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Intracellular changes in Ca^{2+}, K^{+} and pH after sperm motility activation in the European eel (*Anguilla anguilla*)

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Abstract

The goal of this study was to check if intracellular sperm Ca^{2+}, K^{+} and pH changed upon sperm motility activation by hyperosmotic shock in the European eel sperm. It was observed that $[Ca^{2+}]_i$ and $[K^{+}]_i$ increased after the sperm motility initiation, while pH_i showed a gradual decrease over time. An accumulation of Ca^{2+} and K^{+} on the mitochondrion was observed before sperm motility activation. These results are discussed in relation with the current models explaining the sperm motility activation in both marine and freshwater fish.

Keywords

Sperm motility; ion flux; calcium; potassium, pH, flow cytometry

1. Introduction

In marine teleosts, spermatozoa are quiescent in isotonic solutions, such as seminal plasma, and become motile in contact with hypertonic solutions, suggesting that motility is suppressed by the seminal plasma osmolality that is initiated by exposure to hypertonic seawater at spawning (Morisawa and Suzuki, 1980, Cosson, 2004). The osmotic shock faced by the spermatozoa when they are released into the marine environment leads to a rapid flux of ions and water between intracellular and extracellular compartments (Oda and Morisawa, 1993; Zilli et al., 2009). Ca^{2+} and K^{+} ions have been proposed as the main triggers of sperm motility initiation in marine fishes (Morisawa, 2008), but the exact mechanisms are still unknown. Although both in marine and freshwater fish species, an intracellular increase in Ca^{2+} has been observed after activation, it has been never studied in eel sperm. For instance, it has been reported that the seawater tilapia (*Oreochromis mossambicus*) requires extracellular Ca^{2+} as well as osmotic shock for motility activation (Linhart et al., 1999). However, Krasznai et al. (2003) showed that extracellular Ca^{2+} was not necessary for the sperm activation in puffer fish (*Takifugu niphobles*), but a hyperosmotic shock was required to release Ca^{2+} from intracellular stores. In marine fish species is neither clear if K^{+} flows from the sperm to the external environment, like it has been proposed for salmonid and cyprinid fish (Morisawa, 2008), if it flows from the environment into the sperm cell, like it has been suggested in puffer fish (Takai and Morisawa, 1995) or even if it could be released from the intracellular compartment. Probably the differences reported in the literature indicate the species specificity of the mechanisms of motility activation and signal transduction.

On the other hand, intracellular pH has not received much attention as a factor regulating sperm motility in marine fish. It has been demonstrated that a decrease in pH_i can suppress sperm motility in hypertonic solutions (Peñaranda et al., 2009) and, on the contrary, the increase in pH_i plays an important role in the initiation of sperm motility in some marine species (Oda and Morisawa, 1993).

Thus, this study was designed to observe the changes of intracellular Ca^{2+}, K^{+}, and pH after motility activation in European eel spermatozoa. In this study we used flow cytometry to describe the variations of these factors at several moments during the initiation of sperm motility, with the aim of establishing a first hypothesis on the motility activation in this species.

2. Materials and methods

2.1 Fish handling and sampling

Fifteen adult eel males (100±2 g; 40±5 cm) from the fish farm Valenciana de Acuicultura, S.A. (Puzol, Valencia; East coast of Spain) were moved to aquaculture facilities at the Universitad de León (Spain). The fish were distributed in three 60-L aquaria (5 males per aquarium) equipped with separate recirculation systems, thermostats and covered to maintain constant darkness. The eels were gradually acclimatized to artificial seawater (Aqua Medic Meersalz, 37 g/l) and once a week they were anaesthetized with benzocaine (60 ppm) and weighed before receiving the administration of hormone (hCG; 1.5 IU g^{-1} fish; *Argent Chemical Laboratories*, USA) by intraperitoneal injection during 13 weeks. Fish were fasted throughout the experiment and were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC).

