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Carbonic anhydrases and their functional differences in human and mouse sperm physiology

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ABSTRACT

Fertilization is a key reproductive event in which sperm and egg fuse to generate a new individual. Proper regulation of certain parameters (such as intracellular pH) is crucial for this process. Carbonic anhydrases (CAs) are among the molecular entities that control intracellular pH dynamics in most cells. Unfortunately, little is known about the function of CAs in mammalian sperm physiology. For this reason, we re-explored the expression of CAI, II, IV and XIII in human and mouse sperm. We also measured the level of CA activity, determined by mass spectrometry, and found that it is similar in non-capacitated and capacitated mouse sperm. Importantly, we found that CAII activity accounts for half of the total CA activity in capacitated mouse sperm. Using the general CA inhibitor ethoxyzolamide, we studied how CAs participate in fundamental sperm physiological processes such as motility and acrosome reaction in both species. We found that capacitated human sperm depend strongly on CA activity to support normal motility, while capacitated mouse sperm do not. Finally, we found that CA inhibition increases the acrosome reaction in capacitated human sperm, but not in capacitated mouse sperm.

1. Introduction

Before internal fertilization can occur, spermatozoa travel a long stretch inside the female reproductive tract; throughout their journey in this harsh environment, they face important changes in the concentration of different ions. Sperm encounter reduced $[K^+]_e$, increased $[HCO_3^-]_e$ (which in turn increases $[HCO_3^-]_i$) and $[Na^+]_e$, and during capacitation the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) increases [1]. The increases in $[HCO_3^-]_i$ and $[Ca^{2+}]_i$ activate soluble adenylate cyclase (sAC). Therefore, sAC is a likely connection between carbonic anhydrases (CAs; metalloenzymes that turn CO_2 into HCO_3^-) and downstream steps in the sperm signaling pathways. It has been demonstrated that cAMP produced by sAC has different targets, including protein kinase A (PKA), cyclic nucleotide gated (CNG) channels, sperm Na^+/H^+ exchanger (sNHE), and exchange protein directly activated by cAMP (EPAC) [2]. In turn, PKA is a crucial modulator of sperm motility through phosphorylation of different proteins. Sperm are immotile inside the epididymis, in part due to the acidic conditions of the extracellular fluid. Upon ejaculation, spermatozoa are mixed with seminal fluid having an alkaline pH (7.2–8.4) and higher $[HCO_3^-]$. Bicarbonate ions not only help maintain the motility of spermatozoa, but also protect them in the acidic environment of the vagina [3]. These changes constitute the first motility stimulus, and are followed by increases of $[HCO_3^-]$ in upper regions of the female tract, which in addition to further activate motility, contribute to the capacitation process. While the role of HCO_3^- during the acrosome reaction is not as well established, it is now clear that regulation of sperm activity is strongly related to $[HCO_3^-]_i$ levels and pH_i values. However, the identity and interplay of the molecular entities participating in their modulation remain far from clear. For example, despite the

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importance of CAs in the regulation of pH in almost every cell type, little is known about their subcellular distribution and their physiological role in mammalian sperm. CAs are present in the three domains of life, though encoded by six evolutionarily unrelated gene families, namely α, β, γ, δ, ζ and η-CAs [4]. The sixteen isoforms of α-CAs are the only CAs present in mammals, displaying distinct subcellular and tissue distribution, kinetic properties, and sensitivity to inhibitors [5]. Using different biochemical techniques, it has been shown that isoforms CAI [3], CAII [3,6] and CAXIII [7] are present in human sperm, while isoforms CAII [8], CAIV [9–11] and CAXII [12] are found in mouse sperm. Interestingly, it has been demonstrated that isoform CAIV is transferred to the plasma membrane of non-capacitated mouse sperm as they pass through the epididymis, and that both murine and human non-capacitated sperm respond to increases in extracellular CO_2 with an increase in flagellar beat frequency, an effect that can be blocked by the general CA inhibitor ethoxyzolamide (EZA) [11]. Moreover, non-capacitated sperm from CAIV$^{-/-}$ null mice showed a decrease in total CA activity and a reduced response to CO_2 compared to the wild type [11]. Another study led to the proposal that glucose consumption and the regulation of flagellar beat frequency in mouse sperm are interconnected through the generation of HCO_3^- from CO_2, a reaction that is mediated by the activity of CAs [13]. Given that various physiological important differences between human and mouse sperm have been established [1,5], and considering the scarcity of studies on CAs in sperm, in this work we set out to compare their distribution and activity between both species using various experimental techniques, along with general and specific CA inhibitors.

