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Mitochondrial PKA mediates sperm motility

Rashel Mizrahi, Haim Breitbart *

The Mina & Everard Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan 5290002, Israel

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ABSTRACT

Background: Mitochondria are the major source of ATP to power sperm motility. Phosphorylation of mitochondrial proteins has been proposed as a major regulatory mechanism for mitochondrial bioenergetics.
Methods: Sperm motility was measured by a computer-assisted analyzer, protein detection by western blotting, membrane potential by tetramethylrhodamine, cellular ATP by luciferase assay and localization of PKA by immuno-electron microscopy.
Results: Bicarbonate is essential for the creation of mitochondrial electro-chemical gradient, ATP synthesis and sperm motility. Bicarbonate stimulates PKA-dependent phosphorylation of two 60 kDa proteins identified as Tektin and glucose-6-phosphate isomerase. This phosphorylation was inhibited by respiration inhibition and phosphorylation could be restored by glucose in the presence of bicarbonate. However, this effect of glucose cannot be seen when the mitochondrial ATP/ADP exchanger was inhibited indicating that glycolytic-produced ATP is transported into the mitochondria and allows PKA-dependent protein phosphorylation inside the mitochondria.
Conclusions: Bicarbonate activates mitochondrial soluble adenylyl cyclase (sAC) which catalyzes cAMP production leading to the activation of mitochondrial PKA. Glucose can overcome the lack of ATP in the absence of bicarbonate but it cannot affect the mitochondrial sAC/PKA system, therefore the PKA-dependent phosphorylation of the 60 kDa proteins does not occur in the absence of bicarbonate.
General significance: Production of CO_2 in Krebs cycle, which is converted to bicarbonate is essential for sAC/PKA activation leading to mitochondrial membrane potential creation and ATP synthesis.

1. Introduction

Sperm motility describes the ability of sperm to move towards the egg in order to achieve fertilization. Progressive motility is defined as active motion, either linearly or in a large circle, regardless of speed [1]. Sperm progressive motility is essential for the sperm to pass through the cervical mucus [2], as well as to penetrate the zona pellucida both *in vivo* and *in vitro*, and thus this mode of motility is considered one of the most important factors in determining fertilization rates [3–5]. Therefore, the property of progressive motility is an essential parameter for studies related to fertility or to the effects of exogenous factors on sperm motility [6].

After ejaculation, most sperm exhibit typical progressive and linear motility. The activation of the vigorous and asymmetric movement of the flagella occurs as soon as the sperm leave the epididymis, and represents one of the first events in the multiple physiological and biochemical modifications that accompany sperm capacitation. For effective fertilization, sperm cells should reside in the female reproductive tract for a few hours, where they undergo several biochemical processes collectively called capacitation (Rev. by [7,8]). Sperm cells can be capacitated *in vitro* by incubation in a strictly defined capacitation medium in which Ca^{2+} and bicarbonate are two essential components of the capacitation medium [8].

Sperm flagellar motility requires the activation of both energy metabolism and the motility apparatus. Flagellar motion is generated by the motor activities of the axonemal dynein ATPase activity that is localized along the entire length of a flagellum and depends on a constant supply of ATP [5]. Thus, motility is strongly related to the ability of the spermatozoa to manage its energy status.

Oxidative phosphorylation (OXPHOS) and glycolysis supply ATP for sperm motility [9]. Despite extensive research, the regulation of mitochondrial OXPHOS is still incompletely understood. Recently, evidence has emerged suggesting that cAMP-mediated phosphorylation of mitochondrial enzymes plays a role in OXPHOS regulation [10–15]. cAMP is generated by two types of adenylyl cyclases (ACs): transmembrane AC (tmAC), regulated by heterotrimeric G-proteins and stimulated by forskolin [16], and soluble AC (sAC or SACY), stimulated by bicarbonate [17] and Ca^{2+} [18]. The presence of tmAC in mammalian sperm is still controversial [19], and therefore, it is generally accepted that sAC is responsible for most cAMP synthesis in sperm, and its regulation by Ca^{2+} and HCO_3^- is essential to induce cAMP-dependent physiological changes related to sperm capacitation [13,14]. Bicarbonate stimulates sAC

* Corresponding author. Tel.: +972 3 5318201.
E-mail address: haim.breitbart@biu.ac.il (H. Breitbart).

