Role of Carbonic Anhydrase IV in the Bicarbonate-Mediated Activation of Murine and Human Sperm

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Abstract

HCO₃⁻ is the signal for early activation of sperm motility. In vivo, this occurs when sperm come into contact with the HCO₃⁻ containing fluids in the reproductive tract. The activated motility enables sperm to travel the long distance to the ovum. In spermatozoa HCO₃⁻ stimulates the atypical sperm adenyl cyclase (sAC) to promote the cAMP-mediated pathway that increases flagellar beat frequency. Stimulation of sAC may occur when HCO₃⁻ enters spermatozoa either directly by anion transport or indirectly via diffusion of CO₂ with subsequent hydration by intracellular carbonic anhydrase (CA). We here show that murine sperm possess extracellular CA IV that is transferred to the sperm surface as the sperm pass through the epididymis. Comparison of CA IV expression by qRT PCR analysis confirms that the transfer takes place in the corpus epididymidis. We demonstrate murine and human sperm respond to CO₂ with an increase in beat frequency, an effect that can be inhibited by ethoxzolamide. Comparing CA activity in sperm from wild-type and CA IV⁻/⁻ mice we found a 32.13% reduction in total CA activity in the latter. The CA IV⁻/⁻ sperm also have a reduced response to CO₂. While the beat frequency of wild-type sperm increases from 2.86±0.12 Hz to 6.87±0.34 Hz after CO₂ application, beat frequency of CA IV⁻/⁻ sperm only increases from 3.06±0.20 Hz to 5.29±0.47 Hz. We show, for the first time, a physiological role of CA IV that supplies sperm with HCO₃⁻, which is necessary for stimulation of sAC and hence early activation of spermatozoa.

Introduction

Post-testicular maturation of sperm in the epididymis and the female genital tract includes multiple changes in sperm membrane composition and signal transduction [1,2,3,4]. After spermatogenesis sperm travel along the epididymal tract and the female genital tract where they undergo fundamental changes in their motility. During the early passage they have to adjust from immotility to a linear swimming behaviour to travel along the vagina, uterus and the oviduct. Bicarbonate plays a major role in this early activation of spermatozoa and is necessary for successful fertilization [5,6,7]. The activation of soluble adenyl cyclase (sAC) by bicarbonate induces the elevation of intracellular cAMP and subsequent phosphorylation of several proteins by protein kinase A. We have previously shown that bicarbonate in concentrations of 15 mM induces a reversible, robust acceleration of sperm resting beat frequency from 2–3 Hz to 7 Hz in less than 1 min. In addition, acceleration of beat frequency is accompanied by facilitation of Ca²⁺ channels and beat symmetry [8].

Little is known how sperm regulate a rise of intracellular bicarbonate. In addition to bicarbonate transporters using HCO₃⁻ of the oviduct as a substrate, carbonic anhydrases (CAs) can catalyse the equilibrium between CO₂ and HCO₃⁻. More than a dozen CAs have been identified in mammals [9]. In this study we focused on the physiological role of CA IV after it was identified in the male reproductive tract in mouse and rat [10,11,12] and linked it to the early activation of sperm motility by bicarbonate. We show that extracellular carbonic anhydrase IV is a key enzyme in the early activation of sperm. In addition, we demonstrate that sperm do not acquire CA IV during spermatogenesis, but instead CA IV is transferred to the sperm membrane during the passage through the corpus epididymidis.

Materials and Methods

Ethics statement

Killing of animals was applied for and approved by the animal rights office of the Saarland University (ID 18/05). Human sperm were collected from healthy volunteers with approval of the local ethics committee of the Philipps-University of Marburg, Germany (approval Number 105/05). Written and informed consent was obtained from all participants.

