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Characterization of NAADP-mediated calcium signaling in human spermatozoa

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ABSTRACT

Ca^{2+} signaling in spermatozoa plays a crucial role during processes such as capacitation and release of the acrosome, but the underlying molecular mechanisms still remain unclear. Nicotinic acid adenine dinucleotide phosphate (NAADP) is a potent Ca^{2+}-releasing second messenger in a variety of cellular processes. The presence of a NAADP synthesizing enzyme in sea urchin sperm has been previously reported, suggesting a possible role of NAADP in sperm Ca^{2+} signaling. In this work we used *in vitro* enzyme assays to show the presence of a novel NAADP synthesizing enzyme in human sperm, and to characterize its sensitivity to Ca^{2+} and pH. Ca^{2+} fluorescence imaging studies demonstrated that the permeable form of NAADP (NAADP-AM) induces intracellular [Ca^{2+}] increases in human sperm even in the absence of extracellular Ca^{2+}. Using LysoTracker®, a fluorescent probe that selectively accumulates in acidic compartments, we identified two such stores in human sperm cells. Their acidic nature was further confirmed by the reduction in staining intensity observed upon inhibition of the endo-lysosomal proton pump with Bafilomycin, or after lysosomal bursting with glycyl-L-phenylalanine-2-naphthylamide. The selective fluorescent NAADP analog, Ned-19, stained the same subcellular regions as LysoTracker®, suggesting that these stores are the targets of NAADP action.

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1. Introduction

A crucial characteristic of Ca^{2+}-mediated signaling lies in its versatility, as it can invoke a wide range of signaling patterns differing in magnitude, duration and source [1]. It has long been established that there are two basic types of Ca^{2+} sources available to a cell: one provided by intracellular compartments such as the endoplasmic reticulum (ER), and the second one consisting of the extracellular fluid [1]. Currently, there are three main mechanisms known to elicit Ca^{2+} release from intracellular stores. The key molecules participating in each mechanism are inositol 1,4,5-trisphosphate (IP_3), cyclic ADP-ribose (cADPR), and nicotinic acid adenine dinucleotide phosphate (NAADP). The first two target receptors in the ER, while NAADP targets acidic Ca^{2+} stores such as lysosomes and endosomes [2].

NAADP is the most potent among these second messengers, given that it functions as a trigger of the Ca^{2+} signal [3] by first releasing Ca^{2+} from acid stores [4] that result in additional Ca^{2+} release by the calcium-induced calcium release (CICR) process through ryanodine receptors (RyRs) and/or IP_3 receptors (IP_3Rs) located in the ER [5]. The combined release of Ca^{2+} from intracellular stores may in turn activate store-operated calcium channels (SOCs) located on the plasma membrane. These events in coordination greatly amplify the Ca^{2+} signal originated via NAADP.

NAADP is synthesized (*in vitro*) by CD38, a multifunctional ADP-ribosyl cyclase (ADPRC) enzyme that catalyzes a base-exchange reaction whereby the nicotinamide group of NADP is replaced by nicotinic acid, leading to the production of NAADP [6]. CD38 also catalyzes the hydrolysis of $NADP^+$ to adenosine diphosphate ribose (ADPR) – and to a minor extent its conversion to cyclic-ADPR (cADPR) – as well as the degradation of NAADP and cADPR [7]. In summary, CD38 is able to catalyze the synthesis and/or the degradation of cADPR, ADPR and NAADP.

Ca^{2+} signaling is of particular significance in sperm cells, mediating capacitation, hyperactivation and the acrosome reaction (AR). However, the involvement of IP3, cADPR and NAADP during each Ca^{2+} signaling event in spermatozoa is not completely understood. Notably, mature sperm cells lack ER [8] although they nonetheless possess the molecular components known to be functionally significant for the activity of Ca^{2+} stores in somatic cells [9]. Current evidence supports the existence of at least two Ca^{2+} storage organelles in mammalian sperm, namely the acrosome (located in the sperm head), and the redundant nuclear envelope (RNE), consisting of a group of vesicles produced by nuclear condensation during spermatogenesis (located in the sperm neck) [10]. Moreover, the