2. Materials and methods

2.1. Mouse and human sperm preparation

Mouse spermatozoa were obtained from adult (~3 months old) male CD1 or C57BL/6J mice. The CAII$^{-/-}$ mice colony was obtained from the University of Kaiserslautern, Germany. The animals were killed by cervical dislocation and non-capacitated motile spermatozoa were obtained from epididymal caudas in Whitten's medium pH 7.4 using the swim-up technique [14]. Whitten's medium contains (in mM): NaCl 100, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, glucose 5.5, sodium pyruvate 1, Na-L-lactate 4.8, HEPES 20, $NaHCO_3$ 24 and $CaCl_2$ 2. The use of human sperm in this study was approved by the Bioethics Committee at the Biotechnology Institute, UNAM. Ejaculates were obtained by masturbation from healthy donors after 48 h of sexual abstinence. Samples that fulfilled the parameters established by the World Health Organization were used. The semen samples were allowed to liquefy at 37 °C and then the motile spermatozoa were recovered in Ham's F-10 medium pH 7.4 (plus $CaCl_2$ 2 mM) using the swim-up technique [15]. To generate capacitated cells, mouse and human sperm samples were incubated in their respective media, supplemented with bovine serum albumin 5 mg/mL. Mouse and human sperm samples (either capacitated or non-capacitated) were then adjusted to a concentration of 10×10^6 cells/mL, and incubated at 37 °C with CO_2 5%/air 95% during at least 40 min or 4 h, respectively. Before each experiment, capacitated human cells were centrifuged during 5 min at 735× *g* and resuspended in physiological solution pH 7.4, which contains (in mM): NaCl 94, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1, sodium pyruvate 1, $NaHCO_3$ 25, glucose 5, HEPES 30 and Na-L-lactate 10.

2.2. SDS-PAGE and western blot assays

After swim-up, mice and human spermatozoa were washed twice with PBS and centrifuged during 5 min at 735× *g*. Pellets were resuspended in 500 μL of solubilization buffer and the samples were left in constant agitation during 1.5 h at 4 °C. Samples were centrifuged at 16,000× *g* during 15 min and proteins in the supernatant were concentrated to 50 μL with centrifugal filters Amicon Ultra 10K (Merck Millipore, Carrigtwohill, Ireland). The concentrated protein was mixed with loading buffer as described previously [15]. The sample was then heated at 70 °C during 10 min and finally centrifuged at 16,000× *g* for 10 min. A volume equivalent to the protein content of 30×10^6 cells was loaded per lane on a 10% or 15% SDS-PAGE gel, depending on the condition. Proteins were electrotransferred to an Immobilon P membrane (Millipore, Massachusetts, USA) with a semi-dry electrophoretic transfer cell (Bio-Rad, Mexico City, Mexico) and the membrane was blocked with fat-free milk 5% v/v. Membranes were probed with anti-CAII, anti-CAXIII (Sigma Aldrich, Mexico City, Mexico) or anti-CAIV (Santa Cruz Biotechnology, Texas, USA). Immunodetection was performed as described elsewhere [15].