Animals and Cell Preparation

Sperm were isolated from NMRI mice, C57BL/6J mice and CA IV⁻/⁻ (B6.129S1-Car⁻/−/−/−/−/−). After treatment with isoflurane and cervical dislocation, the caput, corpus, cauda epididymidis and vas deferentia were excised from mice, transferred into 1 ml
HS buffer, incised several times, and incubated for 20 min at 37°C in 5% CO2. Released sperm were washed twice (400×g for 3 min) and stored at 1–2×10^7 cells/ml in HCO3−-free HS buffer (in mM): 135 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 20 HEPES, 5 glucose, 10 lactic acid, 1 pyruvic acid, adjusted to pH 7.4 with NaOH. Each experiment was performed with three or more animals.

Preparation of human sperm. Human ejaculates were obtained from healthy volunteers between 20 and 25 years of age. The fresh ejaculates were centrifuged (300×g, 3 min) and the pellet was split into two samples. Each pellet was transferred to 40 ml buffer HS and incubated for 2 hours at room temperature to remove HCO3− from the seminal plasma. After centrifugation, the cells were pooled and re-suspended in 0.5 ml buffer HS and stored at 3–4×10^7 cells/ml.

Immunohistochemistry

Sperm from caput, corpus and cauda epididymis were prepared as described above. After washing in HS buffer, sperm suspensions were diluted to a final concentration of 4×10^6 cells/ml. Sperm were air-dried on cover slips, fixed for 15 min in methanol and again air-dried.

Mouse kidney, testis, epididymis and vas deferens were fixed for 6 h in Bouin solution, dehydrated and embedded in paraffin. For immunohistochemistry, tissue was cut into 5 μm slices and dried on glass slides prior to deparaffinization with xylol and rehydration in water. Hematoxylin (Roth, Karlsruhe, Germany), followed by 5 min incubation in tap water to induce the color reaction. Finally, the slices were incubated overnight at 4°C with goat anti-CA IV IgG (1:1,000 in TBS-T) (R&D Systems, Minneapolis, MN, USA). After washing thrice with TBS-T, the membranes were incubated with HRP-conjugated donkey anti-goat IgG (diluted 1:10 000 in TBS-T) for 1 h at RT. Proteins were detected with an ELC detection reagent (GE Healthcare, Buckinghamshire, UK) on a Chemi-Doc™ XRS+ apparatus (Bio Rad, München, Germany).

qRT PCR

Tissue isolated from kidney, testis, caput, corpus and cauda epididymis was homogenized in 50 μl TriFast™ (PiqLab, Erlangen, Germany) on ice. Total RNA was extracted with the RNeasy Plus™ Micro Kit (Qiagen, Hilden, Germany) and cDNA was prepared with the High Capacity cDNA™ Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). To detect the CA IV gene, 100 ng of total cDNA were processed with a TaqMan® gene expression assay (Applied Biosystems, Foster City, CA, USA). For relative quantitation with the ΔΔCt method [13], we used 18S ribosomal RNA as endogenous control and kidney as reference tissue. All measurements were carried out on a StepOnePlus™ qRT-PCR device from Applied Biosystems (Foster City, CA, USA). Results are presented as mean RQ values ± SEM from three independent preparations.

Assessment of Viability and Motility Parameters

Sperm motility parameters were assessed by means of a computer-assisted sperm analysis (CASA) system (MedeaLAB CASA System, v 5.5, Medical Technology GmbH, Altendorf, Germany). The parameters measured were average velocity [μm/s], motility [%] and the proportions of fast and slow progressive sperm [%].

After washing, sperm were stored in pre-warmed HS buffer containing 5% BSA. For analysis, 20 μl of the sperm suspension was loaded into a pre-warmed (37°C) counting chamber (Makler, Sefi-Medical Instruments ltd., Biosigma S.r.l., Italy). The results are presented as mean ± SEM.