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activity by facilitating active site closure, while calcium promotes activity by increasing the affinity of the enzyme for ATP [20,21].

Although cAMP has been reported to open ion channels or to activate guanine nucleotide exchange factors, most evidence suggests cAMP-dependent protein kinase A (PKA) as the major downstream effector of cAMP signals in sperm. PKA is a tetrameric enzyme consisting of two catalytic (C) and two regulatory domains (R). In mammals, there are three known isoforms of the catalytic subunits (CαCβCγ) and four isoforms of the regulatory ones (RIαRIβRIIαRIIβ). cAMP binding to R releases active C subunits, which phosphorylate key substrates, initiating a cascade of signaling events inside the cell [22,23]. To modulate such a diverse spectrum of events with the required specificity, PKA must be activated at precise cellular locations and at specific times. This spatial–temporal regulation is conveyed in part through interaction of PKA with protein kinase A-anchoring proteins (AKAPs) [24,25]. Although structurally diverse, all AKAPs contain a PKA-anchoring domain and a specific targeting motif that dictates their subcellular localization. AKAPs are scaffolding proteins that sequester not only protein kinases but also phosphatases to coordinate phosphorylation dynamics [26]. For example, besides PKA, most of the AKAPs also anchor phosphodiesterases that degrade cAMP and terminate PKA activity. This allows AKAP to locally regulate the amplitude and duration of PKA activation [27]. Both, PKA (reviewed in [28,29]) and A kinase-anchoring proteins (AKAPs), have been identified in mammalian mitochondria [30,31].

Over the past years, phosphorylation of mitochondrial proteins has emerged as an important player in the modulation of mitochondrial oxidative metabolism. While PKA presents one of the most widely studied kinases, the role of PKA phosphorylation of mitochondrial proteins in the regulation of mitochondrial metabolism remains controversial.

Here we show that sperm motility, dependent on mitochondrial ATP production, is regulated by mitochondrial sAC/cAMP/PKA in response to metabolically generated bicarbonate.

2. Materials and methods

2.1. Materials and antibodies

BSA (Fraction V), dibutyryl cAMP, Antimycin A (from *Streptomyces* sp.), Atractyloside (Atractyloside potassium salt), H89 (N-[2-(p bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide), 2-Hydroxyestradiol and Coomassie Brilliant Blue R Staining Solution were purchased from Sigma Chemical Company (St. Louis, MO). Tetramethylrhodamine methyl ester perchlorate (TMRM) was obtained from Molecular Probes (Eugene, OR, USA).

Antibody against phospho-PKA substrate (RRXS/T) (100G7) was purchased from Cell Signaling (Beverly, MA, USA). Mouse monoclonal β-actin (C4) HRP was purchased from Santa Cruz Biotechnology (California, USA). Goat anti-mouse IgG-HRP conjugated and goat anti-rabbit IgG-HRP conjugated were purchased from Bio-Rad (Richmond, CA, USA). Re-Blot Plus Strong Solution (×10) was purchased from Millipore (Temecula, California, 92590).

All other chemicals were purchased from Sigma (Sigma-Aldrich Israel Ltd. Rehovot, Israel) unless otherwise stated.

2.2. Sperm preparation

Bovine sperm was supplied by the SION Artificial Insemination Center (Hafetz-Haim, Israel). Ejaculated bull spermatozoa were obtained by using artificial vagina, and the "swim up" technique was applied to obtain motile sperm. Sperm cells were washed three times by centrifugation (780 ×*g* for 10 min at 25 °C) in NKM buffer (110 mM NaCl, 5 mM KCl, and 20 mM 3-N-morpholinopropanesulfonic acid (MOPS) (pH 7.4)) and the sperm were allowed to swim up after the last wash. The washed cells were counted and maintained at room temperature until use.

Cryopreserved samples were supplied in 0.25 μl tablets. The semen cryopreserved in tablet form was thawed at 64 °C for 30 s. The cells were washed in NKM medium by three centrifugations at 780 ×*g* for 5 min. After centrifugation, the samples were kept in a water bath at 39 °C for 10 min and only the "swim up" (cells found in the supernatant presumed to be the undamaged cells) fraction was used for the experiment. The final pellets were re-suspended in NKM or mTALP, with the sperm concentration adjusted to 10^8 cells/ml.