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presence of IP_3Rs [11] and RyRs [12] in mammalian sperm cells has been reported. Billington et al. [13] reported micromolar concentrations of cADPR in human sperm cells, which do not change significantly during capacitation. This group also reported the presence of an active cADPR synthesizing machinery in human sperm. On the other hand Park et al. [14] demonstrated that human prostasomes, but not sperm, contain CD38, suggesting the existence of an alternate pathway for cADPR and NAADP production in spermatozoa. Indeed, Vasudevan et al. [15] demonstrated the presence of a NAADP synthesizing enzyme in sea urchin sperm. In this work we aimed to determine whether such an enzyme is also present in human sperm, and whether in these cells NAADP participates in Ca^{2+}-induced fertilization events.

2. Methods

2.1. Human sperm preparation

The use of human sperm in this study was approved by the Bioethics Committee at the Institute of Biotechnology, UNAM. Ejaculates were obtained by masturbation from healthy donors after 48 h of sexual abstinence. Samples that fulfilled the WHO parameters were selected for experiments. Motile spermatozoa were recovered after a swim-up separation for 1 h in Ham's F-10 medium supplemented with 5 mg/mL BSA with incubation at 37 °C in a humid chamber containing 5% CO_2. Using Human Sperm Medium (HSM; in mM: 120 NaCl, 15 $NaHCO_3$, 4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 Na lactate, 5 D-glucose, 1 Na pyruvate, pH = 7.4), the cell concentration was adjusted to 10×10^6 spermatozoa/mL, and the incubation was continued for at least 4 h to promote *in vitro* capacitation [16].

2.2. mNAADP synthase assays

Capacitated sperm (15×10^6 cells) were incubated in Earl's balance salt solution (EBSS, in mM: 116.4 NaCl, 5.4 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5.5 glucose, 25 $NaHCO_3$, Na 2.5 pyruvate, 2.5 Na lactate, 0.8 $MgSO_4$ and 0.3% BSA, pH 7.4) with saturating concentrations of NADP (10 mM) and nicotinic acid (100 mM) for 1 h (37 °C, 5% CO_2) and the substrate/product solution was analyzed by HPLC. The identity of each HPLC peak was determined by comparing their retention times with those of standards. Substrates were also incubated in the absence of sperm as a negative control. Ca^{2+} and pH activity curves were obtained under the same conditions but either changing the EBSS pH value 4.5–8.5, adjusted with HEPES or Tris), or the extracellular Ca^{2+} concentration ($[Ca^{2+}]$ range 10^{-9}–10^{-4} M, adjusted with EGTA). In order to assay the range of reactions catalyzed by mNAADP synthase, the aforementioned base exchange substrates were replaced with either cADPR (10 mM), NAD (10 mM) or NAD (10 mM).

2.3. High performance liquid chromatography (HPLC) analysis and NAADP-AM synthesis

Nucleotides were analyzed on a 150 mm × 2.5 mm column packed with anion-exchange resin (AGMP1, Biorad, USA), using a concave upwards gradient of trifluoroacetic acid (TFA) as previously described [17]. Briefly, samples (100 μL) were injected into a column equilibrated with water. Bound material was eluted with a concave-up TFA gradient, which increased linearly to 2, 4, 8, 16, 32 and 100% at 1.5, 3.0, 4.5, 6.0, 7.5 and 7.51 min, respectively. The flow rate was 4 mL/min. Nucleotides were detected by their UV light absorbance at 254 nm and identified by their retention time as compared to that of standards. NAADP was synthesized from NADP using the base-exchange reaction catalyzed by ADP-ribosyl cyclase as previously described [17]. NAADP-AM and cADPR-AM were synthesized as previously described [18,19].

2.4. Fluorescent dye loading for Ca^{2+} measurements

Capacitated human sperm samples (30×10^6 cells/mL) were incubated for 30 min at 37 °C with 2 μM fluo-3 AM (Invitrogen) in a humid chamber containing 5% CO_2. Cells were centrifuged (750 g) for 5 min. The supernatant was discarded and the pellet was resuspended in the appropriate volume of HSM to achieve the cell concentration required for each technique [16].