Waveform Analysis

The flagellar waveform was analyzed as previously described [14] with a Nikon Diaphot 300 microscope. In brief, images were collected at 150 Hz (murine sperm) and 300 Hz (human sperm) respectively by a M3 high speed camera (IDT; Talahasee, FL, USA). Determination of flagellar beat frequency was performed by...
A

| tissue         | genotype |
|----------------|----------|
| testis         | ++       |
| epididymis     | ++       |
| vas deferens   | +/+      |

CA IV

B

| tissue        | genotype |
|---------------|----------|
| caput         | +/+      |
| corpus        | +/+      |
| cauda         | +/+      |
| vas deferens  | +/+      |

CA IV

C

| tissue         | genotype |
|----------------|----------|
| vas deferens   | untreated |
|                | +/+      |
|                | flashed  |
|                | +/+      |
|                | luminal content |
|                | +/+      |

CA IV

D

| tissue | genotype |
|--------|----------|
| kidney | +/+      |
| brain  | +/-      |

CA IV

E

| tissue  | RQ value |
|---------|----------|
| kidney  | 1.20     |
| testis  | 1.00     |
| caput   | 0.80     |
| corpus  | 0.60     |
| cauda   | 0.40     |

| CA IV -/+ |
|-----------|
| 0.20      |
| 0.10      |
| 0.04      |

CA IV -/+
semi-automated analysis software written in Igor-Pro™ (Wave- metrics, Lake Oswego OR, USA). The data obtained was collected in Sigma Plot (Systat Software, San Jose, CA, USA) and presented as mean ± SEM. Sp-5,6-dichloro-1-β-D-ribofur- anosylbenzimidazole-3',5'-monophosphorothioate (cBIMPS) was supplied by BioMol (Hamburg, Germany), ethoxyzolamide (EZA) and acetazolamide (AZA) were from Sigma-Aldrich (Steinheim, Germany). 2% and 5% CO₂ was supplied by Air Liquide (Duesseldorf, Germany). To maintain CO₂ equilibration during measurements a heated measuring chamber was continuously perfused with CO₂. Where indicated CO₂ was additionally applied to solutions by gas bubbler manifolds (Harvard Apparatus, Kent, UK) to allow equilibration before perfusion.

Determination of CA Activity by Mass Spectrometry

Determination of CA activity was performed as previously described [15]. In brief, we monitored 18O depletion from doubly-labeled 13C18O2 through several hydration and dehydration steps of CO2 and HCO3⁻ at 25°C [16,17]. The loss of 18O from 13C18O18O (m/z = 49) over the intermediate product 13C18O16O (m/z = 47) and the end product 13C16O16O (m/z = 45) was observed with a quadrupole mass spectrometer (MSD 5970; Hewlett Packard, Waldbronn, Germany). The relative 18O enrichment was documented by the constant measurement of the changes in the signals for m/z = 45, m/z = 47, m/z = 49 (a45, a47, a49) over time and was calculated by the following equation:

\[
\log \text{enrichment} = \log \left( \frac{a_{49}+100}{a_{49}+a_{47}+a_{45}} \right).
\]

The linear slope of the log enrichment over time, calculated with OriginPro™ 7 (OriginLab, Northamton, MA), provided the rate of loss of 18O. This was used to calculate the carbonic anhydrase activity, by comparing the rate with the corresponding rate of the non-catalyzed reaction. To calculate the enzyme activity in units, the Badger and Price [18] definition was used, which defines 1 unit of activity as producing a 100% increase in the non-catalyzed rate of 18O depletion from doubly-labeled 13C18O2. For the experiments, a cuvette was filled with 8 ml HS buffer, followed by 100–200 μl sperm suspension (4 × 10⁶ cells). EZA was added after 6 minutes in the respective concentration.

Statistics

Student’s t-Test was used to calculate the significance in differences of mean values. In the figures shown a significant level of p<0.05 is marked with *, p<0.01 is marked with **, and p<0.001 with ***.