2.3. Immunoblot analysis

Sperm cells were washed by centrifugation at 10,000 ×*g* for 5 min at 4 °C, and then the supernatant was discarded, and the pellet was resuspended in Tris-buffered saline (TBS) and centrifuged again to remove excess BSA. Sperm lysates were then prepared by the addition of lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 6% SDS, protease inhibitor cocktail 1:100, 50 mM NaF, 50 mM pyrophosphate, 0.2 mM Na_3VO_4, and freshly added 1 mM phenylmethylsulfonyl fluoride (PMSF)), to the pellet, and the lysate was vortexed vigorously for 15 min at room temperature. Lysates were then centrifuged at 10,000 ×*g* for 5 min at 4 °C, the supernatant was transferred, and the protein concentration was determined by the Bradford method [32] or by the BCA method [33]. Sample buffer ×5 was added to the supernatant, and boiled for 5 min. The extracts were separated on 10 or 12% SDS-polyacrylamide gels, and then electrophoretically transferred to nitrocellulose membranes (300 mA; 1 h) using a buffer composed of 25 mM Tris (pH 8.2), 192 mM glycine, and 20% methanol. Blots were routinely stained with Ponceau solution to confirm equal loading and even transfer. The blots were blocked with 1% BSA in TBS, pH 7.6, containing 0.1% Tween 20 (TBST), for 30 min at room temperature. Phospho-PKA substrates were immunodetected using anti-phospho-PKAs antibody (diluted 1:10,000). The membranes were incubated overnight at 4 °C with the primary antibodies diluted in 1% BSA in TBST. Next, the membranes were washed three times with TBST and incubated for 1 h at room temperature with specific HRP-linked secondary antibodies (Bio-Rad Lab), diluted 1:5000 in TBST and 1% BSA. The membranes were washed three times with TBST and visualized by ECL (Amersham, Little Chalfont, UK). In order to use more than one antibody, the membrane was stripped for 30 min at room temperature under gentle agitation using Re-Blot Plus Strong Solution, then blocked twice with 1% BSA in TBST for 30 min at room temperature and subjected to incubation with another antibody. Each figure in this article represents a single membrane/blot that was subjected to different antibodies using stripping buffer.

2.4. Protein sequencing and mass spectroscopy

Proteins were separated on 12% SDS-polyacrylamide gels, using a large format 1-D electrophoresis system for wide range protein separation. The gel was stained using Coomassie Brilliant Blue R and only the band of interest was excised for protein identification.

Serines and threonines were analyzed for the probability of phosphorylation using mass spectroscopy (Smoler Proteomics Center Faculty of Biology, Technion-Institute of Technology, Israel). The samples were analyzed by LC–MS/MS on LTQ-Orbitrap (Thermo), and identified by Discoverer software against the bovine part of the Uniprot database, and a decoy database.

2.5. Measurement of mitochondrial membrane potential (MMP)

The potentiometric vital dye, tetramethylrhodamine methyl ester (TMRM), which is the indicator of choice in MMP assessment [34] was used to study MMP kinetics. Cells were mixed with TMRM stain (100 nM), and incubated at 37 °C for 15 min. Images were acquired using a motorized upright epifluorescence Olympus BX51 microscope (Olympus, Japan) or an inverted IX81 microscope equipped with an

incubator system for maintaining optimal temperature (37 °C). Cells were illuminated by a Mercury light source. The emitted fluorescence was imaged using a CoolSNAP HQ monochrome CCD camera. Initial MMP was measured using appropriate filters (excitation filter: 540 ± 10 nm, emission filter open from 590 nm). Digital image analysis of cellular fluorescence was performed by Image Pro Plus software (Media Cybernetics, USA) and Olympus Cell^P software.

2.6. Motility measurements using the CASA device

Sperm cells (3×10^7 cells/ml) were incubated for capacitation in mTalp medium. Protemine sulfate (40 μg/1 ml) was added for 5 min before measurement, and samples were vortexed for a few seconds before each measurement to prevent head to head aggregation. Samples (5 ml) were placed in a prewarmed standard count four chamber 20 mm-depth slide (Leja, Nieuw Vennet, Netherlands) at 37 °C, and analyzed using a computer-assisted sperm analysis (CASA) device with IVOS software (version 12, Hamilton-Thorne Biosciences). Up to 10 sequences, each 30 s long, were acquired for each sample. At least 100 cells were analyzed in each sample, using parameters identifying bovine sperm motility. After each scan, the playback facility performed a quality control for the correct acquirement of the field.