2.2 Sperm collection and evaluation

Sperm samples were collected 24 h after the administration of the hormone to obtain the highest quality sperm (Pérez et al., 2000). Before sperm collection and after cleaning the genital area with freshwater and thoroughly drying to avoid the contamination of the samples with faeces, urine and seawater, the sperm was collected by abdominal pressure. A small aquarium air pump was modified to obtain a vacuum breathing force and to collect the sperm in a tube.

Samples were collected between the 6^{th} and the 14^{th} week after the first injection and they were kept in plastic tubes (4 ^{o}C) for 1-2 h before the analyses. Sperm was activated by mixing 1 µl of sperm with 200 µl of artificial seawater (SW; Aqua Medic Meersalz, 37 g/l, with 2% BSA (w/v), pH adjusted to 8.2) and the intracellular concentrations of Ca^{2+}, K^{+} and H^{+} were estimated pre-activation and at 30, 60 and 120 s post-activation. Motility was assessed subjectively for triplicate and only samples having >50% of motile cells were selected for its study.

2.3 Determination of intracellular Ca^{2+}, K^{+} and H^{+}

Fresh sperm samples were diluted 1:100 in P1 medium (in mM: NaCl 125, $NaHCO_3$ 20, KCl 30, $MgCl_2$ 2.5, $CaCl_2$ 1, and pH 8.5; Peñaranda et al., 2010) and the relative

amounts of different ions were determined by flow cytometry using a CyAn ADP Flow Cytometer (Beckman Coulter, Brea, CA). For $[Ca^{2+}]_i$ determination, spermatozoa were loaded with Fluo-4 AM indicator (Invitrogen) for a final concentration of 1 µM during 30 min. For $[K^+]_i$ determination, spermatozoa were loaded with PBFI AM indicator (Invitrogen P1267) for a final concentration of 5 µM during 90 min; and for $[H^+]_i$ determination, spermatozoa were loaded with Snarf-5F AM indicator (Invitrogen S23923) for a final concentration of 5 µM during 45 min. In all the cases sperm incubations were carried out at room temperature (20 ºC). To exclude dead cells from the analysis, spermatozoa were also incubated with TO-PRO®-3 (Invitrogen T7596) for a final concentration of 2 µM. Ions concentration in sperm was measured before and 30, 60 and 120 s after the addition of activation media.

Fluo-4 AM and Snarf-5F AM were excited by the blue laser (488 nm), and their fluorescence was read with the FL1 (530/40BP filter) and FL4 (680/30BP filter) photodetector, respectively. PBFI AM was excited by ultraviolet light (340 nm) and its fluorescence was read with the FL6 photodetector (450/50BP filter). TO-PRO-3 was excited using red laser (635 nm), and its red fluorescence was read with the FL8 photodetector (665/20BP filter). Fluorescence data was displayed in logarithmic mode. Ten thousands events were collected per sample, with a flow rate of 200 cells/s, using a gate in forward and side scatter to exclude debris and aggregates from the analysis.

2.4 Ca^{2+} and K^+ location on quiescent spermatozoa

An aliquot of sperm samples incubated independently with Fluo-4 AM and PBFI AM (see section 2.3) was used to obtain microphotographs through a Nikon Eclipse E600 microscope. UV-2A (ultraviolet excitation and blue emission) and B-2A (blue excitation and green emission) filters were used for Ca^{2+} and K^+ images, respectively.

2.5 Statistical analysis

Weasel software (WEHI, Victoria, Australia) was used to analyze the data obtained by flow cytometry. After removing dead spermatozoa (TO-PRO®-3) from the analysis, the mean fluorescence intensity (MFI, arbitrary units) was obtained from each sample. Statistical analyses were performed using the statistical package SPSS version 19.0 for Windows software (SPSS Inc., Chicago, IL, USA). Shapiro-Wilk and Levene tests were used to check the normality of data distribution and variance homogeneity, respectively. One-way analysis of variance (ANOVA) was used to analyze data with normal

distribution. Significant differences were detected using the Tukey multiple range test (P<0.05). For non-normally distributed populations, Kruskal-Wallis one-way ANOVA on ranks was used.