Results

Wild-type kidney (the positive control) shows immunoreactions with the CA IV antibody in the apical plasma membrane of the proximal tubules in the cortex of the kidney (Fig. 1A/a). A weaker

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Figure 2. Immunoblot and real-time PCR of CA IV. A. Immunoblot of CA IV. A CA IV signal in the range of 38 kDa is present in wild-type corpus and cauda epididymis and vas deferens. No specific CA IV band is detectable in wild-type testis and caput epididymis or in any of the CA IV⁻/⁻ tissues. B. Analysis of sperm protein fractions isolated from the different sections of the epididymis shows a positive signal in corpus and cauda sperm and sperm from vas deferens. No specific signal is present in wild-type caput sperm or in any of the CA IV⁻/⁻ sperm. C. CA IV is present in the whole vas deferens tissue and not present in the flushed vas deferens. With the luminal content only a specific CA IV band can be seen. D. kidney and brain tissue were used as positive control. E, CA IV qRT PCR analysis of wild-type and CA IV⁻/⁻ mice. The diagram shows mean RQ values ± s.e.m. of three independent experiments for each tissue. In relation to wild-type kidney (calibrator) the RQ value of wild-type corpus epididymis averages at 0.54. No CA IV mRNA is detectable in the other wild-type or in any of the CA IV⁻/⁻ tissues (n = 3) of wild-type and CA IV⁻/⁻ mice.

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Figure 3. Response of sperm to bicarbonate from different epididymal segments. Sperm of different segments of epididymis show varying responses to bicarbonate. Values shown are mean ± S.E.M. Sperm in HS buffer (black bars) do not show significant differences in resting beat frequency. Mean values were 2.42±0.13 Hz for caput sperm, 1.95±0.07 Hz for corpus sperm and 2.67±0.13 Hz for sperm of cauda epididymis. Sperm beat frequency in HSB buffer (containing 15 mM HCO3⁻) (gray bars) increases the beat frequencies to 6.79±0.42 Hz for caput sperm, 6.09±0.25 Hz for corpus sperm and 9.13±0.40 Hz for sperm of cauda epididymis. (n=30).

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signal is detectable on the basal plasma membrane of the tubulus. CA IV staining is not found in either wild-type testis or caput epididymidis (Fig. 1C/c; 1E/e). However, immunoreactivity is visible in the apical plasma membrane of epithelial cells in the corpus epididymidis (Fig. 1I/i). At higher magnification (insets) the CA IV signal is located in the stereocilia network. In addition we found immunoreactions with CA IV antibodies in both the stereocilia network and spermatozoa of cauda epididymids (Fig. 1M/m). The CA IV \(^{2/2}\) tissue is completely negative for CA IV (Fig. 1B/b; 1D/d; 1F/f; 1J/j; 1N/n).

Both wild-type tissue and caput sperm show no immunoreactions with CA IV antibodies (Fig. 1G), whereas specific staining is found in corpus and cauda sperm from vas deferens show a prominent 38 kDa immunoreactive CA IV band (Fig. 2B). A small signal is detectable in caput sperm. CA IV \(^{2/2}\) sperm do not show any CA IV signal. Tissue of flushed vas deferens and sperm were examined separately and the results demonstrate, that CA IV is localized in luminal sperm only (Fig. 2C). Kidney and brain were used as positive controls (Fig. 2D).

qRT PCR analysis was used to examine the expression of CA IV mRNA in the male reproductive tract (Fig. 2E). CA IV\(^{+/+}\) and CA IV\(^{2/2}\) tissue from kidney, testis, caput, corpus and cauda epididymids was analyzed. Kidney, as the reference tissue, was assigned a constant RQ value of 1. Wild-type corpus epididymidis shows a significant RQ signal of 0.54. No significant RQ signal was found in the other wild type tissues or in any of the CA IV\(^{2/2}\) tissues.