Spermatozoa with an average path velocity (VAP) ≥ 50 μm/s and straightness (STR, as VSL/VAP, %) ≥ 50% were considered progressive. Motility (%) was defined as the percentage motile cells of the total, and progressive motility (%) was defined as the percentage progressive motile cells of the motile population.

2.7. Cellular ATP determination

Cells (2.5×10^8/ml) were incubated in a final volume of 0.25 ml in medium A with 1.25 mM Pi, 0.2 mM $CaCl_2$ and 10 mM substrate. After 10 min at 37 °C the incubation was terminated by adding 0.25 ml ice cold 20% TCA. The TCA was then extracted with a two volume portion of Freon/tri-N-octylamine (3:1, v/v) and the two phases separated by centrifugation. In this procedure the precipitated protein appeared as a thin layer between the two phases. An aliquot of the aqueous phase was removed and assayed for ATP using the luciferase method.

2.8. Detection of cellular PKA-Cs

For immuno-electron microscopy, anti-PKA-Cs (a gift from G.B. Witman, Department of Cell Biology, University of Massachusetts Medical School, Worcester, MA), was added to 100-Å sections of embedded sperm cells and then incubated with 15 nm gold-conjugated secondary antibody (Zymed). Control experiments produced only background level signals.

3. Results

Today most of the dairy cows in the world are inseminated by frozen sperm from superior bulls. It is well known that cryopreservation retards general sperm motility, and progressive motility, in particular. Thus, we performed this study to understand the causes for motility reduction after freezing and to use the frozen sperm as a model for reduced motility in order to understand the bioenergetic mechanisms that promote sperm motility.

We followed the percentage of motile sperm (total motility) and progressive motility before and after cryopreservation in both non-capacitation and capacitation-supporting conditions. We determined sperm motility incubated in capacitation medium with or without bicarbonate. The data in Fig. 1 show that bicarbonate-deficient medium had a small effect on total motility (22% or 26% reduction after 0.5 h or 4.0 h, respectively) or progressive motility (11% or 36% reduction after 0.5 h or 4.0 h, respectively) of fresh sperm. However cryopreserved sperm exhibited 50% or 100% reduction in total motility after 0.5 h or

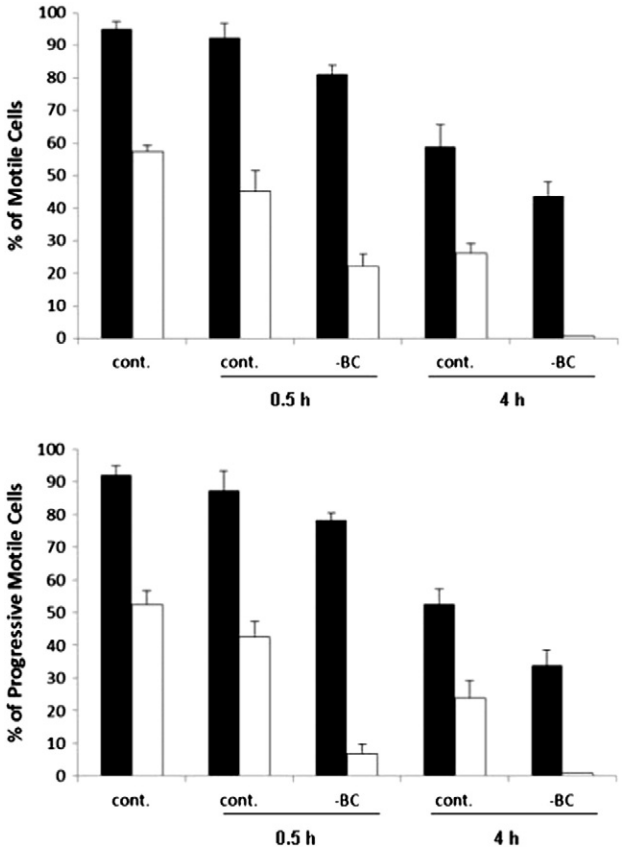

Fig. 1. Effect of bicarbonate on sperm motility. Fresh and cryopreserved sperm were incubated in standard (cont.) or HCO^--deficient (−BC) mTALP. Motility data for both general and progressive motility were collected at time 0, 0.5 and 4 h, and analyzed using CASA-Ivos. Black columns — fresh semen; open columns — cryopreserved semen. The values shown represent the mean ± SD of six experiments.