2.5. Conventional fluorometry and single cell imaging

Spermatozoa previously loaded with fluo-3 AM were used to measure fluorescence intensity vs. time in response to test compounds, and acquired under the appropriate conditions for either conventional fluorometry or single cell imaging, as described by Mata et al. [16]. Raw fluorescence intensity values were exported to Microsoft Excel and normalized using the equation $(F/F_0) - 1$; where F is the fluorescence intensity measured at any given time, and F_0 is the mean basal fluorescence taken during the initial 30 s. The difference between the fluorescence intensity values before and after the addition of the test compounds (ΔF) was plotted vs. time [16].

2.6. Identification of intracellular acid stores and NAADP-binding sites in human sperm

Aliquots of capacitated sperm samples (5.0×10^7 cells/mL) were distributed into separate tubes for the following treatments: (a) untreated control; (b) 30-min incubation with 75 nM LysoTracker Red DND99 (Invitrogen); (c) 1-h incubation with 3 μM Bafilomycin A1 (Sigma) followed by 30-min incubation with 75 nM LysoTracker Red DND99; (d) 1-h incubation with 3 μM Bafilomycin A1; and (e) 4-h incubation with 100 μM Ned19. Round coverslips coated with poly-L-lysine were mounted in an Attofluor Cell Chamber (Invitrogen) and 10 μL of each human sperm suspension were deposited on a separate coverslip. Samples were examined by fluorescence confocal microscopy (excitation 577 nm, emission 590 nm for LysoTracker Red; and excitation 355 nm, emission 415 nm for Ned19).

3. Results

3.1. Human sperm cells are able to synthesize NAADP

A novel enzyme with base-exchange reaction activity is known to be present in sea urchin (*Lytechinus pictus*) sperm [19]; we investigated the presence of a similar enzyme in human spermatozoa. Our first step was to determine whether these cells exhibit the associated base-exchange enzymatic activity. To this end, capacitated sperm cells were incubated with saturating concentrations of the substrates NADP (10 mM) and nicotinic acid (100 mM) for 1 h and the substrate/product solution was analyzed by HPLC. The substrates were also incubated in the absence of sperm cells as a negative control. The resulting HPLC traces are shown in (Fig. 1A). Four distinct peaks were detected in the absence of sperm cells, corresponding to nicotinamide, nicotinic acid, NADP, and ADPR-P. This indicates the presence of some contaminating ADPR-P and nicotinamide but no NAADP in commercial NADP. An NAADP peak was detected only in the presence of sperm, indicating its enzymatic synthesis. This conclusion is reinforced by the concomitant increase in the amount of the second product of the base-exchange reaction (i.e. a larger nicotinamide peak). We will refer to this enzymatic