Figure 4. Effect of CA-Inhibitors on sperm beat frequency and CA-activity. A, The effect of HCO\(_3\)^\textsuperscript{-} und CO\(_2\) on sperm beat frequency. Values shown are mean \(\pm\) S.E.M. Mouse sperm beat frequency was measured in HS buffer, HSB buffer (containing 15 mM HCO\(_3\)^\textsuperscript{-}) and in HS buffer in the presence of 5% CO\(_2\). Mean values are 2.96\(\pm\)0.17 Hz of sperm in HS buffer, 8.48\(\pm\)0.17 Hz of sperm in HSB buffer and 7.94\(\pm\)0.31 Hz for sperm stimulated with CO\(_2\) \((n=10)\). B, Concentration-dependent inhibition of carbonic anhydrase activity was determined by mass spectrometry. The addition of varying EZA concentrations results in a decrease of enzymatic activity of between 35.0% (4.53\(\pm\)0.87 U/ml) for 50 nM EZA and 62.12% (2.72\(\pm\)0.12 U/ml) for 5 \(\mu\)M EZA \((n=6)\). C, Sperm beat frequency was measured in HS and HSB buffer in the absence or presence of different EZA or AZA concentrations. Resting beat frequency in HS is not influenced by EZA or AZA. In the presence of bicarbonate, the addition of 100 \(\mu\)M EZA decreases sperm beat frequency from 7.60\(\pm\)0.28 Hz to 4.26\(\pm\)0.21 Hz, whereas the addition of 100 \(\mu\)M AZA decreases beat frequency from 7.84\(\pm\)0.27 Hz to 5.13\(\pm\)0.31 Hz \((n=10)\). D, The cAMP analogon cBIMPS increases sperm beat frequency by acting downstream of carbonic anhydrases. Sperm measured in HS buffer including cBIMPS in the absence or presence of 10 \(\mu\)M ethoxyzoalide (EZA). \((n=10)\). doi:10.1371/journal.pone.0015061.g004

The effect of HCO\(_3\)^\textsuperscript{-} and CO\(_2\) on sperm beat frequency.

Values shown are mean \(\pm\) S.E.M. Mouse sperm beat frequency was measured in HS buffer, HSB buffer (containing 15 mM HCO\(_3\)^\textsuperscript{-}) and in HS buffer in the presence of 5% CO\(_2\). Mean values are 2.96\(\pm\)0.17 Hz of sperm in HS buffer, 8.48\(\pm\)0.17 Hz of sperm in HSB buffer and 7.94\(\pm\)0.31 Hz for sperm stimulated with CO\(_2\) \((n=10)\). B, Concentration-dependent inhibition of carbonic anhydrase activity was determined by mass spectrometry. The addition of varying EZA concentrations results in a decrease of enzymatic activity of between 35.0% (4.53\(\pm\)0.87 U/ml) for 50 nM EZA and 62.12% (2.72\(\pm\)0.12 U/ml) for 5 \(\mu\)M EZA \((n=6)\). C, Sperm beat frequency was measured in HS and HSB buffer in the absence or presence of different EZA or AZA concentrations. Resting beat frequency in HS is not influenced by EZA or AZA. In the presence of bicarbonate, the addition of 100 \(\mu\)M EZA decreases sperm beat frequency from 7.60\(\pm\)0.28 Hz to 4.26\(\pm\)0.21 Hz, whereas the addition of 100 \(\mu\)M AZA decreases beat frequency from 7.84\(\pm\)0.27 Hz to 5.13\(\pm\)0.31 Hz \((n=10)\). D, The cAMP analogon cBIMPS increases sperm beat frequency by acting downstream of carbonic anhydrases. Sperm measured in HS buffer including cBIMPS in the absence or presence of 10 \(\mu\)M ethoxyzoalide (EZA). \((n=10)\). doi:10.1371/journal.pone.0015061.g004
Figure 3 compares the ability of bicarbonate to increase the flagellar beat frequency of sperm from the caput, corpus and cauda epididymidis. Stimulation with 15 mM bicarbonate leads to an increase in beat frequency of randomly-selected cells from $2.42 \pm 0.13$ Hz to $6.79 \pm 0.42$ Hz for caput sperm and from $1.95 \pm 0.07$ Hz to $6.09 \pm 0.25$ Hz for corpus sperm, whereas for cauda sperm the beat frequency increases from $2.67 \pm 0.13$ Hz to $9.13 \pm 0.40$ Hz.