3. Results

3.1 Intracellular concentrations of Ca^{2+}, K^+ and H^+

Intracellular concentrations of Ca^{2+}, K^+ and H^+ were estimated on pre- and post-activated sperm cells. Intracellular [Ca^{2+}] increased significantly 30 s after the sperm activation respect the baseline levels, and it remained at this level until the end of the activation (120 s) (Figure 1A). $[K^+]_i$ showed a similar pattern (Figure 1B), increasing significantly after the sperm activation and remaining in a similar level. However, $[Ca^{2+}]_i$ increase was higher than the $[K^+]_i$ increase. In contrast to Ca^{2+} and K^+, the pH (Figure 1C) decreased progressively after sperm activation, showing significant differences with pre-activation levels at 60 and 120 s.

3.2 Intracellular distribution of Ca^{2+} and K^+

Images of Figure 2 show the calcium and potassium locations on quiescent eel spermatozoa. In the apical zone of the head higher fluorescence intensity for both Ca^{2+} and K^+ could be observed, which corresponds with the location of the eel mitochondria.

4. Discussion

It is not well known the molecular mechanism generated after the hyperosmotic shock, and several fluctuations of different ions could act like triggers of sperm motility. We have shown by first time that intracellular calcium and potassium increased at hyperosmotic activation of sperm motility in European eel, although. such increases at the osmotic shock have been previously observed in other marine species; puffer fish (Oda and Morisawa, 1993) or not only marine fish but salmonid species (Tanimoto et al., 1994).Therefore, the role of fluctuation of Ca^{2+} and K^+ seems to be also involved in motility initiation in European eel sperm, which agree with the hypothesis from Morisawa (2008) for explaining motility initiation in marine fish.

Regarding Ca^{2+}, it has been reported that this ion plays an important role in the control of the axonemal movement in some marine species (Zilli et al., 2012). The flagellum can change its beating pattern in response to Ca^{2+} concentration, and thus, Ca^{2+}

fluctuations could regulate the spermatozoa kinetic features (Brokaw, 1991; Cosson et al., 2008). Takai and Morisawa (1995) reported in the puffer fish (*Takifugu niphobles*) that the addition of Ca^{2+} ionophore to quiescent spermatozoa induced motility initiation in the same manner as in the seawater, suggesting that $[Ca^{2+}]_i$ increase and fluctuation are sufficient for the induction of sperm motility. Regarding K^+, there are not many studies about the effect of this ion in sperm motility in marine fish species. In Atlantic croaker (*Micropogonias undulatus*), K^+ channel blockers reduced the percentage of motile cells (Detweiler and Thomas, 1998). In puffer fish it has been demonstrated recently that $[K^+]_i$ increased after sperm activation regardless the composition of the activation media (Gallego et al, 2013). Therefore, the increase of this ion at the initiation of sperm motility in the eel could have an important role as it occurs in other marine and freshwater teleosts.

In relation to intracellular pH (pH_i), a gradual decrease was observed after sperm activation. Our results disagree with the results published by Oda and Morisawa (1993) in two marine fish species, in which a transient increase in intracellular pH was observed in hyperosmolar-dependent initiation of sperm motility. The variation of pH_i as a possible mechanism regulating sperm motility has been investigated in several species: in common carp, the duration of the flagellar motion does not depend on the pH_i between 6.5 and 8.5, but it decreases significantly both below and above this range (Márián et al., 1997); in flat fish species (Inaba, 2003), pH_i values higher than 7 are necessary to induce the spermatozoa motion and, in sea urchins, the pH_i seems to be a key factor in the initiation of sperm activation (Christen et al., 1983).

