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Role of calcium on the initiation of sperm motility in the European eel

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ABSTRACT

Sperm from European eel males treated with hCG_{rec} was washed in a calcium free extender, and sperm motility was activated both in the presence (seawater, SW) and in the absence of calcium (NaCl + EDTA), and treated with calcium inhibitors or modulators. The sperm motility parameters were evaluated by a computer-assisted sperm analysis (CASA) system, and changes in the $[Ca^{2+}]_i$ fluorescence (and in $[Na^+]_i$ in some cases) were evaluated by flow cytometry.

After sperm motility was activated in a medium containing Ca^{2+} (seawater, SW) the intracellular fluorescence emitted by Ca^{2+} increased 4–6-fold compared to the levels in quiescent sperm. However, while sperm activation in a Ca-free media (NaCl + EDTA) resulted in a percentage of motility similar to seawater, the $[Ca^{2+}]_i$ levels did not increase at all. This result strongly suggests that increasing $[Ca^{2+}]_i$ is not a pre-requisite for the induction of sperm motility in European eel sperm. Several sperm velocities (VCL, VSL, VAP) decreased when sperm was activated in the Ca-free activator, thus supporting the theory that Ca^{2+} has a modulatory effect on sperm motility. The results indicate that a calcium/sodium exchanger (NCX) which is inhibited by bepridil and a calcium calmodulin kinase (inhibited by W-7), are involved in the sperm motility of the European eel. Our results indicate that the increase in $[Ca^{2+}]_i$ concentrations during sperm activation is due to an influx from the external medium, but, unlike in most other species, it does not appear to be necessary for the activation of motility in European eel sperm.

1. Introduction

In teleost, spermatozoa are immotile in the testis and sperm duct, and in general they become motile when released into the surrounding water. Hyperosmotic sea water induces sperm motility in marine fish sperm, while hypo-osmotic freshwater induces sperm motility in freshwater fish species. Apart from the hyper- or hypo-osmotic shock, there are other factors involved in sperm motility acquisition, including the ion Ca^{2+} (Morisawa., 2008). Studies on the effect of this ion on sperm motility, including measurements of $[Ca^{2+}]_i$ levels, have primarily focused on freshwater fish species, such as rainbow trout (*Onchorynchus mykiss*; Cosson et al., 1989; Boitano and Omoto, 1992; Tanimoto et al., 1994; Takei et al., 2012) carp (*Cyprinus carpio*; Krasznai et al., 2000, 2003b) and tilapia (*Oreochromis mossambicus*, Morita et al., 2003). Studies of sperm $[Ca^{2+}]_i$ in marine fish species are even more scarce, restricted to pufferfish (*Takifugu niphobles*; Oda and Morisawa, 1993; Gallego et al., 2013b) and pacific herring (*Clupea pallasi*; Cherr et al., 2008), the latter of which is an unusual case, as sperm activation is triggered by two egg molecules, one of which induces an influx of Ca^{2+} into the sperm cell. Recently it was demonstrated that, similarly to these marine species, European eel (*Anguilla anguilla*) sperm experienced an increase in $[Ca^{2+}]_i$ during sperm activation in seawater (Gallego et al., 2014). However, it is not known if this increase in $[Ca^{2+}]_i$ is the trigger for sperm motility in this species.

Cosson et al. (1989) demonstrated that there is an increase in $[Ca^{2+}]_i$ in rainbow trout sperm when sperm cells start to move, whereas in conditions where the spermatozoa are immotile, for example after being washed in a Ca-free extender and activating in a Ca-free activator, the $[Ca^{2+}]_i$ levels did not increase. In other studies, an increase in $[Ca^{2+}]_i$ post-activation has been observed even in the absence of external Ca^{2+}, indicating that the increase in $[Ca^{2+}]_i$ is due to it being released from intracellular stores (rainbow trout, Boitano and Omoto, 1992; puffer fish, Oda and Morisawa, 1993; tilapia, Morita et al., 2003). In rainbow trout and carp sperm (Cosson et al., 1989; Krasznai et al., 2000) the increase in $[Ca^{2+}]_i$ required an influx from the external medium, as sperm cells were immotile in the Ca-free activator. In some cases, Ca-free extenders or activators had not been used, like in the study carried out by Tanimoto et al. (1994) on salmonids. In this case, the external or internal origin of the increase in $[Ca^{2+}]_i$ could not be discovered.