Figure 4A shows that both bicarbonate and CO$_2$ increase flagellar beat of cauda sperm similarly. Sperm accelerate their beat frequency from $2.96 \pm 0.17$ Hz to $8.48 \pm 0.17$ Hz when treated for 5 min with HS medium containing 15 nM bicarbonate. The application of 5% CO$_2$ to sperm in HS medium alone increases the beat frequency within 5 minutes to $7.94 \pm 0.31$ Hz. The total carbonic anhydrase activity of $\sim4 \times 10^8$ cells was determined by mass spectrometry before and after the application of different concentrations of the carbonic anhydrase inhibitor EZA (Fig. 4B). The addition of 50 nM EZA significantly decreases enzymatic activity from $7.01 \pm 0.46$ U/ml to $4.53 \pm 0.87$ U/ml (35.38% reduction), 100 nM EZA leads to a highly significant decrease
the action of HCO3⁻ on the flagellar beat. Figure 4C shows that after treatment with 5 nM or 500 nM EZA, bicarbonate increases the beat frequency from 2.63±0.15 Hz to 5.71±0.23 Hz and from 2.61±0.15 Hz to 5.01±0.16 Hz. The addition of HCO3⁻ in the presence of 100 μM EZA results in a <1.5-fold increase in sperm beat frequency (from 2.92±0.24 Hz to 4.26±0.21 Hz) only. Treatment with AZA in the same concentrations as EZA shows nearly the same inhibitory effect (5 nM AZA: from 2.63±0.10 Hz to 6.47±0.41 Hz; 500 nM AZA: from 2.68±0.10 Hz to 5.83±0.34 Hz; 100 μM AZA: from 3.01±0.25 Hz to 5.13±0.31 Hz). To examine if EZA has actions downstream of bicarbonate, which was applied for 60 s, an accelerated beat frequency from 6.23±0.26 Hz to 13.00±0.56 Hz is apparent in the presence of EZA (dashed box). In the following two-minute period the slope of beat frequency is increased to 1.83 Hz/min (ΔAm₄) (n = 10).

Flagellar movement of human sperm was analyzed in the same way as for murine sperm. With 2% CO₂, the resting beat frequency of 7.51±0.56 Hz speeds to 14.62±0.70 Hz (Fig. 5C) after 40 s, whereas in the presence of 1 μM EZA, beat frequency rises from 6.26±0.81 Hz to 10.39±1.50 Hz. Also with 15 mM bicarbonate, which was applied for 60 s, an accelerated beat frequency from 6.23±0.26 Hz to 13.00±0.56 Hz is apparent (Fig. 5D).

Figure 6 compares the motility parameters of CASA determined for sperm of wild type and of CA IV⁻/⁻ mice in the absence of bicarbonate. In CA IV⁻/⁻, the total motility is significantly decreased (35.75±7.46%; CA IV⁻/⁻: 26.38±3.93 μm/s). There are also no significant changes in slow progressive sperm from wild-type mice and CA IV⁻/⁻ mice (wild-type: 22.13±1.97%; CA IV⁻/⁻: 15.50±2.65%).
In comparison to wild type sperm, the enzymatic activity of CA IV\(^{-/-}\) animals is decreased highly significantly by 32.13% (from 5.26\(\pm\)0.34 U/ml to 3.57\(\pm\)0.25 U/ml) (Fig. 7A). Waveform analysis was performed to analyze the effect of HCO\(_3\)^{-} and CO\(_2\) on the beat frequency of CA IV\(^{-/-}\) sperm (Fig. 7B, C). Fig. 7B shows that there is only a slight difference in the first 20 seconds between the sperm of wild-type and CA IV\(^{-/-}\) animals in the response to HCO\(_3\)^{-}. Perfusion of sperm of wild-type and CA IV\(^{-/-}\) animals with buffer containing 15 mM HCO\(_3\)^{-} leads to an increase of sperm beat frequency within 20 seconds from 2.81\(\pm\)0.17 Hz to 6.13\(\pm\)0.35 Hz and from 2.92\(\pm\)0.17 Hz to 5.57\(\pm\)0.18 Hz, respectively (n = 10). Sperm of CA IV\(^{-/-}\) mice show a decreased response to CO\(_2\). Through the application of 2% CO\(_2\) in the measuring chamber and the manifolds wild-type sperm beat frequency (solid line) increase within the first 20 seconds from 2.86\(\pm\)0.12 Hz to 6.87\(\pm\)0.34 Hz, while the beat frequency of CA IV\(^{-/-}\) sperm takes 1 min to rise from 3.06\(\pm\)0.20 Hz to 5.29\(\pm\)0.47 Hz (n = 10). Sperm of CA IV\(^{-/-}\) mice show the same response to the cAMP analogon cBIMPS as wild-type sperm. 20 seconds after stimulation with 5% CO\(_2\), the sperm of CA IV\(^{-/-}\) mice show a 20.48% reduction in beat frequency compared to wild-type sperm. By contrast, after 10 minutes stimulation with 50 \(\mu\)M cBIMPS wild-type and CA IV\(^{-/-}\) sperm speed their beat to comparable values (from 2.89\(\pm\)0.16 Hz to 5.71\(\pm\)0.24 Hz and from 2.80\(\pm\)0.15 Hz to 5.83\(\pm\)0.30 Hz, respectively) (n = 10).