4.0 h, respectively, and 84% or 100% reduction in progressive motility after 0.5 h or 4.0 h respectively, in bicarbonate deficient medium. Thus, although fresh sperm show relatively high motility, even in bicarbonate-deficient medium, there is complete inhibition of motility after 4 h incubation of cryopreserved sperm in medium without added bicarbonate. We found that 10 or 25 mM HCO_3^- induced identical stimulatory effects on cryopreserved sperm motility, but 5 mM bicarbonate was not effective (not shown). Bicarbonate can penetrate the cell and alkalize the cytosol. Intracellular alkalization has been proposed as the mechanism responsible for the initiation of motility in mammalian sperm [35,36]. However, alkalization of the intracellular space by

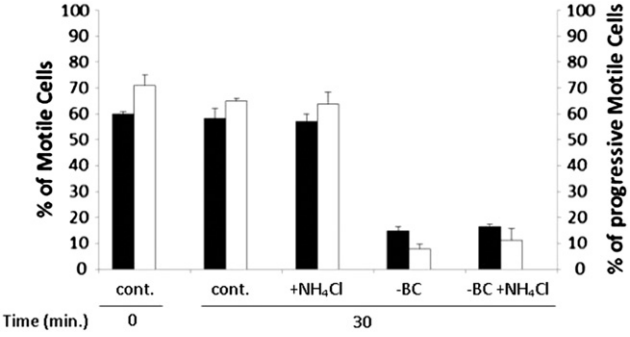

Fig. 2. Effect of NH_4Cl on total and progressive sperm motility. Cryopreserved sperm were incubated for 30 min in standard (cont.) or bicarbonate deficient (−BC) mTALP, with or without NH_4Cl (25 mM). Motility was determined by CASA-Ivos, immediately and after 30 min of incubation. Black columns — total motility; open columns — progressive motility. The values represent the mean ± SD of six experiments.

incubating the cells with NH_4Cl did not restore the motility of cryopreserved sperm in bicarbonate deficient medium (Fig. 2), and thus, bicarbonate mediates motility through another mechanism.

In mammalian sperm, most of the cAMP is produced by the soluble-adenylyl-cyclase (sAC), known to be activated by Ca^{2+} and bicarbonate [17]. To test the possibility that bicarbonate deficiency prevents cAMP production, we added the membrane permeable 8Br-cAMP, to cryopreserved sperm incubated in bicarbonate deficient medium and followed its effect on motility. As shown in Fig. 3 although 8Br-cAMP stimulated an increase of 2.0 or 3.5 folds total or progressive motility respectively, in bicarbonate deficient medium, there is only 56% or 40% restoration of total or progressive motility, respectively, by 8Br-cAMP compared to bicarbonate containing medium. Thus, the action of bicarbonate in promoting sperm motility is not mediated solely through cAMP.

We further measured motility in medium without glucose, to test the assumption that motility is largely dependent on mitochondrial activity. Indeed we found that the respiratory-specific inhibitor, Antimycin A, caused complete inhibition of the motility parameters tested (Fig. 4). Moreover, glucose, which provides ATP *via* glycolysis, could replace bicarbonate in restoring the motility of cryopreserved sperm (Fig. 4). These data suggest that insufficient levels of cellular ATP are the cause for poor motility in cryopreserved sperm in the absence of added bicarbonate.

To test our assumption, we have measured cellular ATP levels. Antimycin A treated cells or cryopreserved cells incubated in bicarbonate-deficient media showed very low ATP content (Table 1). This loss of ATP levels was completely restored by the addition of bicarbonate or glucose (Table 1).

Thus, mitochondrial activity appears to be defective in cryopreserved sperm. Indeed, we found that the mitochondrial membrane potential (MMP) (Fig. 5), which is essential for ATP synthesis, and cellular ATP levels (Table 1) are very low in cryopreserved sperm incubated without added bicarbonate, and the addition of bicarbonate elevated the MMP and ATP to the level found in fresh sperm (Fig. 5 and Table 1). In fact, the MMP and ATP levels in cryopreserved sperm without bicarbonate was comparable to the MMP and ATP levels in Antimycin A treated sperm, in which the respiration is blocked and apparent MMP cannot be created. In order to investigate the involvement of PKA in MMP creation we used an anchoring disrupting peptide, Ht31, which contains the anchoring motif present in all AKAPs and competes with AKAPs for binding the regulatory subunit (RII) of PKA. The data reveal complete inhibition of MMP (Fig. 5) and immediate inhibition of sperm motility (not shown).

