The Fish Biology Journal MEDAKA, 7: 21–24 (1995)

Inorganic contents of the medaka egg before and after cortical reaction

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(Received July 3, 1995)

Abstract Major anionic and cationic contents of medaka *Oryzias latipes* eggs were measured by ion chromatography and atomic absorption spectrophotometry, respectively. Differences in the total amounts of respective inorganic ions as determined with the ashes of eggs were hardly recognizable before and shortly after exocytosis. However, a decrease in the content of free Ca^{2+} and Mg^{2+} was recognized in dialysates of egg homogenates shortly after the initiation of exocytosis. The present data for *O. latipes* eggs suggest that changes in these cationic contents may be associated with the process of egg activation.

Introduction

It is generally known that Ca^{2+} is released from cytoplasmic compartments into the cytosol at an early stage of fertilization (cf. Jaffe, 1983, 1985) at the same time that other ions such as Na^+, K^+ and H^+ move across the plasma membrane (Girard *et al.*, 1982). The mobility of ions is especially critical for metabolic activation of fertilizing eggs in marine invertebrates (Epel, 1978). In yolk-rich eggs such as those of fishes and amphibians, mobility of ions has also been recognized early in the fertilization process. If the increase in cytoplasmic Ca^{2+} is inhibited by a Ca-chelator such as ethyleneglycol-bis-(β-amino-ethyl ether) N,N'-tetraacetic acid (EGTA), no exocytosis of cortical granules or vesicles (alveoli) occurs (Iwamatsu *et al.*, 1988). These facts lead to the conclusion that cytoplasmic Ca^{2+} plays an important role in the exocytosis process. However, the mechanism of exocytosis of cortical alveoli in the fish egg remains to be demonstrated.

The process of exocytosis in the fish egg should be analyzed using an *in vitro* model, as proposed by Vacquier (1975) for the sea urchin egg. Before the process of exocytosis of cortical alveoli can be studied *in vitro*, it will be necessary to clarify the intracellular ionic composition of unfertilized eggs. In the medaka *Oryzias latipes*, the metal content of eggs before and after fertilization has been measured so far only by Hori (1965). We repeated this study to ascertain in more detail the major anionic and cationic contents in fish eggs before and just after exocytosis. On the whole, the results confirm the data on cationic contents previously reported by Hori (1965). In addition, we have also obtained data on the change in free dialyzable ions within eggs upon exocytosis. The present paper reports the results.

Materials and Methods

Preparation of eggs before and after fertilization

Unfertilized medaka (*Oryzias latipes*, orange-red type) eggs were obtained from females which had spawned every day under artificially controlled conditions (14-h light, 10-h dark; 26–28°C) which were optimum for reproduction. Within 2 h after ovulation, females that had spawned every day were pithed and laparotomized. Ovaries were isolated into a saline solution (Iwamatsu *et al.*, 1976). Unfertilized eggs were released into saline from the lumina of the isolated ovaries by tearing the ovarian sac with fine forceps. Eggs undergoing fertilization were prepared by artificially inseminating them in a suspension containing sperms at a concentration of 2-3 × 10^7/ml (25°C). Activated eggs which had completed exocytosis were immediately transferred into a glass basket with vinyl netting at the bottom (Iwamatsu *et al.*, 1985) 10 min after insemination, and carefully rinsed 6 times for about 2 min in double-distilled water (100 ml) in separate Petri dishes. Cytolyzed eggs were quickly identified under a binocular dissecting microscope and discarded. This was necessary because eggs that had just completed exocytosis were fragile and sometimes collapsed during rinsing in double-distilled water.

Sampling of ashes for measurement of total ion content

Every group of 50 eggs rinsed as described above was immediately transferred into a Potter-Elvehjam homogenizer with redistilled water. This

water was completely removed by pipetting before the eggs were homogenized in 5 ml of freshly added double-distilled water. The egg homogenate was dried in a heat-dry chamber, laid in ashes brought to 140°C with a heat mantle, and oxidized twice by pouring on a few drops of H_2O_2. The ashes of one group of eggs were dissolved in 50 ml of 4 mM carbonate buffer (pH 10.2) and then analyzed for the determination of anions by ion chromatography. For another group, the ashed sample was dissolved in 5 ml of 0.5 N HCl for measurement of cation contents.