Indirect evidence of the importance of Ca^{2+} fluxes on fish sperm motility comes from studies with calcium channel inhibitors. In some marine species inhibitors of voltage-gated calcium channels reduced or suppressed sperm motility (Atlantic croaker *Micropogonias undulatus*, Detweiler and Thomas, 1998; Pacific herring, *Clupea pallasi*, Vines et al., 2002). In addition, inhibitors of voltage-gated calcium channels inhibited sperm motility in other freshwater species, including the bluegill (*Lepomis macrochirus*; Zuccarelli and Ingermann, 2007) and

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sterlet (*Acipenser ruthenus*; Alavi et al., 2011), and reduced sperm curvilinear velocity (VCL) in redside dace (*Clinostomus elongatus*); (Butts et al., 2013).

Calcium has been linked to the flagellar beating pattern, inducing asymmetric beating, or circular motility, in several freshwater fish species, including rainbow trout (Cosson et al., 1989; Boitano and Omoto, 1992), and sterlet (Alavi et al., 2008); and in marine fish species, such as European sea bass (*Dicentrarchus labrax*; Cosson et al., 2008) and hake (*Merluccius merluccius*; Cosson et al., 2010). At the same time, sperm motility activation in a Ca^{2+}-free medium reduced VCL in gilthead seabream (*Sparus aurata*) and stripped seabream (*Lithognathus mormyrus*; Zilli et al., 2008). Likewise, in marine invertebrates, such as sea urchins or ascidians (Brokaw et al., 1974; Shiba et al., 2006) the degree of flagellar beating asymmetry is linked to Ca^{2+} concentrations, and in mammals, hyperactivated sperm motility, characterized by high amplitude and asymmetrical flagellar waveform, is Ca^{2+}-dependent. In mammals, sperm activation occurs in two stages: firstly, straight motility (*activated* stage) occurs during ejaculation, and later, *hyperactive*, more circular motility occurs as part of the capacitation process, in the female tract. Both *active* and *hyperactive* motility are calcium-dependent (Wade et al., 2003; Darszon et al., 2011), with hyperactive motility being mediated by an influx of Ca^{2+} through a sperm specific calcium channel (CatSper) which is a pH-dependent (Carlson et al., 2003).

In the present study the European eel was used as the experimental organism. They could be considered a marine species, as their spawning grounds are in the sea, presumably in the Sargasso Sea (Tesch, 1977; Van Ginneken and Maes, 2005). This species has a particular life cycle and this, coupled with its phylogenetic position as an ancient teleost, makes it an interesting model for the investigation of the regulatory mechanisms of reproductive physiology, and for providing insights into ancestral regulatory functions in teleost. Eel species do not mature spontaneously in captivity, but spermatogenesis and spermiation can be obtained in males after long-term treatment with human chorionic gonadotropin (hCG) (Pérez et al., 2000; Peñaranda et al., 2010). Our research group recently demonstrated (Gallego et al., 2012) that treatment with recombinant hCG (hCG_{rec}) gave better results in terms of milt quality and production levels than the traditional urine-purified hCG. Using this treatment it is possible to obtain good sperm quality (motility >60%) for at least 6 weeks from the 8th week of hormonal treatment, if the eels are injected weekly. This feature makes it a useful model for the study of sperm physiology.

In this paper, the role of calcium ions on European eel sperm motility has been studied by testing the effect of several calcium channel inhibitors or modulators on sperm motility and kinetics. Flow cytometry has been used to measure variations in $[Ca^{2+}]_i$ levels in different conditions (with or without external calcium).

2. Material and methods

2.1. Chemicals and solutions

Bepridil hydrochloride, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7), A-23187, EDTA, and Bovine Serum Albumin (BSA) were purchased from Sigma (St. Louis, MO, USA). Fluo-4AM, CoroNa Green AM, Pluronic F-127, and propidium iodide (PI) were purchased from Life Technologies (Madrid, Spain). Salts were of reagent grade.