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**Discussion**

This work analyses the distribution and physiological activity of CA IV in the murine male genital tract. The results show that CA IV is involved in the regulation of intracellular bicarbonate concentration and early activation of spermatozoa by bicarbonate. Bicarbonate as an important factor for sperm maturation and storage in the male reproductive tract, is responsible for acceleration of sperm beat frequency and calcium channel activation [3,8,19].

**CA IV has a distinct location in the male reproductive tract**

By immunohistochemistry we show that CA IV is not a constituent that is acquired during spermatogenesis. CA IV was not detectable at all stages of sperm maturation in testis. However,
in caput, corpus and cauda epididymidis, CA IV appears in the stereocilia of epithelium and from there on also in isolated spermatozoa. Localization in the plasma membrane and in the stereocilia network are in accordance with the findings that CA IV is an extracellular GPI-anchored protein [20]. This supports the proposal of Ekstedt and co-workers [12] who recognized the possible transfer of CA IV during sperm passage through the epididymal tract. In rat, a different distribution of CA IV was found in the epididymis, where only epithelial cells of the corpus epididymidis showed CA IV immunoreactivity [21]. A post-testicular transfer of other proteins was demonstrated for sperm adhesion molecule1 (Spam1), which is secreted in epididymosomes released by the epithelial cells to the luminal fluid and integrated into the sperm surface [22,23]. Such a possible transfer of CA IV is supported by two other findings of the present study. Western-blot and real-time PCR show CI IV mainly in the corpus epididymidis. First western blot analysis shows CA IV only in corpus, cauda and vas deferens and secondly real time-PCR detects mRNA for CA IV only in corpus epididymidis. The amount of transcripts of CA IV mRNA in corpus epididymidis is comparable to that of the kidney, which served as reference. In relation to the mouse kidney, the CA IV mRNA in the corpus epididymidis averages at 54.30% compared to kidney.

