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Regulation of salmonid fish sperm motility by osmotic shock-induced water influx across the plasma membrane

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ABSTRACT

The motility of salmonid fish sperm is initiated by a decrease in the extracellular K^+ concentration. However, our previous studies revealed that salmonid fish sperm motility could be initiated in the presence of an inhibitory concentration of K^+ by drastic osmotic shock induced by suspension in a hypertonic glycerol solution and subsequent dilution in a hypotonic solution (glycerol-treatment). In the present study, we examined if an osmotic shock-induced water influx is involved in the regulation of salmonid fish sperm motility. $HgCl_2$, a common inhibitor of aquaporins (AQPs), decreased the duration of salmonid fish sperm motility. Dilution of sperm cells in a hypotonic solution increased the cellular volume, whereas $HgCl_2$ inhibited such an increase in cellular volume. Furthermore, the expression of AQP 1a and 10 in rainbow trout testes was confirmed. In contrast, $HgCl_2$ did not affect glycerol-treated sperm motility, indicating that AQPs are not involved in glycerol-treated sperm motility. We also explored the possibility of aquaporin-independent water influx in glycerol-treated sperm by assessing the sperm membrane permeability using propidium iodide. The plasma membrane of glycerol-treated sperm was considerably permeabilized. The cellular volume was decreased in a hypertonic glycerol solution and increased upon subsequent hypoosmotic shock, indicating an AQP-independent water flux across the plasma membrane upon glycerol-treatment. Taken together, these results showed that water influx across the plasma membrane via AQP is crucial for the maintenance of salmonid fish sperm motility under normal conditions, whereas water influx by osmotic shock-induced membrane permeation is critical for the initiation of glycerol-treated sperm motility.

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1. Introduction

Fish spermatozoa from many species are kept immotile in the testis and initiate motility by a drastic change in the extracellular environment, such as osmotic pressure during spawning (Alavi and Cosson, 2006). In marine teleosts such as flounder, *Kareius bicoloratus*, and puffer fish, *Takifugu niphobles*, the release of spermatozoa into a hyperosmotic environment upon spawning causes hypertonic-induced Ca^{2+} mobilization from the intracellular store, leading to the initiation of sperm motility (Oda and Morisawa, 1993). Conversely, spermatozoa of freshwater teleosts, such as carp and zebrafish, initiate motility by hypotonic shock-induced Ca^{2+} influx when released into a hypotonic environment upon spawning (Takai and Morisawa, 1995; Krasznai et al., 2000).

In contrast to the teleosts mentioned above, salmonid fish sperm motility is suppressed by a high concentration of K^+ in the testis, for example, 37 mM in rainbow trout (Morisawa et al., 1983), and is initiated by a decrease in extracellular K^+ upon spawning (Morisawa and Suzuki, 1980). However, our previous study revealed that salmonid fish sperm motility is initiated even in the presence of inhibitory extracellular K^+ when sperm were treated with a 10% glycerol solution (Morita et al., 2005). We have further investigated the mechanism of glycerol-treatment caused motility initiation in salmonid fish sperm and showed that glycerol treatment caused osmotic shock-induced transient Ca^{2+} mobilization from the intracellular Ca^{2+} store, thereby initiating sperm motility (Takei et al., 2012). This result suggested that salmonid fish sperm motility could also be initiated by osmotic shock-triggered Ca^{2+}-dependent signalling pathways, but the mechanisms underlying glycerol-treated sperm motility initiation still remain to be elucidated.

Osmotic shock consequently induces a water influx/efflux across the plasma membrane along the anti-osmotic gradient. Indeed, a hypotonic shock caused swelling of the cellular volume in carp and freshwater-

Abbreviations: AQP, aquaporin; ASP, artificial seminal plasma; LOS, low osmotic solution; PI, propidium iodide; PTS, potassium test solution; STS, sodium test solution

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acclimated tilapia spermatozoa, suggesting an active water influx across the plasma membrane along the anti-osmotic gradient (Perchec-Poupard et al., 1997; Morita et al., 2003). The events downstream of water permeation are well investigated: Downstream of such water-permeation-induced cell volume expansion (or shrinkage), the opening of mechano-sensitive ion channels is common in various cell types (Lansman et al., 1987; Liedtke et al., 2000). In sperm cells, the involvement of mechano-sensitive channels in sperm motility initiation has been reported by several authors (Rossato et al., 1996; Krasznai et al., 2003). However, very few reports have focused on the effect of water permeation itself on sperm motility and the molecules responsible for water permeation.

The influx/efflux of water could be mediated by aquaporins (AQPs), a well-known water channel that allows rapid water transport across cell membranes (Preston et al., 1992a). Several authors have reported the relevance of AQPs to sperm physiology in sea bream (*Sparus aurata*) (Zilli et al., 2009), mouse (Chen et al., 2011), and human sperm (Saito et al., 2004; Yeung et al., 2010). It has been reported that AQPs in mammalian (mouse and human) spermatozoa are involved in volume regulation (Yeung et al., 2010; Chen et al., 2011), while AQPs in sea bream spermatozoa are involved in motility activation (Zilli et al., 2009). There are various subtypes of AQPs: AQP 1 in canine sperm (Ito et al., 2008); AQP 3, 7, 8, 9 in mouse sperm (Sohara et al., 2007; Yeung et al., 2009; Chen et al., 2011); AQP 7 in human sperm (Saito et al., 2004); and AQP 1a and aquaglyceroporin (later identified as AQP 10b; Cerdà and Finn, 2010) in sea bream sperm (Zilli et al., 2009). Meanwhile, neither the existence of AQPs in salmonid fish sperm nor their physiological role has been investigated.