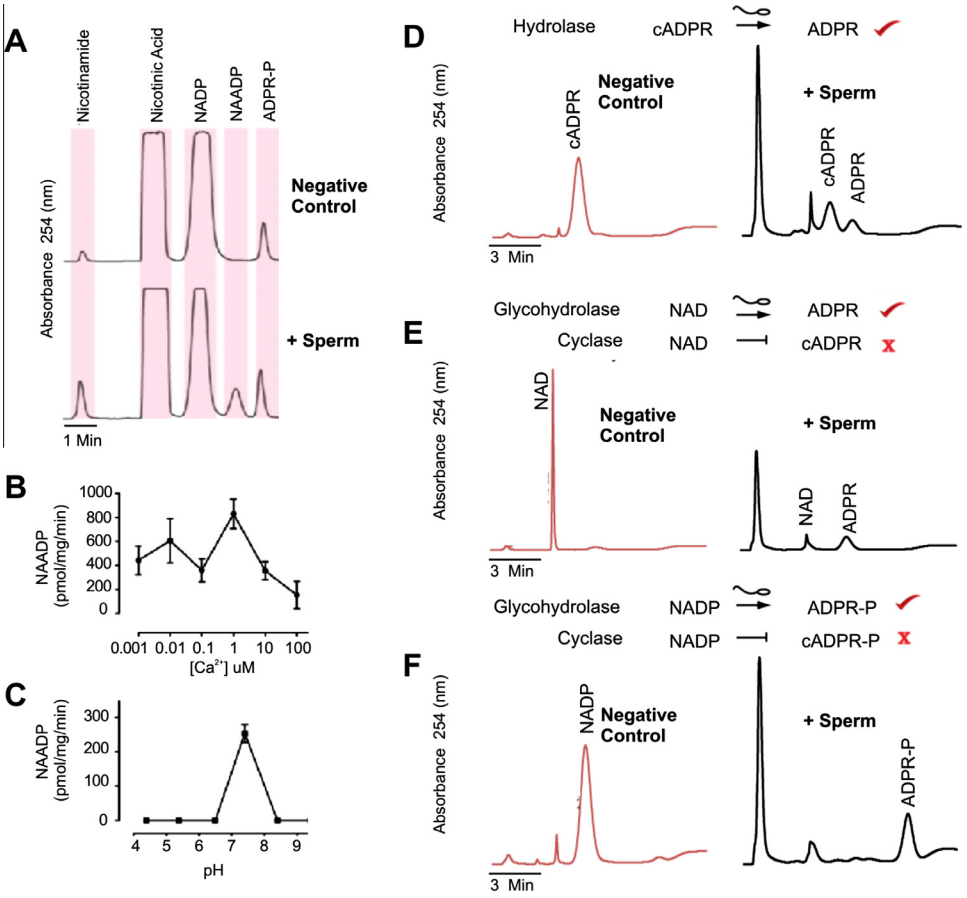

Fig. 1. NAADP synthesis in human spermatozoa. (A) HPLC traces after incubation of the base-exchange substrates nicotinic acid and NADP in the absence (upper trace) and presence (lower trace) of sperm. (B) Ca^{2+}- and (C) pH-dependence of the base-exchange activity; mean rate (±S.E.M.) ($n = 3$). HPLC traces after incubation of cADPR (D), NAD (E) or NADP (F) in the absence (left traces) or presence (right traces) of sperm.

activity detected in sperm cells as mammalian NAADP synthase (mNAADP synthase). Given that we used intact sperm and the cell membrane is not permeable to the substrates, the enzyme is presumably located on the plasma membrane. To explore whether spermatozoa also posses intracellular mNAADP synthase activity, cells were permeabilized either by the addition of the detergent digitonin (50 μM) or by a freeze–thaw cycle (−80 °C, 10 min) prior to incubation with the substrates for NAADP production. There was no significant difference in the amount of NAADP synthesized in permeabilized compared to non-permeabilized sperm (data not shown). We therefore conclude that mNAADP synthase is localized on the extracellular side of the plasma membrane, just as reported for sea urchin sperm [19].

It has been shown that Ca^{2+} and pH regulate the activity of *L. pictus* NAADP synthase [19]. Therefore, we explored the influence of Ca^{2+} and pH on NAADP production in mammalian sperm. The Ca^{2+} dependence displayed a weak bell-shaped curve, with a maximum of NAADP production around 1 μM [Ca^{2+}] (Fig. 1B). The pH dependence for the base exchange activity was very clear, displaying a sharp peak in the pH 7–8 range. At pH values <6 or >8 the activity was negligible (Fig. 1C).

3.2. Human sperm mNAADP synthase demonstrates multi-functional enzyme activity

All enzymes thus far shown to have base exchange activity are known to posses multi-functional enzyme activity [20]. Therefore we assessed a range of reactions that may be catalyzed by mNAADP synthase (Fig. 1D–F). The initial substrates cADPR, NAD and NADP were incubated in the absence or presence of human sperm. Following incubation, the substrate/product solutions were analyzed by HPLC. (Fig. 1) shows the resulting HPLC traces, along with the corresponding reactions to indicate the substrate selectivity and versatility of mNAADP in terms of substrates and types of reaction catalyzed, which are as follows: hydrolase activity on cADPR (Panel 1D) and glycohydrolase acitivity on NAD and NADP (Panels 1E and F, respectively), but no cyclase activity on NAD or NADP (Panels 1E and F, respectively).

