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Speract, a sea urchin egg peptide that regulates sperm motility, also stimulates sperm mitochondrial metabolism

Juan García-Rincón, Alberto Darszon *, Carmen Beltrán *

Departamento de Genética del Desarrollo y Fisiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Apdo. Postal 510-3, Cuernavaca, Morelos C.P. 62210, México

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ABSTRACT

Sea urchin sperm have only one mitochondrion, that in addition to being the main source of energy, may modulate intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) to regulate their motility and possibly the acrosome reaction. Speract is a decapeptide from the outer jelly layer of the *Strongylocentrotus purpuratus* egg that upon binding to its receptor in the sperm, stimulates sperm motility, respiration and ion fluxes, among other physiological events. Altering the sea urchin sperm mitochondrial function with specific inhibitors of this organelle, increases $[Ca^{2+}]_i$ in an external Ca^{2+} concentration ($[Ca^{2+}]_{ext}$)-dependent manner (Ardón, et al., 2009. BBActa 1787: 15), suggesting that the mitochondrion is involved in sperm $[Ca^{2+}]_i$ homeostasis. To further understand the interrelationship between the mitochondrion and the speract responses, we measured mitochondrial membrane potential ($\Delta\Psi$) and NADH levels. We found that the stimulation of sperm with speract depolarizes the mitochondrion and increases the levels of NADH. Surprisingly, these responses are independent of external Ca^{2+} and are due to the increase in intracellular pH (pH_i) induced by speract. Our findings indicate that speract, by regulating pH_i, in addition to $[Ca^{2+}]_i$, may finely modulate mitochondrial metabolism to control motility and ensure that sperm reach the egg and fertilize it.

1. Introduction

Mitochondria are multifunctional organelles deeply involved in cell metabolism [1]. They possess an outer and an inner membrane (IMM) harbouring, amongst many components, complexes I–IV of the electron transport chain (ETC) and the FoF_1-ATPsynthase [2]. The hydrogen donors NADH and FADH2 transfer their electrons to complex I and II of the ETC respectively [3]. The output of protons (H^+) through complexes I, III and IV at the IMM generates an electrochemical proton gradient comprising the mitochondrial pH gradient ($\Delta pH_{mit;}$ a pH gradient across the IMM [4]) and the mitochondrial membrane potential ($\Delta\Psi$) [4,5].

Ca^{2+} entry into mitochondria depends on $\Delta\Psi$ [6,7] and alters the activity of the Ca^{2+}-sensitive intramitochondrial dehydrogenases and the FoF_1-ATPsynthase [8,9]. Ca^{2+} fluxes from this organelle, together with those in the endoplasmic reticulum, contribute to the spread and synchronization of Ca^{2+} signals throughout the cytoplasm in many cell types [10–12]. In sperm, which lack endoplasmic reticulum, the intracellular Ca^{2+} signals modulate motility, chemotaxis and acrosome reaction, all essential events required for homologous egg fertilization [13].

The single mitochondrion of sea urchin sperm, located at the base of the head, is important for motility [14], and possibly for Ca^{2+} homeostasis and the acrosome reaction [15]. It is known that sea urchin sperm obtain their energy for swimming from the oxidation of endogenous phospholipids [14,16]. Long-chain fatty acids (LCFA) generated by the hydrolysis of triglycerides are incorporated from the cytosol into the mitochondrial matrix through the carnitine palmitoyltransferase (CPT) shuttle system which consists of CPT-I, acylcarnitine translocase (IMM), and CPT-II (reforms the acyl-CoA moiety in the mitochondrial matrix). The pH-sensitive CPT-I which spans the outer mitochondrial membrane and catalyzes the transfer of long-chain fatty acyl groups, from free coenzyme A (CoASH) to carnitine, is considered the rate-limiting step in the transfer of LCFA into the mitochondria [17–20]. The re-esterified acyl-CoA in the mitochondria (by CPT-II), undergo fatty acid β-oxidation [21] that produces NADH and FADH2 yield their electrons to the ETC that provides the $\Delta\Psi$ required for the synthesis of

Abbreviations: ASW, artificial sea water; BCECF-AM, 2,7,-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethylester; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CCCP, carbonyl cyanide m-chlorophenylhydrazone; CPT-I, carnitine palmitoyl transferase-I; 0CaASW, is ASW lacking $CaCl_2$ containing 30 μM EGTA yielding a final extracellular Ca^{2+} concentration ($[Ca^{2+}]_{ext}$) of 110 nM; ΔpH_i, intracellular pH change; $\Delta\Psi$, mitochondrial membrane potential; Em, membrane potential; ETC, electron transport chain; FFA, free fatty acids; IMM, inner mitochondrial membrane; $[K^+]_{ext}$, extracellular K^+ concentration; Rhod-123, Rhodamine 123.