Sampling of dialysates for measurement of free ion content

The egg homogenate was dialyzed for 24 h in a cellulose tubing (retention more than M_r 10,000: Viskase Scales Corp.) against 95 ml of deionized, distilled water for measurement of cationic contents or against 95 ml of 4 mM carbonate buffer (pH 10.2) for measurement of anionic contents with continuous gentle stirring. The ion content of the dialysate was measured as the quantity of free ions.

Measurements of ion content

Cationic contents were measured using a beam atomic absorption spectrophotometer (Hitachi 207), as described elsewhere (Iwamatsu *et al.*, 1985).

Contents of anions such as F^-, Cl^- and SO_4^{2-} in the dialysate against carbonate buffer were measured simultaneously using a rapid, efficient and interference-free method (Hall *et al.*, 1986). For the determination of anionic contents, Dionex Model 12 equipped with a 6 × 50 mm precolumn (HPLC-AG 3), a 6 × 250 mm anion separation column (HPLC-AS 3), and an anion fibre suppressor were used. Experiments on each group were repeated at least 3 times. The content of each ion per egg was expressed as μg/egg, and ionic concentrations within an egg were calculated using 0.922 μl to represent the volume of an unfertilized egg. The volume of the egg including the chorion was calculated assuming the egg to be a perfect sphere, although the egg was actually an oblate spheroid measuring about 1.24 mm in horizontal diameter and a little less in vertical diameter (Iwamatsu, 1994). The resulting error was probably slight.

Results

The concentrations of Na^+, K^+, Mg^{2+} and Ca^{2+} in a medaka egg were measured before and shortly after cortical alveolar exocytosis following insemination. The results are shown in Table 1. No significant changes upon exocytosis (fertilization) were found in these ion contents of ashed groups. In dialysate groups, the contents of Ca^{2+} and Mg^{2+} underwent a small but statistically significant decrease shortly after exocytosis. A slight reduction in the K^+ contents of the dialysate was observed shortly after exocytosis but it was not statistically significant.

The contents of Cl^-, F^-, NO_2^-, NO_3^-, SO_4^{2-} and PO_4^{3-} in an medaka egg before and after fertilization were also measured and the result is summarized in Table 2. In ash groups, the contents of anions in unfertilized eggs did not significantly differ from that of fertilized eggs just after exocytosis. F^-, Cl^- and NO^{2-} were nondetectable or present in very small amounts in ash groups, compared with those in dialysate groups. In contrast, the contents of NO_3^-, PO_4^{3-} and SO_4^{2-} were significantly less in dialysates than in ashed groups before and shortly after exocytosis. In particular, NO_3^- was nondetectable in the dialysates.

Group of eggs (No. of experiments)		Na^+ (μg/egg)	K^+ (μg/egg)	Ca^{2+} (μg/egg)	Mg^{2+} (μg/egg)
Before exocytosis	A (5)	2.89 ± 0.11 (136.3 mM)	2.58 ± 0.04 (71.6 mM)	0.72 ± 0.04 (19.5 mM)	0.37 ± 0 (16.5 mM)
	B (3)	2.49 ± 0.14 (117.5 mM)	2.57 ± 0.06 (71.5 mM)	0.80 ± 0.12 (21.6 mM)	0.30 ± 0.01 (13.4 mM)
After exocytosis	A (8)	2.99 ± 0.23 (141.1 mM)	2.37 ± 0.05 (65.7 mM)	0.46 ± 0.01* (12.4 mM)	0.28 ± 0.01* (12.5 mM)
	B (7)	2.40 ± 0.05 (113.2 mM)	2.58 ± 0.09 (71.6 mM)	0.70 ± 0.06 (18.9 mM)	0.35 ± 0.02 (16.0 mM)

Table 1. Cationic content of *Oryzias latipes* egg. A: Dialysate group. B: Ash group. Each value is expressed as mean ± SE. *A significant decrease after exocytosis ($P < 0.05$). The values in parentheses (mM) indicate the respective cationic concentrations in the egg.