2.3. Immunocytochemistry

Mouse and human sperm cells were attached to glass slides and fixed with paraformaldehyde/PBS 4% during 1 h at room temperature (RT), and then washed 3 times with PBS. Cells were permeabilized with Triton X-100/PBS 0.1% for 10 min and washed 3 times with PBS at room temperature. Non-specific sites were blocked with BSA/PBS 5% during 2 h at RT and then incubated overnight at 4 °C in a 1:100 dilution of anti-CAI, anti-CAII or anti-CAXIII. Cells were washed 3 times with PBS at RT, incubated with a secondary antibody coupled to Alexa 488 (Invitrogen, Mexico City, Mexico), and washed again 3 times with PBS; finally, the samples were mounted with Citifluor (Electron Microscopy Sciences, Pennsylvania, USA). The fluorescence of cells was observed in a confocal microscope Zeiss LSM510 META with a 100X objective.

2.4. Determination of CA catalytic activity

Activity of CAs was determined by measuring the ^{18}O depletion of doubly labeled $^{13}C^{18}O_2$ through several hydration and dehydration steps of CO_2 and HCO_3^- at RT. The reaction sequence of ^{18}O loss from $^{13}C^{18}O^{18}O$ ($m/z = 49$) over the intermediate product $^{13}C^{18}O^{16}O$ ($m/z = 47$) and the end product $^{13}C^{16}O^{16}O$ ($m/z = 45$) was monitored with a quadrupole mass spectrometer (OmniStar GSD 320; Pfeiffer Vaccum, Asslar, Germany). The relative ^{18}O enrichment was calculated from the measured 45, 47 and 49 abundance as a function of time according to the equation: log enrichment = log [49 × 100/(49+47+45)]. For the calculation of CA activity in capacitated mouse spermatozoa, the rate of ^{18}O degradation was obtained from the linear slope of the logarithmic enrichment over time, using the analysis software OriginPro 9.1. This rate was compared with that of the non-catalyzed reaction. Enzyme activity in units (U) was calculated from these two values as defined previously [16]. For each experiment the cuvette was filled with 6 mL of Whitten's medium without $NaHCO_3$ and 5×10^6 (either capacitated or non-capacitated) cells. Measurements were performed either in the absence or presence of CA inhibitors.

2.5. Motility assays

Motility of capacitated human and mouse sperm was measured in a Sperm Class Analyzer (SCA Microptic, Barcelona, Spain). Aliquots (10×10^6 cells/mL) were incubated during 30 min at 37 °C with CO_2 5%/air 95% either with vehicle (DMSO) or with increasing concentrations of the general CA inhibitor EZA. 10 μL of cell sample were placed inside a MicroCell® chamber (mouse) or between a glass slide (human) and a coverslip, and the motility parameters

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VCL (curvilinear velocity), VSL (straight line velocity), VAP (average path velocity), LIN (linearity), STR (straightness), WOB (wobble), ALH (amplitude of lateral head displacement) and BCF (beat cross frequency) of at least 200 cells per experiment were calculated by the SCA motility software from a stack of images acquired at 50 Hz and with a 10X objective. Analyses of numerical data for trajectories were done with the software SigmaPlot 10 (California, USA).

2.6. Acrosome reaction assays

Acrosome reaction (AR) was promoted in capacitated human and mouse sperm by incubation at 37 °C with CO_2 5%/air 95% for 30 min with physiological inductors of the AR, namely progesterone (10 μM) for human or *zona pellucida* (ZP; 5 *ZP* equivalents/μL) for mouse, with or without 15 min pre-incubation in the presence of the general CA inhibitor, EZA 30 μM. Sperm were also incubated with either a Ca^{2+} ionophore ionomycin 10 μM or without any inductor of AR, as positive and negative controls, respectively. Human and mouse sperm were fixed, stained with FITC-PSA (25 μg/mL, PBS pH 7.4) or Coomassie Blue G-250 0.22% v/v, respectively, and AR was evaluated as previously described [17,18].

2.7. Statistical analyses

All statistical analyses were performed with the freeware KyPlot 2.0 (Tokyo, Japan), and statistical values are shown as mean ± standard error of the mean (S.E.M.). Student's *t* tests were used for calculation of significant differences and are represented in the figures and the table as: $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$.