The inflow of bicarbonate into the cell can either occur by anion transporters across the cell membrane, or via diffusion of CO₂, which is then hydrated by intracellular CA. We were able to show CO₂-induced acceleration of sperm beat frequency in a dose-dependent manner and that treatment with carbonic anhydrase inhibitors EZA or AZA slows acceleration of beat frequency, which shows that CAs are involved in the control of flagellar beating presumably due to hydration of CO₂ to supply sperm with HCO₃⁻. The inhibitory effect of AZA in the luminal fluid of epididymis in rat has been investigated in other studies. It was found that AZA depresses the luminal acidification by 80% in rat cauda epididymidis [24]. Murine sperm show significant CA IV activity. In our study, we were primarily interested in characterizing the physiological role of CA IV in murine epididymal spermatozoa. By measuring the response as acceleration of beat frequency of single cells to CO₂, we show that carbonic anhydrases are involved in bicarbonate supply. The speed of response to CO₂ could be reduced either by using carbonic anhydrase inhibitors or using sperm of mice with a targeted mutagenesis of the CA IV gene. In spite of other carbonic anhydrases, which are mainly located intracellularly, a state of equilibrium between HCO₃⁻ and CO₂ in the cells of CA IV⁻/⁻ animals takes longer to develop than in sperm of wild-type animals. In addition, the free diffusion of CO₂ through the sperm membrane appears to be faster than the import of HCO₃⁻ by anion transporters, which might create a local disequilibrium near the cell surface. When sperm lack CA IV, the reestablishment of the uncatalyzed equilibrium is slower, and acceleration of flagellar beat is delayed. This delay is even more evident when 2% CO₂ is used for stimulation instead of 5% CO₂. The presence of other CAs presumably explains why CA activity in sperm of CA IV⁻/⁻ animals measured by mass spectrometry is reduced only by one third, whereas inhibition of CAs using 5 μM EZA leads to a decrease in enzymatic activity of 62.12%. We discuss this difference either as being an inhibitory effect of EZA towards other CA-isoforms [25], or as a compensatory effect by upregulation of other CA-isoforms during spermatogenesis. It is even more notable that the lack of CA IV is compensated only partially and underlines the importance of CA IV for spermatozoa. It was not surprising that we did not observe fundamental changes in the response to bicarbonate in CA IV⁻/⁻ animals, since the transport of bicarbonate through anion transporters is not affected in CA IV⁻/⁻ sperm.

cAMP acts downstream of CA

The application of cAMP analog cBIMPS in wild-type and CAIV⁻/⁻ animals increases flagellar beat frequency to a similar
ext. As expected, treatment of wild-type sperm with EZA, did not lead to a difference in eBIMPS-induced acceleration of sperm beat, demonstrating that eBIMPS bypasses the action of HCO3− on soluble adenylyl cyclase.

CO2 and HCO3− show similar effects on human sperm beat frequency

It was previously shown that also human sperm speed flagellar beat in response to HCO3− from 6.60 Hz to 12.20 Hz [26]. We extended our studies and successfully demonstrate for the first time that human spermatozoa also respond to CO2 with an approximately doubled increase of beat frequency from 7.51 Hz to 14.62 Hz. This response can be reduced with EZA about 27.56% and elucidates carbonic anhydrases to be active in these cells. Bering responsible for one third of the whole CA activity, CA IV is comparable to membrane bound CA in other cells like astrocytes [27]. However, the fact that CA IV is transferred in the corpus epididymids to the sperm surface accentuates the importance for post-testicular regulation of CO2 and HCO3− and therefore motility.

The use of computer-assisted sperm analysis (CASA) demonstrates that basic motility parameters of a whole sperm population can be significantly different compared to the evaluation of motility parameters on a single cell level. We found significant differences in the amount of motile and fast progressive spermatozoa between wild-type and CA IV−/− sperm. Determination of the basal beat frequency of motile sperm did not reveal any alterations in CA−/− animals.

Model of CA IV action in murine sperm

In conclusion we would postulate the role of CA IV in spermatozoa as follows: CA IV, as an external carbonic anhydrase, equilibrates HCO3− and CO2 near the sperm surface, so that an increase in external CO2 rapidly replenishes HCO3− at the extracellular membrane face. Bicarbonate can enter the cell by anion transporters. In sperm carbonic anhydrases located in the cytoplasm, such as CA II, use CO2 to provide HCO3− by catalytic fast equilibration, resulting in early activation of spermatozoa by sAC. A targeted disruption of CA IV inhibits fast equilibration between HCO3− and CO2 near the cell membrane and leads to a transient disequilibrium, and delayed restoration of the HCO3− concentration resulting in decreased HCO3− influx and hence smaller activation of HCO3−−dependent sAG-mediated activation of flagellar beat (Fig. 8).

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Author Contributions

Conceived and designed the experiments: GW JWD HMB. Performed the experiments: PMW MR NM WSS. Analyzed the data: GW MR NM PMW HMB. Contributed reagents/materials/analysis tools: WSS HMB JWD. Wrote the paper: GW MR PMW NM.