3.3. NAADP-AM but not cADPR-AM mobilizes Ca^{2+} in human sperm

In order to determine whether NAADP and/or cADPR may have a physiological effect in human sperm, we measured changes in $[Ca^{2+}]_i$ in cells exposed to these second messengers. Capacitated sperm cells loaded with fluo3-AM were exposed to different concentrations of the permeable forms of NAADP or cADPR (NAADP-AM or cADPR-AM, respectively), and the $[Ca^{2+}]_i$ changes in the sperm population were recorded by conventional fluorometry (Fig. 2). Each trace also records the effect of 4 μM progesterone, added at the end of each experiment as a positive control to verify that the cells were capable to respond to a known agonist. Addition of cADPR-AM provoked no $[Ca^{2+}]_i$ increases in the tested concentration range (Fig. 2A). In contrast, NAADP-AM produced transient $[Ca^{2+}]_i$ increases (Fig. 2B). A plot of the mean fluorescence amplitude for each of the different concentrations of NAADP-AM reveal that this second messenger induces $[Ca^{2+}]_i$ increases in a dose-dependent manner (Fig. 2C).

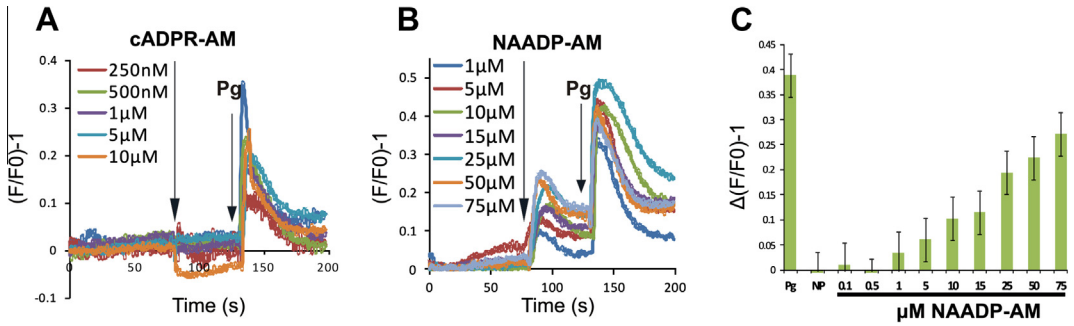

Fig. 2. NAADP-AM but not cADPR-AM mobilizes Ca^{2+} in human sperm. Fluorescence changes vs. time were recorded before and after addition of increasing concentrations of cADPR-AM (A) or NAADP-AM (B), followed by progesterone (Pg, positive control). Average fluorescence changes observed in (B) are plotted as a function of NAADP-AM concentration (C); non-permeable NAADP (NP) does not elicit fluorescence changes (negative control) ($n \geqslant 5$).