3. Results and discussion

3.1. Human and mouse sperm express different CA isoforms

The physiological importance of CAs has been clearly demonstrated in several types of cells [19]; however, little is known about their presence in mammalian sperm and even less is known about their participation in sperm physiology [5]. Some α-CA isoforms have been detected in mammalian sperm (human and mouse) through very different experimental techniques [3,6–9,11,12]; for this reason, in this study we re-explored in a more systematic and uniform approach the presence of the isoforms CAI (cytosolic), CAII (cytosolic), CAIV (GPI-anchored) and CAXIII (cytosolic) in human and mouse sperm through western blot and immunocytochemistry assays. Western blot experiments allowed us to confirm the presence of CAII and to detect for the first time CAXIII in human sperm (Fig. 1A,C), while CAIV, which had not been explored before in these cells, was not found (Fig. 1B). On the other hand CAII, CAIV, and also for the first time CAXIII, were detected in mouse sperm (Fig. 1D–F). CAI was also assayed in both species, but no band with the appropriate molecular weight was detected. On the other hand, our immunocytochemistry assays revealed that CAI, CAII and CAXIII localized to the flagellum of human sperm (Fig. 1G–I). While these three antibodies were also tested in mouse sperm samples (Fig. 1J–L), only CAII was found in this species, both in the flagellum and in the acrosomal region (Fig. 1K).

3.2. Capacitated mouse sperm possess CA activity

A previous study demonstrated that CAs are active in non-capacitated mouse sperm [11]. Given that there are important physiological differences between non-capacitated and capacitated spermatozoa [1], we decided to explore whether CAs are also active in capacitated cells, and if so, whether there is any difference in the total activity of CAs between the two physiological states. Fig. 2A shows for the first time that CAs are also active in capacitated mouse sperm, and that the magnitude of CA activity is similar to that of non-capacitated cells. Considering that only capacitated cells have the ability to fertilize [1], we decided to use only capacitated mouse cells in all further experiments. As a first approach, ethoxyzolamide (EZA), a general inhibitor of CAs, was used at various concentrations, aiming to inhibit CA activity in capacitated mouse cells. As seen in Fig. 2B, EZA significantly diminished CA activity in a dose-dependent manner. In these trials we also tested six different recently designed permeable and impermeable CA inhibitors [19,20] whose expected specific targets are CAVII, CAIX and CAXII. The inhibitory constants of these compounds were determined only for the human CA isoforms [19,20]. In our experiments, the concentrations needed to obtain a significant inhibition of CA activity were above 100 μM in all cases (data not shown), which makes EZA the most potent CA inhibitor in our model among the ones tested. Considering that CAII was the only isoform we detected by both western blot and immunocytochemistry in mouse sperm, and given the availability of $CAII^{-/-}$ null mice, we also explored the contribution of CAII to the total CA activity in these cells (Fig. 2C). We found that total CA activity decreases by 50% in $CAII^{-/-}$ compared to WT cells, which means that CAII contributes to half of the total activity in capacitated mouse cells; and thus other isoforms, such as CAIV [11], are responsible for the remaining activity. Human CA activity could not be measured due to restrictions on the use of human sperm at the University of Kaiserslautern.

3.3. Human sperm motility strongly depends on CA activity

Motility is modulated by different factors including pH_i [5]. It was previously demonstrated that general CA inhibitors such as EZA and acetazolamide decrease beat cross frequency (BCF) in a dose-dependent manner in non-capacitated mouse sperm [11]. In this work we explored the possible participation of CAs in capacitated human and mouse sperm motility analyzing seven different parameters, in addition to BCF. All eight parameters were affected by EZA in a dose-dependent manner in capacitated human cells, and the observed changes were statistically significant starting at EZA 10 nM (Fig. 3A–H). Interestingly, and in contrast to results reported for non-capacitated mouse sperm, among the eight motility parameters analyzed in capacitated mouse sperm, five of them decreased significantly only at EZA 100 μM (Table 1).