At this point, we attempted to identify the mechanism by which bicarbonate affects MMP. Mitochondria contain sAC and PKA, which can affect respiratory chain activity [37]. To test the possibility that

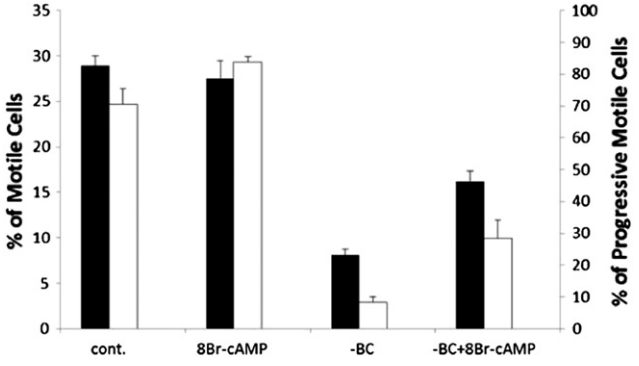

Fig. 3. Effect of cAMP on total and progressive motility of cryopreserved sperm. Cryopreserved sperm were incubated for 30 min in standard capacitation mTALP (cont.) and mTALP devoid of HCO_3^- (−BC), with or without 8Br-cAMP (10 μM). Motility was measured using CASA-Ivos. Black columns — total motility; open columns — progressive motility. The values represent the mean ± SD of three experiments.

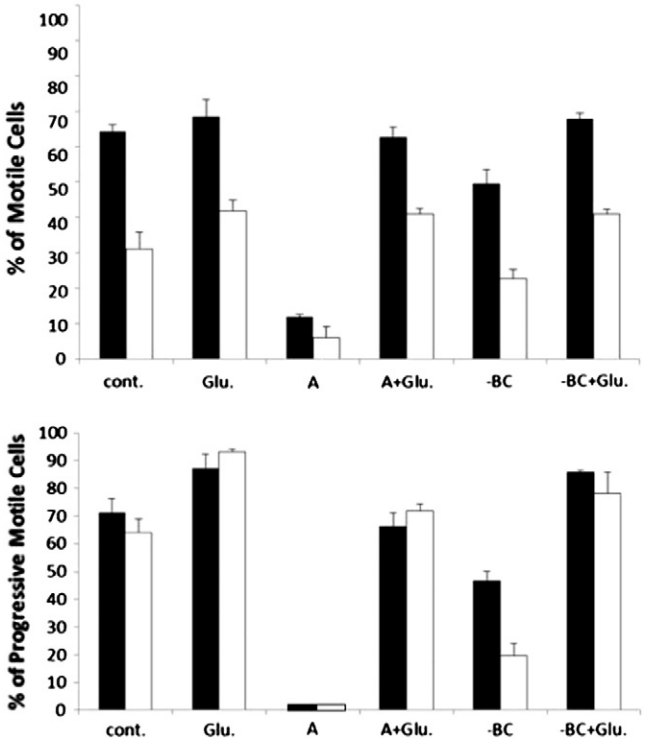

Fig. 4. Effect of bicarbonate on mitochondrial dependent motility. Fresh and cryopreserved sperm were incubated for 5 min in standard (cont.) or HCO^- deficient (−BC) mTALP with and without Antimycin A (A) (1 μM) or glucose (Glu.) (10 mM). Motility analysis was performed using CASA-Ivos. Black columns — fresh semen; open columns — cryopreserved semen. The values represent the mean ± SD of six experiments.