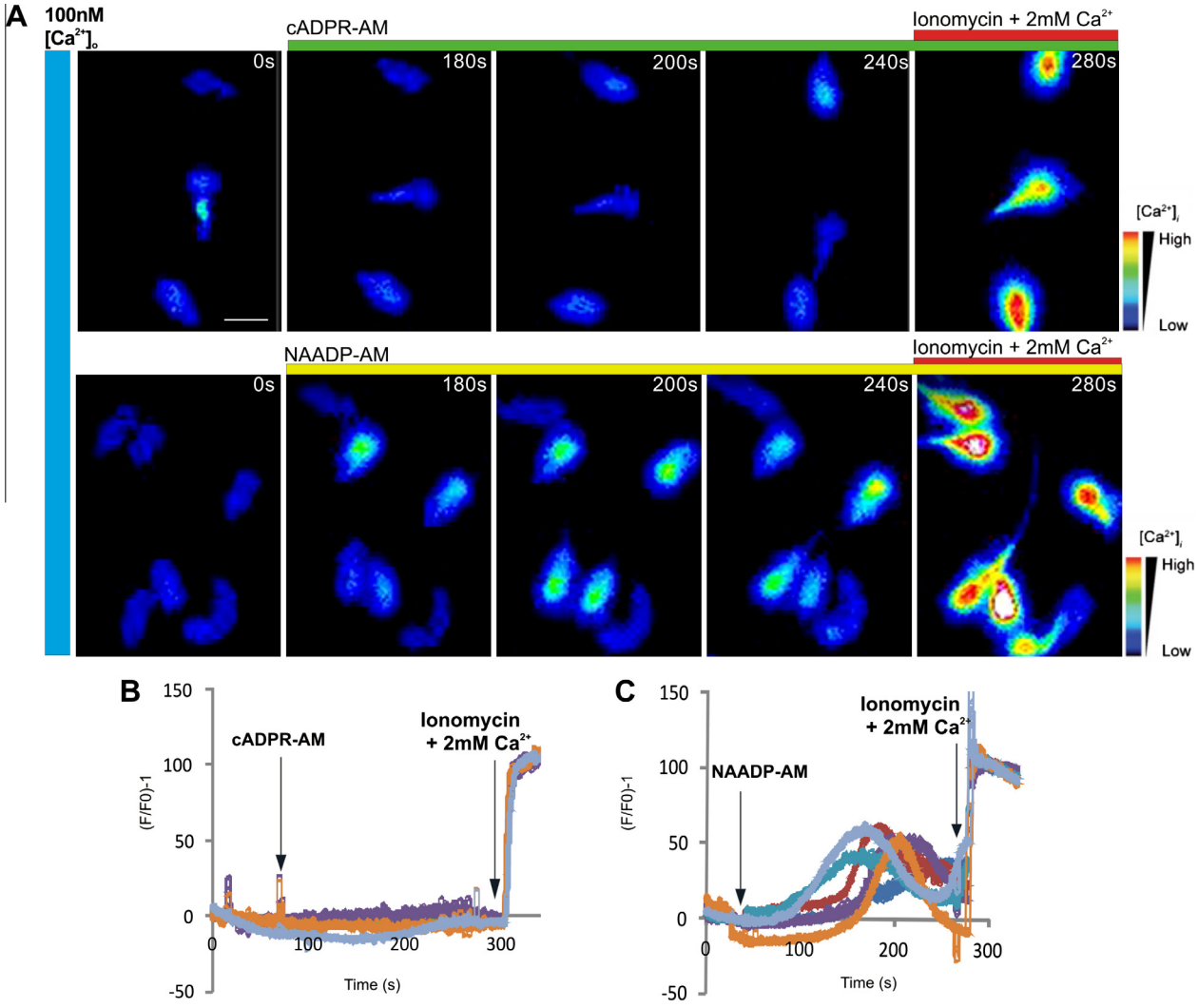

Fig. 3. NAADP-AM but not cADPR-AM increases $[Ca^{2+}]_i$ in human sperm at low $[Ca^{2+}]_o$. (A) Representative single-cell spatiotemporal fluorescence changes before and after addition of cADPR-AM (top) or NAADP-AM (bottom); pseudocolor from black to red represents low to high $[Ca^{2+}]_i$, respectively; time frames are indicated in seconds. Representative fluorescence traces before and after addition of cADPR-AM (B) or NAADP-AM (C) of individual cells from (A). Ionomycin and Ca^{2+} are added as a positive control. ($n \geqslant 5$).

3.4. NAADP-AM mediates $[Ca^{2+}]_i$ increases in human sperm in the absence of $[Ca^{2+}]_o$

As mentioned before, NAADP is the most potent second messenger because it triggers CICR and stimulates SOC channels, thus amplifying the Ca^{2+} signal. The presence of SOC channels in sperm cells has not been unequivocally demonstrated, and in fact Catsper is thus far the only Ca^{2+} channel whose activity in sperm has been electrophysiologically confirmed [21]. Catsper is a highly promiscuous channel known to be activated by a wide range of compounds, including some that have been chemically modified to make them permeable [22]. Given that our experiments