Altogether, these data demonstrate that CAs play a preponderant role in the regulation of capacitated human sperm motility; and although we demonstrated that CAs are active in capacitated mouse sperm, they do not seem to play a major role in the regulation of motility (at least under our experimental conditions). These results in capacitated mouse sperm were surprising, considering that inhibition of CAs in non-capacitated cells had been previously reported to alter at least BCF [11]. The difference in the results obtained for mouse sperm might very well be caused by the different physiological state of cells (non-capacitated *versus* capacitated), but it could also be due to the differences in the composition of media (the concentration of pyruvate is five times higher in the medium used in this work).

3.4. CA inhibition potentiates acrosome reaction in capacitated human sperm

We explored the possible participation of CAs in both species during the AR in response to physiological inductors. As shown in Fig. 4A, progesterone 10 μM promotes the AR in capacitated human sperm, albeit small, at a level consistent with previous reports [21], and pre-incubation of 15 min with EZA 30 μM causes a two-fold potentiation effect in the AR. Regarding these results,

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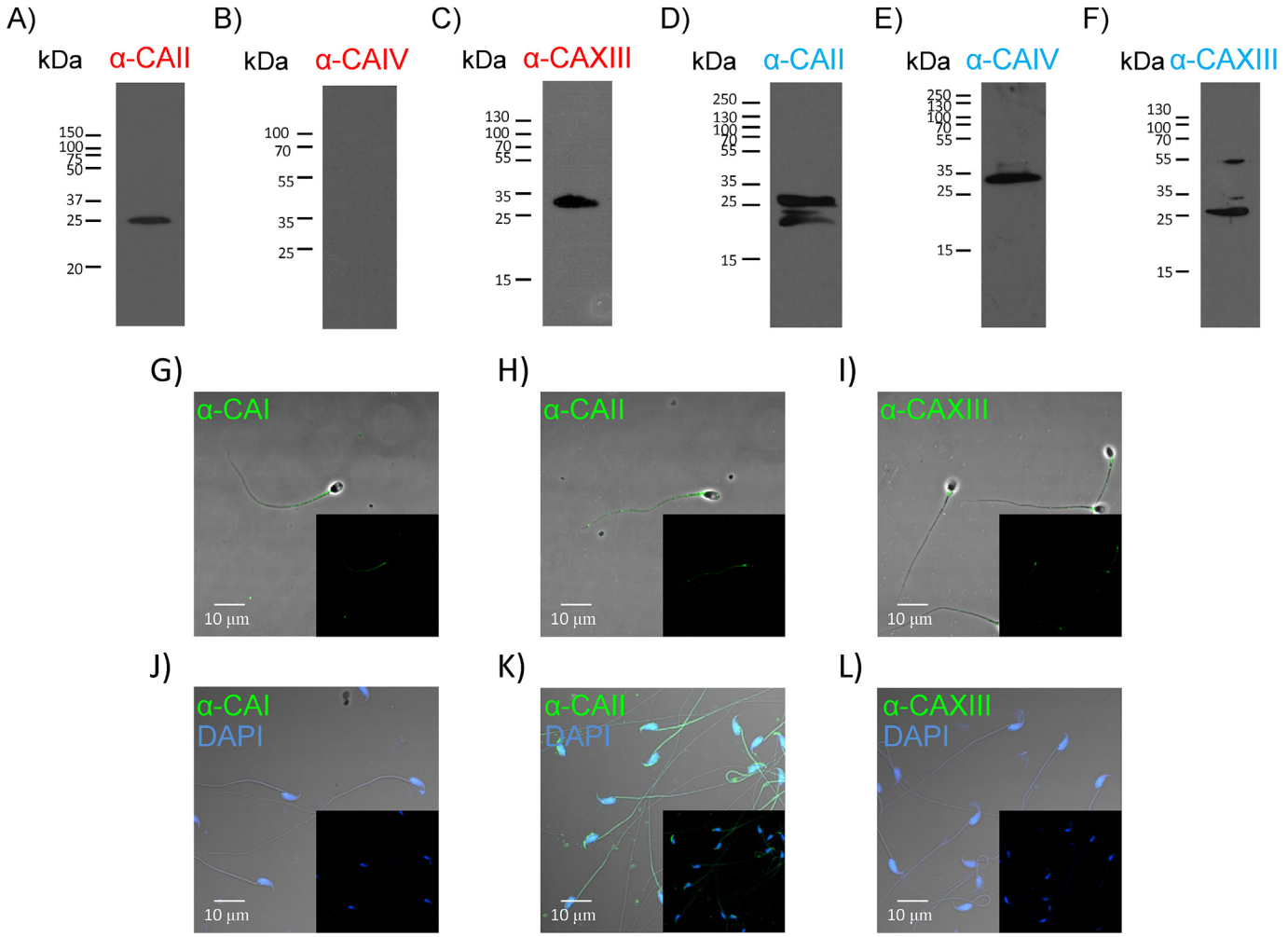