Finally, regarding the ion distribution in the quiescent European eel spermatozoa, fluorescence images showed that Ca^{2+} and K^+ are located mainly into the mitochondrion, a single, small and round organelle, located in the apex of the spermatozoa head opposite to the axoneme (Marco-Jiménez et al., 2006). The accumulation of calcium stores on the mitochondrion has been previously reported in human sperm (Costello et al., 2009), but there are not evidences about potassium accumulation in any species studied so far. The ion accumulation in this organelle suggests, therefore, an important role of this cellular compartment on the activation mechanism on eel spermatozoa.

In conclusion, intracellular concentration of Ca^{2+} and K^+ increase after sperm activation in European eel, with a progressive decrease of intracellular pH. Fluorescence images suggest an accumulation of both ions on the mitochondrion, showing ion´s stores in this

cellular compartment. However, further studies (using ion channels blockers, non-electrolyte activation media, or changes in cell volume) may be necessary to determine the fluxes of these ions and this role on motility initiation on the European eel sperm.

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Figure legends

Figure 1. Intracellular ion concentrations on pre- and post-activation times (30, 60 and 120 s) in European eel spermatozoa: a) Ca^{2+}; b) K^{+} and c) pH. Asterisks indicate significant differences with baseline pre-activation levels.

Figure 2. Pictures show (a) Ca^{2+} and (b) K^{+} distribution on quiescent European eel spermatozoa. Arrows indicate mitochondrion. Scale bar = 5 µm.

299 **Figure 1**

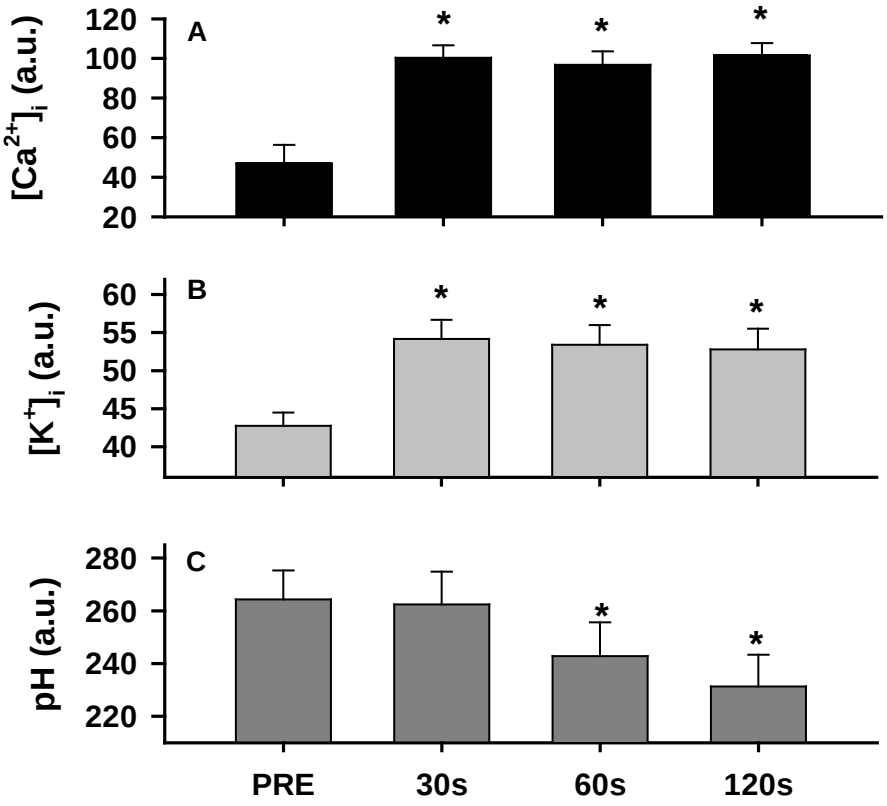

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Figure 2