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Review

Zn^{2+} induces hyperpolarization by activation of a K^+ channel and increases intracellular Ca^{2+} and pH in sea urchin spermatozoa

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ABSTRACT

Zinc (Zn^{2+}) has been recently recognized as a crucial element for male gamete function in many species although its detailed mechanism of action is poorly understood. In sea urchin spermatozoa, Zn^{2+} was reported as an essential trace ion for efficient sperm motility initiation and the acrosome reaction by modulating intracellular pH (pH_i). In this study we found that submicromolar concentrations of free Zn^{2+} change membrane potential (Em) and increase the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) and cAMP in *Lytechinus pictus* sperm. Our results indicate that the Zn^{2+} response in sperm of this species mainly involves an Em hyperpolarization caused by K^+ channel activation. The pharmacological profile of the Zn^{2+}-induced hyperpolarization indicates that the cGMP-gated K^+ selective channel (tetraKCNG/CNGK), which is crucial for speract signaling, is likely a main target for Zn^{2+}. Considering that Zn^{2+} also induces $[Ca^{2+}]_i$ fluctuations, our observations suggest that Zn^{2+} activates the signaling cascade of speract, except for an increase in cGMP, and facilitates sperm motility initiation upon spawning. These findings provide new insights about the role of Zn^{2+} in male gamete function.

Introduction

Although Zn^{2+} is a trace element, it is known to be essential for a wide range of biological processes: transcription, cell division, proliferation, immune function, defense against free radicals and fertilization (Pluth et al., 2011). In mammals, it has been known for long time that Zn^{2+} concentration in the seminal fluid is notably high (Hidiroglou and Knipfel, 1984; Mawson and Fischer, 1953). In fact, the highest concentration in the human body is in the seminal plasma (3 mM; (Saaranen et al., 1987)). Zn^{2+} has also been proposed to stabilize chromatin (Bjorndahl and Kvist, 2010) and regulate sperm capacitation (Andrews et al., 1994; Aonuma et al., 1978; Lishko et al., 2010). Furthermore, it was recently reported that Zn^{2+} is also important for male gamete motility regulation in the nematode *Caenorhabditis elegans* (Liu et al., 2013).

Upon dilution of sea urchin sperm in seawater, metal chelators such as EDTA, EGTA, phenantroline and cysteine, at concentrations that do not alter the Ca^{2+} or Mg^{2+} levels (0.1–1 mM), delay motility initiation and inhibit the AR induced by egg jelly (Clapper et al., 1985; Johnson and Epel, 1983). Among the trace ions in seawater (such as Cu^{2+} and Ni^{2+}), only Zn^{2+} can rescue these chelator inhibitory effects, indicating a crucial role of this divalent cation in sea urchin sperm physiology. In addition, Zn^{2+} is able to induce the AR of *Lytechinus pictus* spermatozoa, although its physiological relevance is unknown. The action of Zn^{2+} in sea urchin spermatozoa has been attributed to pH_i regulation (Clapper et al., 1985).

It is well established that echinoderm spermatozoa respond to sperm-activating peptides (SAPs) derived from the egg jelly of the same species which regulate motility (Suzuki, 1995). Speract is the first structurally identified SAP from the egg jelly of *Hemicentrotus pulcherrimus* (Suzuki et al., 1981) and *Strongylocentrotus purpuratus* (Hansbrough and Garbers, 1981), and also stimulates *L. pictus* spermatozoa (Guerrero et al., 2010; Nishigaki and Darszon, 2000). Speract binding to its receptor in the flagella plasma membrane stimulates the synthesis of cGMP (Garbers, 1989) which directly activates the cGMP-gated K^+ selective channel (tetraKCNG/CNGK) inducing a K^+ efflux and a membrane hyperpolarization (Babcock et al., 1992; Bonigk et al., 2009; Galindo et al., 2007). This hyperpolarization leads to increases in pH_i (Lee, 1984), $[Ca^{2+}]_i$, and cAMP levels (Beltran et al., 1996). All these changes induced by

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speract, except for the cGMP increase, are inhibited by elevating the K^+ concentration in seawater (Harumi et al., 1992).

In this study we show that submicromolar free Zn^{2+} concentrations induce similar sperm responses as speract does including Em changes, cAMP elevation and $[Ca^{2+}]_i$ fluctuations except for the cGMP increase in *L. pictus* spermatozoa. We discuss the molecular mechanisms and physiological significance of Zn^{2+} for sea urchin spermatozoa.

Material and methods

Gametes and reagents

L. pictus sea urchins were obtained from Marinus (Long Beach, CA, USA). Dry sperm were collected after intracelomic injection of 0.5 M KCl and kept on ice until used. The fluorescent dyes 3,3′-dipropylthiadicarbocyanine iodide (DiSC3(5)), Fluo-4-AM, Quin-2 and 5-(and-6)-Carboxyfluorescein diacetate were obtained from Molecular Probes (Eugene, OR, USA). Anhydrous dimethylsulfoxide (DMSO), tolbutamide, glibenclamide, were from Sigma-Aldrich. $ZnSO_4$ was from Merck. Charybdotoxin and Iberiotoxin were from Alomone Labs. The Kits to measure cAMP (TRK 432) and cGMP (TRK 500) were from Amersham. Speract was synthesized in Professor Possani´s Laboratory (IBT-UNAM) and fucose sulfate polymer (FSP) was prepared according to the previous report (Garbers et al., 1983). The rest of the reagents used were of the highest quality available.