Fig. 1. Different α-CA isoforms are expressed in human and mouse sperm. Solubilized total protein of human and mouse sperm were used for western blot assays and primary antibodies against different α-CAs were tested. A) CAII and C) CAXIII were detected in human sperm, while B) CAIV was not. On the other hand, D) CAII, E) CAIV and F) CAXIII are present in mouse sperm. Immunocytochemistry assays were also performed in sperm from both species. In human sperm G) CAI, H) CAII and I) CAXIII localized to the flagella, and for mouse sperm, only K) CAII was detected in the flagellum as well as in the acrosomal region. Neither J) CAI nor L) CAXII were detected in these cells. The fluorescent dye DAPI stains the nuclei (blue) in mouse sperm only. Images are representative of at least 3 experiments.

progesterone is known to activate CatSper directly in human sperm [22,23] and promote hyperactivated motility, but so far it is unknown whether this activation of CatSper is somehow related to the AR. Presumably, CA inhibition increases the pH_i enough to activate CatSper, and this in turn allows enough Ca^{2+} to enter the cells, inducing the AR. On the other hand, previous exposure of capacitated mouse cells to EZA 30 μM did not change the level of the AR induced by *zona pellucida* (Fig. 4B). Although we detected

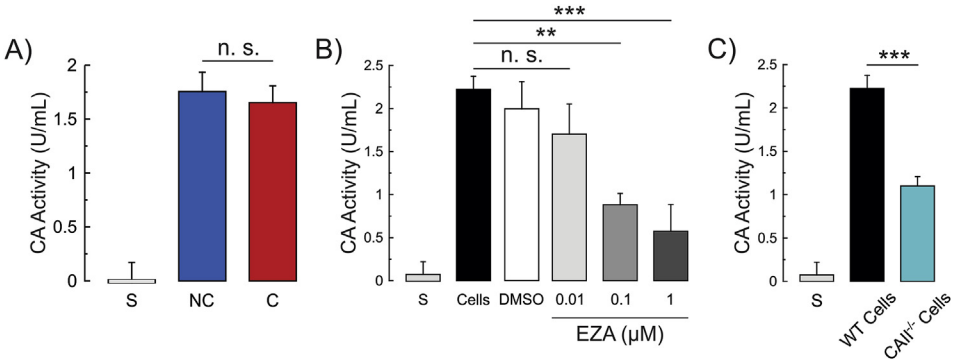

Fig. 2. Capacitated mouse sperm possess CA activity. Motile mouse sperm were obtained by the swim-up technique and CA activity was measured in a quadrupole mass spectrometer in non-capacitated (NC) or capacitated (C) cells. In all the experiments, substrate alone (S), vehicle (DMSO) or both, are shown as controls. A) Non-capacitated and capacitated mouse sperm present CA activity of the same magnitude. B) CA activity decreases in a dose-dependent manner in capacitated mouse sperm when exposed to increasing concentrations of the general CA inhibitor ethoxyzolamide (EZA). C) CAII contributes to half of the global CA activity in capacitated mouse sperm. All values shown are mean ± S.E.M. of at least 3 experiments.

