large differences in the rates of protein synthesis, a relatively constant energy cost per unit mass of proteins synthesized was found during phases of sea urchin development (Pace and Manahan 2006).

Under extreme temperature conditions, Antarctic sea urchins showed an increase in the metabolic rate from

blastula to late gastrula stage (Shilling and Manahan 1994). In the early development of these organisms, the activity of the Na^+/K^+-ATPase required about 40% of the total metabolic energy, maintaining intracellular ionic gradients and generating secondary active transport processes of ions and small organic molecules. The maintenance of ionic gradients in the cells is one of the most energy-consuming processes, expanding to 20–30% of the total energy. For the early developmental stages that are dependent on internal food supplies, this process could be of immense importance (Marsh et al. 1999, 2000).

Similar patterns of Na^+/K^+-ATPase enzyme activity were found in early developmental stages of the sea urchin *Hemicentrotus pulcherrimus* (Mitsunaga-Nakatsubo et al. 1992). According to these authors, the RNA encoding Na^+/K^+-ATPase accumulates in ectodermal cells prior to the formation of the mesenchyme in the blastula stage. The activity increased in the mesenchyme blastula stages and reached its maximum in the gastrula stage (Mitsunaga-Nakatsubo et al. 1996). Northern blot analysis using a fragment of the *Hemicentrotus* Na^+/K^+-ATPase α-subunit cDNA revealed that the mRNA is about 4.6 Kb long and is maximally expressed in the mesenchyme blastula and the gastrula stage (Mitsunaga-Nakatsubo et al. 1992).

For late stage plutei, we found that saponin-treated homogenates showed a Na^+/K^+-ATPase activity that was nearly twice higher than that of untreated homogenates (increase from 0.89 to 1.89 μmol P_i h^{-1} mg $protein^{-1}$; $N = 6$; $P < 0.001$). Such treatment with a detergent permeabilizes the membranes of intracellular organelles containing the pool of Na^+/K^+-ATPase activity, thereby allowing for the measurement of its maximum activity (Chibalin et al. 1999).

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