In the present study, we examined the effect of $HgCl_2$, a conventional potent inhibitor of AQPs (Preston et al., 1992a, 1992b), on salmonid fish sperm motility and searched for AQPs in salmonid fish testes. Furthermore, we investigated whether water permeated across the plasma membrane when sperm were exposed to osmotic shock upon motility initiation, using intact sperm and glycerol-treated sperm that we previously investigated (Takei et al., 2012). Finally, we showed that water-influx either via AQPs or via a hole made by glycerol treatment played an important role in the regulation of salmonid fish sperm motility.

2. Materials and methods

2.1. Chemicals and solutions

Dithiothreitol (DTT) was purchased from Sigma chemical company (St Louis, MO, USA). EGTA, EDTA and Hepes buffer were purchased from Dojindo (Kumamoto, Japan). All other chemicals were of reagent grade and were purchased from Wako Pure Chemicals (Osaka, Japan).

The glycerol solution consisted of 1.3 M glycerol, 150 mM KCl, 0.5 mM DTT, 0.5 mM EDTA and 20 mM Hepes–NaOH, pH 8.0. The sodium test solution (STS) contained 100 mM NaCl and 10 mM Hepes–NaOH, pH 8.0, and the potassium test solution (PTS) contained 100 mM KCl and 10 mM Hepes–NaOH, pH 8.0. Artificial seminal plasma (ASP) consisted of 130 mM NaCl, 40 mM KCl, 2.5 mM $CaCl_2$, 1.5 mM $MgCl_2$ and 10 mM Hepes–NaOH, pH 8.3, as reported previously (Kho et al., 2001; Takei et al., 2012). The extremely low osmotic solution (LOS) contained 10 mM Hepes–NaOH, pH 8.0.

2.2. Experimental animals and collection of sperm

In the present study, we used spermatozoa from two species of fishes for salmonid fish sperm. The Donaldson strain of rainbow trout (*Oncorhynchus mykiss*), which matures from late November to early January, was kindly provided by the Oshino Branch of Yamanashi Prefectural Fisheries Experiment Station and the National Research Institute of Aquaculture. The rainbow trout were kept in a laboratory aquarium at 12 ± 1 °C with controlled lighting in a 12 h light/12 h dark cycle. Seven males in total were used for the motility assay, the membrane permeability assay and measurement of cellular volume. Rainbow trout for RT-PCR were reared at the Nikko Branch of the National Research Institute of Aquaculture, Fisheries Research Agency, Tochigi, for successive generations in outdoor concrete ponds supplied with flow-through spring water at 10 °C.

Chum salmon (*Oncorhynchus keta*) returning to the Otsuchi River in the Iwate prefecture, Japan, were caught at the mouth of the river from late November to December and were kindly provided by the Otsuchi fisherman's union.

Milt was collected by inserting a glass pipette directly into the sperm duct from the cloaca to avoid contamination of the urine and was stored on ice until use within 4 h.

2.3. Motility initiation

Motility initiation in collected sperm was carried out by two methods as reported previously (Takei et al., 2012). First, milt was diluted directly into a 2000 volume of potassium-free STS (intact sperm).

Second, the milt was diluted 100-fold with ice-cold 1.3 M glycerol solution for 15 s, as described above; then, 2 µl of the sperm suspension was resuspended in 40 µl of PTS on a glass slide (glycerol-treated sperm).

Sperm movements were recorded from sperm dilution in experimental media to the cessation of flagellar beating with a video recorder (HR-G11, Victor JVC, Kanagawa, Japan) and a CCD camera (WAT-902H ULTIMATE, Watec, Yamagata, Japan) mounted on a phase-contrast microscope (OPTIPHOTO; Nikon, Tokyo, Japan). A time counter was connected to the video recorder so that the exact time was always displayed on the lower part of the screen. Sperm motility (% of motile sperm) was assessed by means of the methods previously described by Takei et al. (2012) and was determined within 5 s post-activation. The duration of motility was defined as the period from the time the sperm was diluted in the experimental solution to the time when >95% of the spermatozoa stopped flagellar beating. The duration of spermatozoa motility was determined from time counter on the lower part of the video recordings.

2.4. Effect of mercury chloride (II)

Intact sperm or glycerol-treated sperm was diluted into STS or PTS containing 1–10 µM $HgCl_2$, and sperm motility was analysed as described above. To determine whether the mercurial effect was reversed by a reducing agent (Preston et al., 1992a, 1992b; Zilli et al., 2009), 0.5 mM dithiothreitol (DTT), was added to each solution, and changes in sperm motility were analysed.

2.5. Membrane permeability assessment

Membrane permeability was assessed using two methods. First, sperm membrane permeability was assessed by fluorescence labelling using propidium iodide (PI). Intact sperm or glycerol-treated sperm were suspended in either STS or PTS containing 7.5 µM of PI to initiate motility. Fluorescent images were digitally recorded using an EMCCD camera (Luca (S), ANDOR Technology, Belfast, Northern Ireland) mounted on a fluorescence microscope (BX51, Olympus, Tokyo, Japan), and the percentage of PI-stained cells was evaluated.

Second, sperm membrane permeability was assessed by membrane-impermeable dynein inhibitors such as $NiCl_2$ and vanadate. Glycerol-treated sperm were diluted into PTS containing vanadate or $NiCl_2$, and the duration of sperm motility was determined. $NiCl_2$ and vanadate did not shorten the duration of the motility of intact sperm (data not shown).

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