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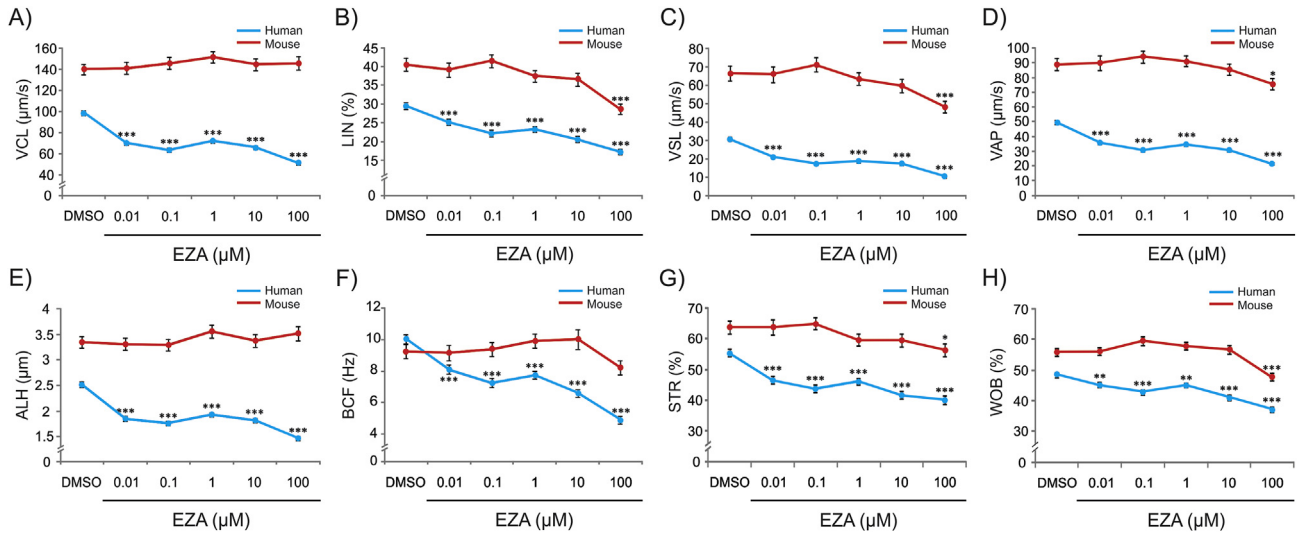

Fig. 3. Motility is affected differentially in capacitated human and mouse sperm by the general CA inhibitor EZA. Motile human and mouse sperm were recovered by the swim-up technique and capacitation was promoted as described previously. Capacitated cells from both species were incubated during 30 min with increasing concentrations of the general CA inhibitor ethoxyzolamide (EZA) or vehicle (DMSO) as control. Motility was measured in a Sperm Class Analyzer. Capacitated human sperm showed a potent decrease in the presence of EZA (from 10 nM) of the eight parameters analyzed: A) Curvilinear velocity (VCL), B) Linearity (LIN), C) Straight line velocity (VSL), D) Average path velocity (VAP), E) Lateral head displacement (ALH), F) Beat cross frequency (BCF), G) Straightness (STR) and H) Wobble (WOB). On the other hand, only the highest concentration of EZA (100 μM) had an inhibitory effect in five of the parameters measured in capacitated mouse sperm (Fig. 3B–D, G and H). All values shown are mean ± S. E. M of at least 3 experiments.

Table 1
Mean values for motility parameters in capacitated human and mouse sperm. All values represent the mean ± S.E.M. of at least 3 experiments (at least 200 cells per experiment) after being exposed during 30 min to vehicle (DMSO) or EZA 100 μM. Significant differences are represented in the table as: $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$.