Composition of artificial seawater (ASW)

Normal ASW was prepared with the following composition (in mM): 465 NaCl, 26 $MgCl_2$, 10 KCl, 30 $MgSO_4$, 10 $CaCl_2$, 2.5 $NaHCO_3$ and 0.1 EDTA and 10 Hepes (or otherwise indicated) with pH 8.0 adjusted with NaOH. Ca^{2+}-free pH 8.0 (0CaSW) or 1 mM Ca^{2+} pH 7.0 (1CaSW) ASWs were the same except for the indicated $CaCl_2$ concentration and pH. High K^+ ASW (50KSW) contained 50 mM KCl. Cl^--free ASW (0ClSW) was prepared by substituting NaCl and KCl with each metanesulphonate salt and substitution of $MgCl_2$ and $CaCl_2$ with each sulfate salt. In all cases the osmolarity was 950–1000 mOsm.

Fluorescence measurements with sperm suspension

Fluorometric measurements were performed in an OLIS-upgraded SLM 8000 Aminco spectrofluorometer with a temperature-controlled cell holder (14 °C) and a magnetic stirrer. Spermatozoa $[Ca^{2+}]_i$ and pH_i measurements were done according to Rodríguez and Darszon (Rodriguez and Darszon, 2003). Briefly, diluted (1:5) dry sperm in 1CaSW containing 20 μM of Quin-2-AM or Fluo 4-AM without or with 0.5% Pluronic F-127 or 10 μM Carboxyfluorescein diacetate were incubated for 3 h at 14 °C in the dark. After loading with these fluorescent dyes, 10 ml of 1CaSW were added to the sperm suspension. Coelomocytes and spines were precipitated by mild centrifugation (121 g for 7 min at 4 °C) and the sperm suspension was applied to further centrifugation (1000 g for 8 min at 4 °C) to eliminate the dyes remaining in the media. The sperm pellet was resuspended in the original volume of 1CaSW and kept on ice in the dark until used. A 10 μl aliquot of the loaded sperm was added to a flat-bottom glass tube containing 800 μl ASW. After 30 s, an agonist was added using a Hamilton syringe. Fluorescence intensities (excitation/emission wavelength) for Quin-2 (340/490 nm), Fluo-4 (505/525 nm) and Carboxyfluorescein diacetate (490/535 nm) were recorded every 0.5 s.

For Em experiments, 10 μl of diluted (1:10 in 1CaSW) sperm were added to 800 μl of ASW plus 0.7 mM EDTA and 0.5 μM DisC3(5). The EDTA concentration for Em measurements was increased because DisC3(5) induces the acrosomal reaction in ASW with 0.1 mM EDTA, but not in 0.8 mM EDTA. Fluorescence intensities of DisC3(5) (640/670 nm) were registered every 0.5 s. After 2–3 min, a protonophore (CCCP 1 μM) was added to avoid mitochondrial membrane potential interference. Once the fluorescence reached the equilibrium, an agonist was added. DisC3(5) fluorescence was calibrated using 1 μM valinomycin and sequential additions of KCl. Em was calculated according to the Nernst equation assuming that the intracellular K^+ concentration is 180 mM (Babcock et al., 1992). For fluorometric experiments, stock solutions of Fluo-4-AM (1 mM) and Carboxyfluorescein diacetate (1 mM) and DiSC3 (5) (0.8 mM) were made in anhydrous DMSO.

Single cell fluorescence imaging

Fluorescence imaging was carried out as previously reported (Nishigaki et al., 2004). Briefly, Fluo-4-labeled spermatozoa were adhered to glass coverslips coated with 50 μg/ml poly-L-lysine solution (Sigma) and mounted into a micro incubator, PDMI-2 (Harvard Apparatus, Holliston, MA, USA) maintained at 14 °C. Fluorescence images were acquired using a Nikon DIAPHOT 300 inverted microscope with a Nikon Plan Apo 60X objective lens (1.4 NA) and a custom-built stroboscopic illumination system (Nishigaki et al., 2006) with a Chroma filter set (Ex, HQ470/40x; DC, 505DCXRU; Em, HQ510LP (Chroma Technology)). Images were acquired with a Quantix 57 camera (Photometrics Inc.) under the continuous (stream) acquisition mode.

Determination of cAMP and cGMP levels

Sperm diluted (1:200) in 0CaSW with 1 mM 3-isobutyl-1-methylxanthine (IBMX) were incubated with a ligand (Zn^{2+} or speract) for 1 min at 14 °C. Subsequently, the sperm suspension was transferred to a boiling bath to quench the enzymatic reaction and extract nucleotides. TRK 432 or TRK 500 Kits of Amersham were used to measure cAMP or cGMP concentration, respectively.

Estimation of free Zn^{2+} concentration

The free Zn^{2+} concentration in ASW after various Zn^{2+} additions was estimated using a WinMaxC 2.4 software package (Stanford University, Chris Patton http://www.stanford.edu/~cpatton/downloads.htm). DisC3(5) contains a certain amount of Zn^{2+} as a contaminant and though small amount of DisC3(5) (0.5 μM) should not significantly affect the total Zn^{2+}, in most Em experiments ASW containing 0.8 mM EDTA was used. Addition of 10 μM $ZnSO_4$ to ASW containing 0.8 mM EDTA yields ~0.2 nM free Zn^{2+}.

Data normalization

Although the absolute changes in the sperm Em obtained within one season are very reproducible, there is variability between experiments with cells from different seasons. For this reason we normalized the data in order to compare them. Furthermore, in experiments where high K^+ (50KSW) was used to block the speract responses it is difficult to calibrate Em using the Nernst equation. Therefore, Em changes in 50KSW were expressed as F/F_0, where F is fluorescence intensity of DisC3 (5) and F_0 is a F value just before addition of Zn^{2+}.