* Corresponding authors at: Instituto de Biotecnología, Universidad Nacional Autónoma de México, Apdo. Postal 510-3, Av. Universidad # 2001, Cuernavaca, Morelos, México, CP 62210.

E-mail addresses: darszon@ibt.unam.mx (A. Darszon), beltran@ibt.unam.mx (C. Beltrán).

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ATP [22]. The pH-sensitive dynein-ATPases at the axoneme (the flagellar motor apparatus), the main ATP-consumers [23], are finely coupled to mitochondrial respiration and pH_i [23,24].

Sperm activating peptides (SAPs) modulate motility in a species-specific manner. Speract was the first SAP isolated from the egg jelly of *Strongylocentrotus purpuratus* [25,26]. Speract binding to its receptor, which is coupled to a guanylyl cyclase (GC) in the sperm flagellum [27], stimulates enzyme activity, increasing the levels of cGMP. The cGMP increase activates the tetra-KCNG K^+ channel, inducing a transient membrane potential (Em) hyperpolarization [28,29]. This hyperpolarization activates, among others, the Na^+/H^+ exchanger and, indirectly, adenylyl cyclase (AC) activity, leading to increases in pH_i and cAMP, respectively [30–32]. The pH_i increase and subsequent Em depolarization elevate $[Ca^{2+}]_i$ by activating pH-sensitive Ca^{2+} channels [33,34], the main candidate being CatSper, a sperm-specific, mildly voltage and pH_i dependent Ca^{2+} channel [35,36]. High extracellular KCl (40 mM) inhibits this speract-activated signaling cascade, except the cGMP increase [37].

Our group reported that mitochondrial inhibitors cause a $[Ca^{2+}]_i$ increase in sea urchin sperm that depends on external Ca^{2+} [15]. As speract induces $[Ca^{2+}]_i$ oscillations [38,39], it could alter mitochondrial function and this contributes to modulate sperm swimming. To better understand how the mitochondrion impinges on the sperm responses to speract, we examined if this decapeptide affects $\Delta\Psi$, using Rhodamine-123 (Rhod-123) and NADH levels by recording its autofluorescence. Our results indicate that speract induces a mitochondrial depolarization ($\Delta\Psi$ decrease) and increases the levels of NADH.

From our previous findings we expected that the speract-induced decrease in $\Delta\Psi$ and the increase in NADH levels would depend on the $[Ca^{2+}]_i$ changes triggered by speract, but to our surprise we discovered that they were independent of $[Ca^{2+}]_{ext}$. Since speract induces a pH_i increase that precedes the $[Ca^{2+}]_i$ elevation [36,40], we explored if an artificial intracellular alkalinization would mimic the effects of speract on the $\Delta\Psi$ and NADH levels. Indeed, we observed that even at nM $[Ca^{2+}]_{ext.}$, adding 10 mM NH_4Cl to *S. purpuratus* sperm caused a decrease in $\Delta\Psi$ and an increase in the NADH levels. Notably, we also document that exposure of the cells to perhexiline or etomoxir, two inhibitors of CPT-I, partially prevents mitochondrial depolarization and the speract-induced increases in NADH levels.

Taken together, our results suggest that speract, by elevating pH_i, increases mitochondrial lipid oxidation and NADH. This increases respiration, ATP production and diminishes $\Delta\Psi$, allowing sperm to regulate their swimming mode in their search for the egg.

2. Experimental procedures

2.1. Materials

Ammonium chloride (NH_4Cl), carbonyl cyanide m-chlorophenylhydrazone (CCCP), cinnamic acid, digitonin, etomoxir, gossypol, nigericin, oligomycin, perhexiline, rotenone, sodium octanoate, valinomycin and the rest of the salts were from Sigma. Rhodamine-123 (Rhod-123) and 2,7,-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethylester (BCECF-AM), were purchased from Life Technologies. Except for KCl, NH_4Cl and etomoxir, the reagents were dissolved in anhydrous dimethylsulfoxide (DM), which in our experimental conditions reached a maximum final concentration of 0.125% *v/v*. All stock solutions were stored at −20 °C until use. Artificial seawater (ASW) contains (in mM): 485 NaCl, 10 KCl, 10 $CaCl_2$, 26 $MgCl_2$, 30 $MgSO_4$, 2.5 $NaHCO_3$, 10 Hepes, 0.1 EDTA, pH 7.8. The 1CaASW is ASW pH 7.0, containing 1 mM $CaCl_2$ and 0CaASW is ASW, pH 7.8 lacking $CaCl_2$ supplemented with 30 μM EGTA, yielding a final $[Ca^{2+}]_{ext.}$ of 110 nM (estimated using the software package WinMaxC 2.4 (Stanford University, Chris Patton http://www.stanford.edu/~cpatton/downloads.htm)). In all cases the osmolarity was 950–1000 mOsm. *S. purpuratus* sea urchins were obtained from Pamanes S.A. de C.V. (Ensenada, Baja California, Mexico). Sperm were collected by intracoelomic injection of the sea urchins with 0.5 M KCl, collected and kept on ice as dry sperm until used (within one day).