Motility parameter	Capacitated human sperm		Capacitated mouse sperm	
	DMSO	EZA 100 μM	DMSO	EZA 100 μM
VCL	98.65 ± 2.34 μm/s	50.78 ± 1.58 μm/s***	140.19 ± 4.96 μm/s	145.85 ± 6.30 μm/s
VSL	30.84 ± 1.03 μm/s	10.55 ± 0.61 μm/s***	66.63 ± 3.98 μm/s	48.61 ± 3.25 μm/s***
VAP	49.67 ± 1.24 μm/s	21.69 ± 0.84 μm/s***	88.85 ± 4.11 μm/s	75.81 ± 3.77 μm/s*
LIN	29.51 ± 0.84%	17.24 ± 0.75%***	40.53 ± 1.78%	28.65 ± 1.39%***
STR	55.40 ± 1.15%	40.09 ± 1.31%***	63.78 ± 2.14%	56.33 ± 2.06%*
WOB	48.46 ± 0.76%	37.21 ± 0.92%***	55.90 ± 1.13%	47.43 ± 1.30%***
ALH	2.52 ± 0.05 μm	1.46 ± 0.04 μm***	3.35 ± 0.11 μm	3.52 ± 0.14 μm
BCF	10.03 ± 0.27 Hz	4.90 ± 0.23 Hz***	9.25 ± 0.43 Hz	8.23 ± 0.44 Hz

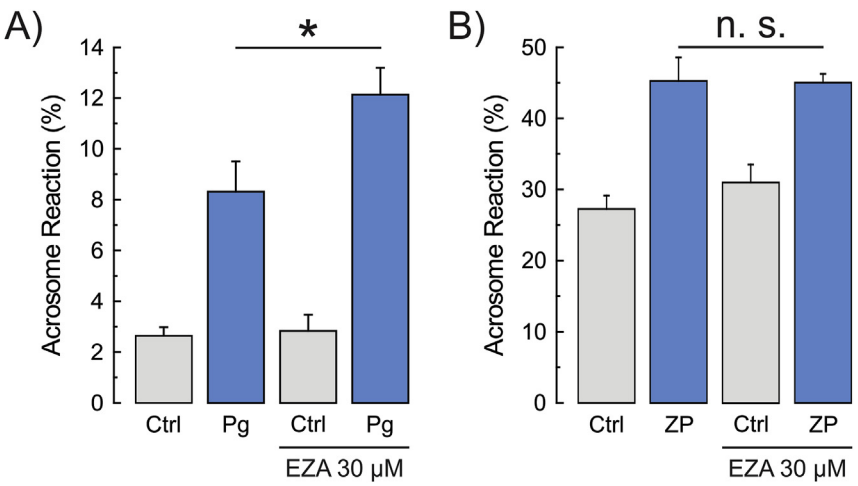

Fig. 4. Acrosome reaction is potentiated in capacitated human sperm through CA inhibition. Motile human and mouse sperm were obtained and capacitated as described previously and then incubated either with progesterone (Pg 10 μM) or 5 *ZP* equivalents/μL, respectively, during 30 min to promote the acrosome reaction (AR) with or without previous 15 min incubation with ethoxyzolamide (EZA) 30 μM. Control (Ctrl) bar represents spontaneous AR. A) Progesterone 10 μM promoted the AR in human sperm, while the preincubation of capacitated cells with EZA increased the percentage of AR. On the other hand, although B) ZP induced the AR in mouse sperm, there was no difference when the cells were pre-incubated with EZA. All values shown are mean ± S.E.M. of at least 3 experiments.

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CAII by immunocytochemistry at the acrosomal region of mouse sperm, these results rule out the possible participation of this isoform in the AR induced by *ZP*. Taken together, these results suggest that the AR signaling pathways are different in human and mouse sperm, although the differences may also be attributable to the different molecules used to induce the AR (a hormone *versus* proteins).

It is also important to mention that CAs can form either physical or functional metabolons with many different anion exchangers, which makes the effect of these enzymes in mammalian sperm physiology even more complex. Although mouse sperm are often used as an experimental model, and the information thus gathered is often extrapolated to human sperm physiology, our results highlight the importance of using both species as experimental models. Indeed, differences between the two species such as the ones we found in this work have also been recently reported elsewhere. Among them, the differential function of certain ion channels and transporters must be mentioned [1,5]. Finally, knowing that CAs play an important role in mammalian sperm physiology, further studies must be done to investigate the presence of others isoforms in sperm, to continue testing specific inhibitors for the different isoforms, and when possible, to elucidate their particular roles in sperm physiology with the use of KO mice.

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Transparency document

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