Group of eggs (No. of experiments)		F^- (μg/egg)	Cl^- (μg/egg)	NO_2^- (μg/egg)	NO_3^- (μg/egg)	PO_4^{3-} (μg/egg)	SO_4^{2-} (μg/egg)
Before exocytosis	A (5)	0.21 ± 0.10 (12.0 mM)	1.95 ± 0.43 (59.6 mM)	0.16 ± 0.07 (3.8 mM)	ND	0.15 ± 0.12 (1.7 mM)	2.01 ± 0.09 (22.7 mM)
	B (3)	ND	0.11 ± 0.01* (3.4 mM)	ND	2.73 ± 0.28* (47.8 mM)	2.79 ± 0.35* (31.9 mM)	4.07 ± 0.11* (45.9 mM)
After exocytosis	A (8)	0.23 ± 0.13 (13.1 mM)	1.59 ± 0.18 (48.6 mM)	0.11 ± 0.05 (2.6 mM)	ND	0.20 ± 0.08 (2.3 mM)	1.65 ± 0.26 (18.6 mM)
	B (7)	ND	0.13 ± 0.04* (4.0 mM)	0.06 ± 0.03	2.21 ± 0.56* (1.4 mM)	3.33 ± 0.94* (38.0 mM)	4.67 ± 0.11* (52.7 mM)

Table 2. Anionic content of *Oryzias latipes* egg. *Significantly different values between the dialysate (A) and ash (B) groups ($P < 0.05$). Each value is expressed as mean ± SE. ND: Non-detectable values. The values in parentheses (mM) indicate the respective anionic concentrations in the egg.

Discussion

The present measurements of cationic content in the medaka egg indicate that free Mg^{2+} is reduced shortly after exocytosis, although the total Mg^{2+} content (ash group) remains unchanged. A slight decrease in bound Mg content upon fertilization has been reported previously (Hori, 1975). A decrease in intracellular Mg following fertilization has been described in *Arbacia* eggs by Monroy-Oddo (1946) and Azarnia and Chambers (1976). In experimental investigations in which a 10 mM Mg^{2+} solution was microinjected into a restricted region of the cortical cytoplasm in unfertilized eggs (Iwamatsu and Ito, 1986; Iwamatsu *et al.*, 1988), the region injected failed to undergo not only the increase in cytoplasmic free Ca^{2+} but also exocytosis. This reveals that a non-physiologically high concentration of Mg^{2+} seems to inhibit these phenomena. Therefore, the decrease in intracellular Mg^{2+} may effectively accelerate the process of egg activation.

The content of free Ca^{2+} as well as Mg^{2+} diffusible through a cellulose membrane tended to decrease shortly after exocytosis, and the change was statistically significant. Investigations on egg homogenates (Masia, 1937; Nakamura and Yasumasu, 1974) and intact eggs (Azarnia and Chambers, 1976) of the sea urchin have demonstrated an increase in free Ca^{2+} following fertilization. Substitution of Ca^{2+} for Mg^{2+} is required for the hardening of the chorion (egg membrane) (Yamagami *et al.*, 1992) and exocytosis (Yamamoto, 1961; Gilkey, 1981) in fertilization. Transiently increased free Ca^{2+} and Mg^{2+} during exocytosis may be sequestered by binding to proteins of the chorion, the plasma membrane and the cortical cytoplasm. Consequently, diffusible Ca^{2+} decreases upon the initiation of fertilization. During cortical reaction, the Ca^{2+} content of the intact eggs increases, while that of naked eggs does not (Iwamatsu *et al.*, 1985). As pointed out by Azarnia and Chambers (1976) for the sea urchin, the initial increase in Ca content represents the absorption of extracellular Ca^{2+} by cortical granule materials discharged following fertilization. At present, the ratio of the non-dialyzable Ca^{2+} in the perivitelline fluid to that of the cytoplasm is unclear.