2.2. Distribution of Rhod-123 in intact sperm

Diluted sperm (1:10 in 1CaASW) were incubated with different concentrations (5, 10 and 20 μM) of the $\Delta\Psi$ fluorescent indicator Rhod-123 at 14 °C during 3 h in the dark (similar results were obtained by incubating 30 min). The Rhod-123 distribution in the whole cell was recorded by confocal microscopy (confocal microscope Zeiss, LSM510).

2.3. Fluorometric determinations of $\Delta\Psi$, pH_i and NADH in sperm populations

Sperm population fluorescence measurements were done according to Rodríguez & Darszon [41] (2003). Briefly, diluted sperm (1:10 in 0.25 ml 1CaASW) were incubated at 14 °C during 2–3 h in the dark in the presence of 10 μM Rhod-123 (for $\Delta\Psi$; λ_{ex} 497 and λ_{em} 523 nm [42]) or 0.5 μM BCECF-AM (for pH_i; λ_{ex} dual 438 and 500; λ_{em} 550 nm [40]). The dye remaining in the media was eliminated from the supernatant by centrifugation (1000 *g*/5 min at 4 °C) and the sedimented spermatozoa were resuspended in the original volume of 1CaASW (except when cells were incubated in the absence of fluorophore, which were diluted 1/3) and kept on ice in the dark until used.

Rhodamine 123 (Rhod 123), a cationic fluorescent dye with a bright emission at 523 nm, is quenched as the dye accumulates within mitochondria according to the negative membrane potential across the IMM. Loss of potential will result in loss of dye from the mitochondria into the cytosol, the dye de-quenches with an overall cellular fluorescence increase, while the dye concentration inside mitochondria decreases indicating a mitochondrial depolarization [43,44].

In this work we use the intrinsic fluorescence of reduced pyridine nucleotides as an indicator of the mitochondrial metabolism. Since the spectroscopic properties of NADH and NAD(P)H are very similar [45], when we record the sperm autofluorescence (λ_{ex} 350; λ_{em} 460 nm) we collect fluorescence from both nucleotides. However, since in sea urchin sperm there is no glycolysis [14,46,47] and its energetic metabolism is oxidative [14], the fluorescence signal corresponds mainly to NADH.

Lactate dehydrogenase (LDH) is a cytosolic enzyme that catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of nicotinamide adenine dinucleotide (NADH) and NAD^+, when oxygen is absent or in short supply [48]. Sea urchin sperm depend on mitochondrial lipid metabolism for energy production [16,49], a pathway which does not involve LDH. Indeed, in *Paracentrotus* and *Arbacia*, it has been demonstrated that this is so [50]. In order to show that the NADH increases triggered by speract in *S. purpuratus* sperm are coming from the mitochondria, we determined the levels of NADH induced by speract in the absence and presence of two LDH inhibitors, cinnamic acid and gossypol. For this we pre-incubated sperm with different concentrations (0.1, 1.0 and 10 μM of gossypol or 0.1, 1.0 and 10 mM of cinnamic acid) of these LDH inhibitors. Supplementary Fig. 1 shows that neither of the two inhibitors did significantly affect the sperm response to speract, confirming that the NADH increase triggered by this decapeptide comes mainly from the sperm mitochondrion.

NADH determinations were performed measuring autofluorescence—λ_{ex} 350; λ_{em} 460 nm—[43] in non-labeled spermatozoa obtained as above. In all assays a 10 μl aliquot of the sperm was added to a round cuvette containing 800 μl ASW or 0CaASW at 14 °C under constant stirring in a SLM 8000 Aminco spectrofluorometer. After recording 10 s, different concentrations of speract, NH_4Cl, or inhibitors were added for $\Delta\Psi$, pH_i or NADH measurements. Fluorescence data (F) were normalized by the average fluorescence value before the addition of agonists or inhibitors (F_0) and are expressed as $F/F_0 - 1$.