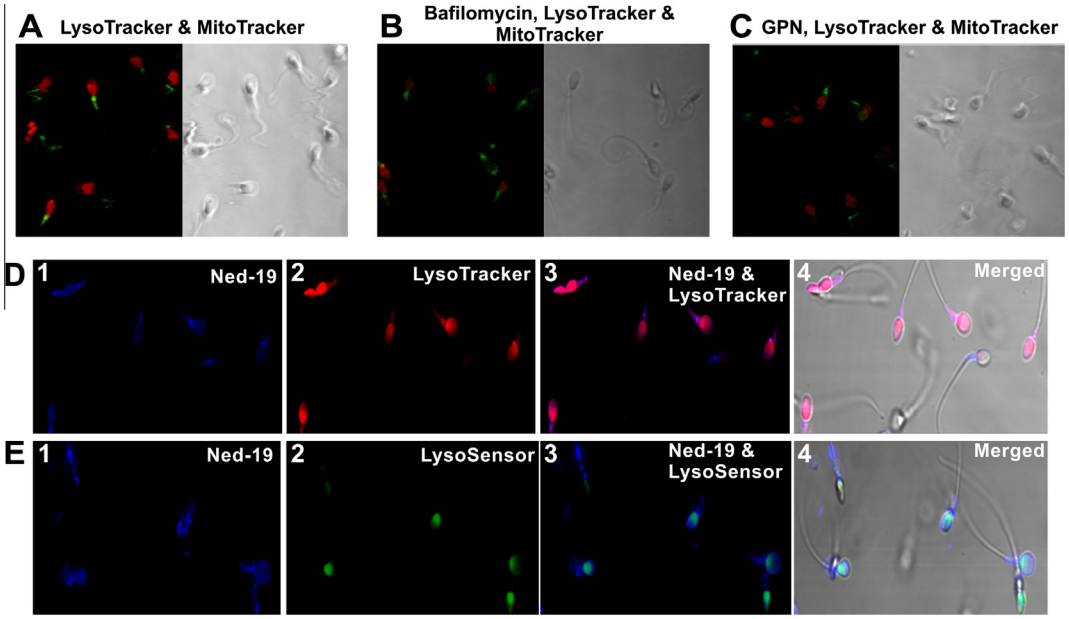

Fig. 4. NAADP-analog (Ned-19) accumulates in human sperm acidic stores. Confocal fluorescence and phase contrast micrographs of spermatozoa incubated with LysoTracker and MitoTracker (A), Bafilomycin, LysoTracker and MitoTracker (B), or GPN, LysoTracher and MitoTracker (C). Confocal fluorescence micrographs of spermatozoa incubated with Ned19 plus LysoTracker (D) or Ned19 plus LysoSensor (E), visualized individually (D1–D2, and E1–E2), in combination (D3, E3), and merged with phase contrast micrographs (D4, E4). ($n = 3$).

required the use of a permeable form of NAADP, we wanted to confirm that the observed increases in $[Ca^{2+}]_i$ were due to Ca^{2+} mobilization from intracellular stores, rather than from non-specific activation of Catsper by NAADP-AM. To this end, we measured NAADP-AM-induced $[Ca^{2+}]_i$ changes using single cell imaging, under conditions where the $[Ca^{2+}]_o$ available for Catsper-mediated import was eliminated through chelation with EGTA. In order to avoid store depletion prior to the experiment, the $[Ca^{2+}]_o$ concentration was decreased by chelation to ~100 nM, which is close to the intracellular resting value. Our results show that NAADP-AM (15 μM) mediates intracellular Ca^{2+} increases in 60% of the cells, under lowered $[Ca^{2+}]_o$ conditions (Fig. 3A bottom series and Fig. 3C). We next investigated whether we could detect a signal using cADPR under the same conditions. (Fig. 3A (top series) and Fig. 3B) show that cADPR could not elicit a Ca^{2+} increase. The sharp increase of fluorescence at the end of the experiments obtained with addition of a known agonist (Ionomycin) and restoring Ca^{2+} in the extracellular medium, demonstrates that all cells maintained responsiveness under our experimental conditions.

3.5. Lysotracker and Ned-19 staining identifies acidic compartments located in the sperm head and midpiece

It has been established that NAADP activates Ca^{2+} release from acidic stores [4]. We used LysoTracker Red DND-99, a dye that accumulates in acidic organelles, in order to identify the presence of such stores in human sperm. As shown in (Fig. 4A), LysoTracker stained two potential acidic compartments located in the head and neck regions of spermatozoa; MitoTracker Green was used as a counterstain to localize mitochondria. To corroborate the acidic nature of these compartments, sperm cells were incubated with Bafilomycin, a proton pump inhibitor, whose disruption dissipates the proton gradient, thus preventing dye accumulation. As expected, preincubation with Bafilomycin diminished the staining pattern obtained with LysoTracker, but did not affect MitoTracker Green staining (Fig. 4B). An alternative method to disrupt the proton gradient of acidic stores is the use of glycyl-L-phenylalanine-2-naphthylamide (GPN), a membrane-permeant agent that accumulates in lysosomes, where it is converted into an impermeant product by a cathepsin-mediated enzymatic cleavage, resulting in osmotic swelling and lysis of the organelle [23]. Consistent with the results obtained for Bafilomycin, preincubation with 100 μM GPN also diminished the staining pattern obtained with LysoTracker Red (Fig. 4C).