The Ca concentration of 20 mM (ca. 18 mM in Hori, 1973) in the medaka egg seems to be very high, if free Ca^{2+} exists in the cytoplasm at this concentration. During exocytosis of cortical alveoli in medaka eggs the concentration of free Ca^{2+} in the cortical cytoplasm increases transiently from 0.1 μM to about 30 μM (Gilkey, 1981; Gilkey *et al.*, 1978). This disagreement in values for the concentration of free Ca^{2+} may be reconciled if (1) a large amount of Ca^{2+} is contained in the yolk mass, which is compartmented away from the cortical cytoplasm, or (2) most Ca^{2+} binds to Ca-binding proteins with molecular weights low enough to be dialyzable. The present data on ion content should be clarified by further investigations on the Ca^{2+} distribution within the cortical cytoplasm and the yolk mass of the egg.

The concentration of K^+ (approx. 72 mM) in eggs of the orange-red type medaka that was measured in the present study coincides with that reported by Ikeda (1937), in which the concentrations of K^+ in the newly spawned medaka egg were 62.6 mM (wild medaka) and 72.1 mM (orange-red type medaka). The values of both Na^+ and K^+ are, however, lower in comparison with those reported by Hori and Kohno (1974). This difference may be attributable to differences in technique and preparation procedures, such as differences in the duration and temperature during the rinsing of eggs, which cause deviation in the

egg size (volume of yolk content). The present data are useful for physiological studies of the medaka egg. However, a problem remains as to whether or not the concentrations of K^+ and Ca^{2+} represent those in the cortical cytoplasm or in the yolk mass of the medaka egg.

The concentration ratio of K^+ to Na^+ (0.5–0.6) in unfertilized medaka eggs in the present study is quite similar to that (about 0.8) described by Hori (1958). The K^+/Na^+ ratio in this fish is also similar to 1.0 of the hen (Morrill and Kostellow, 1991) and about 1.6 of the frog *Rana pipiens* (Morrill, 1965), in contrast with about 4.0 (Rothschild and Barnes, 1953), 10 (Hori, 1965) and 6.05 (Girard *et al.*, 1982) in sea urchin eggs before fertilization, and 9.0 after fertilization (Cameron *et al.*, 1988). The content of neither Na^+ nor K^+ in ashes of medaka eggs changed in the early step of fertilization, although the K^+ content slightly fluctuated in dialysate groups. This change, however, was not statistically significant. Electrical investigations (Ito, 1962; Nuccitelli, 1980a,b) revealed increased permeability (efflux) to K^+ due to opening of the K^+ channels in the plasma membrane during a transition period of fertilization, suggesting a transient decrease in the intracellular K^+ content during the short hyperpolarization period. A few minutes after sperm stimulation both the K^+ conductance and membrane potential recover to the same or a slightly higher level than those of the unfertilized egg, synchronous with the completion of exocytosis. Therefore, the present data probably indicate that K^+ levels were measured after K^+ conductance and membrane potential levels recovered to the same levels as those of unfertilized eggs. K^+ content reaches a plateau 1 h after fertilization at 20% above the level of the unfertilized egg (Hori, 1958).

The present measurements reveal that the total levels of anions examined do not fluctuated upon fertilization, except that free SO_4^{2-} is apt to decrease shortly after exocytosis. The cause for the fluctuation in the content of free SO_4^{2-} is unclear, although in the sea urchin egg, sulfate is released at fertilization by splitting it off from mucopolysaccharides contained in cortical granules (Aketa, 1962, 1963). NO_3^-, PO_4^{3-} and SO_4^{2-} may bind to non-dialyzable substances because most of the ions did not appear in the dialysate. Little or no F^- and Cl^- ions were detected in ash groups. This means that these anions may be easily volatilized during preparation of ash samples.

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