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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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