bicarbonate stimulates sAC/PKA, we examined the effect of the PKA inhibitor, H89, on mitochondrial and glycolytic-dependent sperm motility. H89 inhibited motility only in the presence of bicarbonate, whether or not glucose was present in the medium (Fig. 6). In addition, no effect of H89 was observed in the presence of glucose in Antimycin A treated cells, conditions under which motility is completely dependent on glycolytic activity. In bicarbonate containing medium, H89 caused 30% or 22% inhibition in progressive motility in fresh cells, and 41% or 43% inhibition in cryopreserved sperm, in the presence or absence of glucose, respectively (Fig. 6). Thus, the data show no effect of H89 on motility when Antimycin A and glucose are present or in the absence of bicarbonate whereas partial inhibition was observed only when bicarbonate was present in the incubation medium. The inhibitor H89 did not affect motility when glucose was present, in the absence of bicarbonate, or in Antimycin A treated sperm, suggesting that cytosolic PKA does not mediate sperm motility. Indeed we found that bovine (Fig. 7) and mouse sperm [38] contain mitochondrial localized PKA, therefore we tested whether PKA can phosphorylate mitochondrial proteins. Using anti-phospho-PKA-substrate antibodies, we found complete inhibition of the phosphorylation of a 60 kDa protein band by H89

Table 1
Effect of bicarbonate on ATP production in sperm cells.

Treatment	Fresh cells	Cryopreserved cells
	ATP (μmol/10^8 cells)	
Control	36.1	33.7
Glucose	32.6	32.3
Antimycin A	1.5	1.4
Antimycin A + Glucose	27.8	28.0
−Bicarbonate	30.1	2.5
−Bicarbonate + Glucose	32.5	32.1

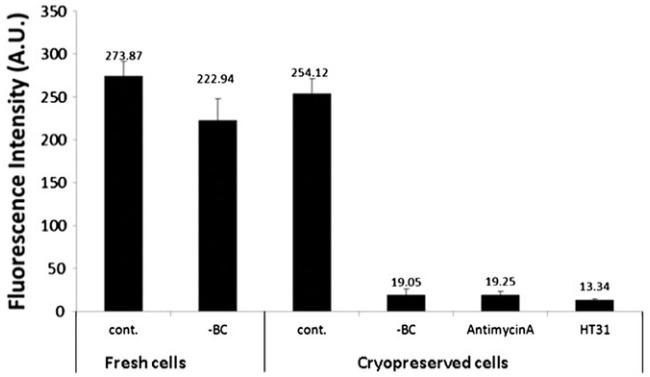

Fig. 5. Sperm mitochondrial membrane potential determined using tetramethylrhodamine-methyl ester (TMRM). Fresh and cryopreserved sperm were incubated for 20 min in standard (cont.) or HCO^--deficient (−BC) mTALP with Antimycin A (1 μM), or HT31 (5 μM). MMP was determined using TMRM (100 μM). Pixel intensity for TMRM fluorescence of mitochondrial regions (midpiece) was analyzed with Image Pro Plus software (Media Cybernetics, USA) and Olympus Cell^P software by the selection of mitochondrial regions (midpiece) from 25 cells from each of four images, for a total of 100 cells in each experiment. The values shown represent the mean ± SD of three experiments.

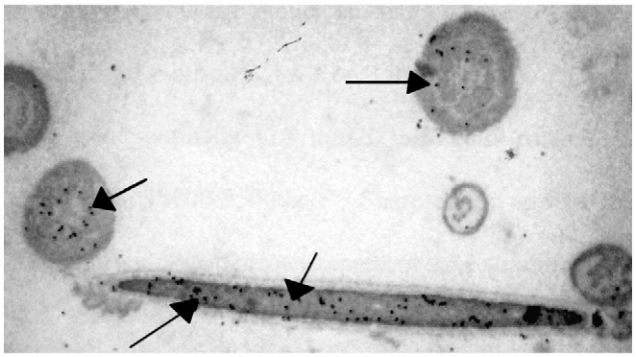

Fig. 7. Immunogold localization of PKA-Cs protein in bovine sperm by electron microscopy. Cross-section is through the acrosome and the midpiece of bovine sperm, and the arrows indicate gold particles localized inside the acrosome (bottom cell) and mitochondria (left and upper right cells). Bar — 0.2 μm.