DMSO stocks 100 mM bepridil, 100 mM W-7, 10 mM A-23187 were prepared, diluted in ultrapure water at a ratio of 1:10 (v/v). Each product was aliquoted and frozen (−20 °C). Each aliquot was used only once after being thawed. For use with the sperm, each product was thawed, and mixed with the sperm to final concentrations of 100 μM (bepridil, W-7) or 10 μM (A-23187). DMSO stocks 1 mM Fluo-4AM, 1 mM CoroNa Green AM, were prepared and used as described in Sections 2.7 and 2.8.

2.2. Preparation of Ca-free solutions

Two Ca-free solutions were prepared: a Ca-free extender (125 mM NaCl, 20 mM $NaHCO_3$, 2.5 mM $MgCl_2{}^*$ $6H_2O$, 30 mM KCl, 5 mM EDTA, 20 mM TAPS, pH adjusted to 8.5), and a Ca-free activation media (550 mM NaCl, 5 mM EDTA, 20 mM TAPS, pH adjusted to 8.2).

To avoid Ca^{2+} contamination of these solutions the glass materials were autoclaved, and then rinsed in a solution of ultrapure milliq water plus 5 mM EDTA (Yoshida et al., pers. com.). The rest of the laboratory materials were also cleaned and rinsed in ultrapure milliq water plus 5 mM EDTA. The osmolality of these solutions was checked with an Osmomat050 (Gonotec, Germany), being 325 ± 10 and 1100 ± 20 mOsm, for Ca-free extender and Ca-free activation media respectively.

2.3. Fish and hormone treatment

Eighty adult male European eels from the fish farm Valenciana de Acuicultura, S.A. (Puzol, Valencia; East coast of Spain) were moved to our facilities, in the Aquaculture Laboratory of the Universitat Politècnica de València, Spain. The fish were distributed in four 200-L aquaria (approximately 20 male eels per aquarium) equipped with separated recirculation systems, thermostats, and coolers, and covered with black panels to maintain constant darkness. The eels were gradually acclimatized to sea water (salinity 37 ± 0.3 g/L) and once a week they were anesthetized with benzocaine (60 ppm) and weighed before being administered with hCGrec (Ovitrelle®, Merck Serono; 1.5 IU per g of fish body weight) by intraperitoneal injection (as described by Gallego et al., 2012).

The fish were fasted throughout the experiment and were handled in accordance with the European Union regulations regarding the protection of experimental animals (Dir 86/609/EEC). In addition, this project received the approval of the Ethics Committee of the Polytechnic University of Valencia (Spain).

2.4. Sperm collection and sampling

The sperm samples were collected 24 h after the administration of hCG because previous studies (Pérez et al., 2000) have demonstrated that this is the moment when the best sperm quality is found. Before sperm collection, the fish were anesthetized, and the genital area was cleaned with freshwater, and carefully dried to avoid contamination with feces, urine, or sea water. The sperm was then collected in plastic tubes, by exerting abdominal massage, and refrigerated (4 °C) until the motility analyses, which took place within the first hour after collection.

2.5. Sperm motility evaluation

Sperm motility activation was carried out as per the method described by Gallego et al. (2013a); by mixing 1 μl of diluted sperm (dilution 1/25 in Ca-free extender; 125 mM NaCl, 20 mM $NaHCO_3$, 2.5 mM $MgCl_2 * 6H_20$, 30 mM KCl, 5 mM EDTA; based on P1 extender, Peñaranda et al., 2010) with 4 μl of artificial seawater (SW; Aqua Medic Meersalz, 37 g/l, with 2% BSA (w/v), pH adjusted to 8.2). The mixture was made in a SpermTrack-10® chamber, 10 μm depth (Proiser, Paterna, Spain) and observed using a Nikon Eclipse 80i microscope, with a 10× objective lens (Nikon phase contrast 10 × 0.25, Ph1 BM WD 7.0). Motility was recorded 15 s after the sperm was mixed with SW, using a high-sensitivity video camera (HAS-220) and ISAS software (Proiser, Paterna, Spain). For each motility test, samples were evaluated in triplicate. Both the sperm and the SW were maintained at 4 °C in a water bath during the sperm motility evaluation. In some cases sperm motility was activated with a Ca-free activator (550 mM NaCl, 5 mM EDTA), but SW activation was always used as a control.