In order to determine whether the sites of action of NAADP in human sperm were acidic stores, we stained spermatozoa with LysoTracker or LysoSensor together with Ned-19, a fluorescent molecule similar to NAADP and capable of binding to the same target sites. Co-incubation of live human sperm cells with Ned-19 and Lysotracker (Fig. 4D) or LysoSensor (Fig. 4E) show that these dyes stained the head and neck regions of spermatozoa with a high degree of co-staining. These results suggest that Ned-19 is binding to the acid stores detected with Lysotracker and Lysosensor, and therefore those may be the sites for NAADP action.

4. Discussion

While there is no doubt that Ca^{2+} signaling is vital for sperm physiology, the molecular entities involved in its mobilization are currently under debate. Patch clamp approaches have so far revealed the presence of Catsper as the main Ca^{2+} channel present in human sperm plasma membrane. In these cells, Catsper is activated with progesterone but is plausible that mechanisms involving Ca^{2+} mobilization from intracellular stores are also operating during progesterone signaling [24]. In this work we explored whether NAADP is synthesized by human sperm, and whether this second messenger induces Ca^{2+} mobilization in these cells.

We report that human sperm cells exhibit NAADP synthesizing capability just as previously described in sea urchin sperm. The pH dependence of mNAADP synthase was very clear, with maximal activity between pH 4–6; this is in contrast to the maximal activity observed in sea urchin at pH = 7–8 [19]. While the Ca^{2+} dependence for mNAADP synthase was not as clear as in sea urchin, the possibility remains for other metabolites (such as cGMP and/or cAMP) to concomitantly influence its activity, as is the case for other ribosyl cyclases [25]. The spatial orientation of the catalytic activity is also of interest since it is now known that ribosyl

cyclases are expressed both on the cell surface and on the cytoplasmic side of intracellular organelles [20], generating a topological paradox. There are currently two proposals to explain this; one presupposes the existence of substrate/product transporters in the plasma and organelle membranes, ensuring their availability in the appropriate side of the membrane. Alternatively, the enzyme may be inserted with opposite orientations into different membranes [20]. Our results show that in human spermatozoa the catalytic site faces the extracellular side.

Regarding substrate selectivity (cADPR, NAD or NADP), we conclude that mNAADP synthase appears to be unrestricted, and that in addition to having base-exchange activity, it possesses hydrolase and glycohydrolase activities. However, unlike conventional ADPR-cyclases, our results rule out cyclase activity for mNAADP synthase. We are unable to discern from our results whether the enzyme activity comes from one or multiple enzymes.

A role for NAADP in fertilization has only been proposed for sea urchin sperm [15]. In this work we report that NAADP, but not cADPR, mobilizes Ca^{2+} in human sperm. Since we used a modified version of NAADP to render it permeable, the question arises as to whether this observed $[Ca^{2+}]_i$ increase was in fact due to a non-specific activation of Catsper channels, as previously reported for several other molecules [22]. We used single cell imaging the absence of extracellular Ca^{2+} in order to establish that at least part of the observed $[Ca^{2+}]_i$ increase was not due to Catsper activation. The results showed that under these condition, NAADP still induces an $[Ca^{2+}]_i$ increase in at least 60% of the cells. It is interesting that the $[Ca^{2+}]_i$ increase observed under these conditions occurred with a 40-s delay as compared to the experiments conducted in the presence of extracellular Ca^{2+}.

To determine the presence of acid stores in human sperm cells, we carried out cell staining with the acidic sensors Lysotracker Red and LysoSensor. We show that both compounds identify acidic compartments located in the head and neck of the sperm. Interestingly, this staining correlates with the location of Ca^{2+} stores in human sperm cells [26] and also with the observed Ned-19 fluorescence, associating NAADP's binding site to the Ca^{2+} stores. Sperm preincubation with Bafilomycin diminished Lysotracker Red and LysoSensor staining, confirming the acidic nature of these compartments. Based on the presented data, we propose that NAADP may be a player during Ca^{2+} signaling in human spermatozoa.

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