in the presence (Fig. 8 lane 7, the control is lane 3) or absence (Fig. 8 lane 6, the control is lane 2) of glucose, whereas Antimycin A caused complete inhibition of phosphorylation in the absence of glucose (Fig. 8 lane 4, the control is lane 2), and no effect in the presence of glucose (Fig. 8 lane 5, the control is lane 3). In bicarbonate deficient medium, the phosphorylation of the 60 kDa protein was normal in fresh sperm (Fig. 8A lanes 8, 9; the controls are lanes 2 and 3, respectively); however, no phosphorylation was observed in cryopreserved sperm even in the presence of glucose in the incubation medium (Fig. 8B lanes 8, 9; the controls are lanes 2 and 3, respectively). In Table 1 we see that no ATP is synthesized inside the mitochondria in Antimycin A treated cells; however, following the addition of glucose, ATP is synthesized by the glycolytic pathway. This cytosolic ATP can penetrate into the mitochondria through the ADP/ATP exchanger and activate the mitochondrial sAC/PKA system in fresh but not in cryopreserved sperm. To further substantiate this possibility, we attempted to block the ADP/ATP exchanger of the inner-mitochondrial membrane by the specific inhibitor, atractyloside. As shown in Fig. 9, the glucose-dependent phosphorylation of the 60 kDa PKA-substrate in Antimycin A treated cells was completely blocked by atractyloside, suggesting that glycolytic-ATP penetrates the mitochondria and activates the sAC/PKA system leading to phosphorylation of the 60 kDa protein. However, the

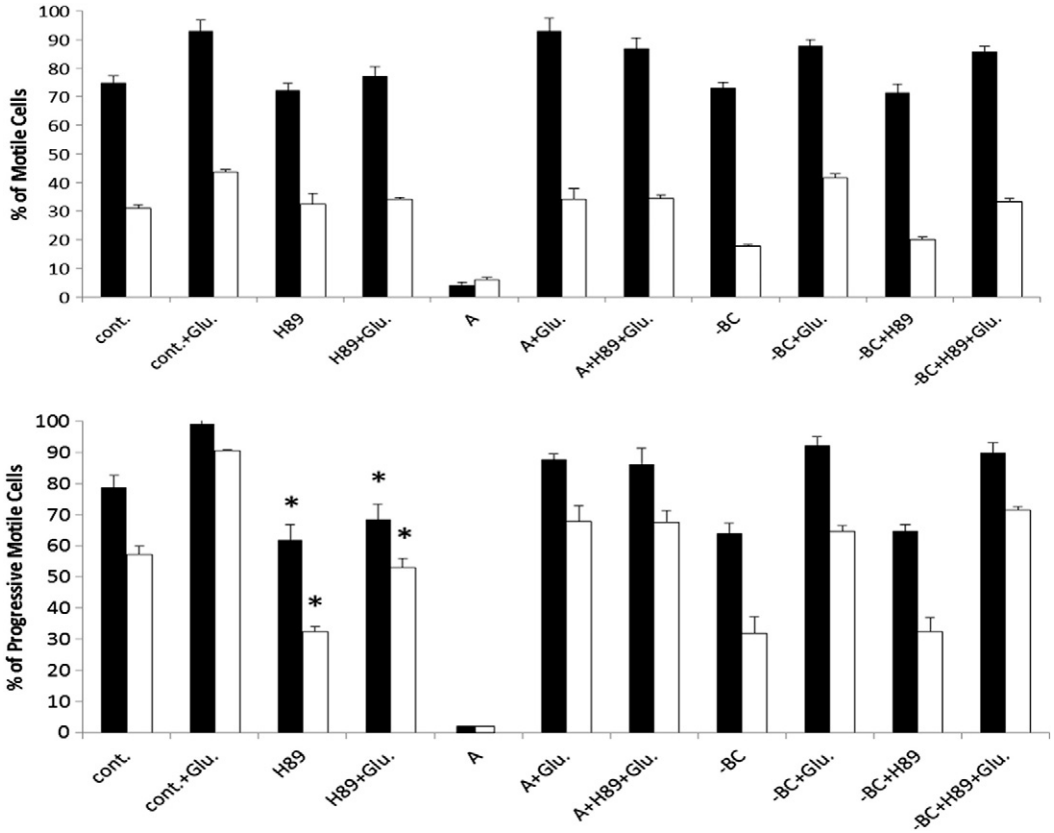

Fig. 6. Effect of PKA inhibition on mitochondrial-dependent motility. Fresh and cryopreserved sperm were incubated for 5 min in standard (cont.) or HCO^--deficient (−BC) mTALP with or without H89 (50 μM), Antimycin A (A) (1 μM), or glucose (Glu.) (10 mM). Data were collected and analyzed using CASA-Ivos. Black columns — fresh semen; open columns — cryopreserved semen. The values represent the mean ± SD of six experiments. Asterisk (*) represents significant difference between treatment and control ($P < 0.01$).

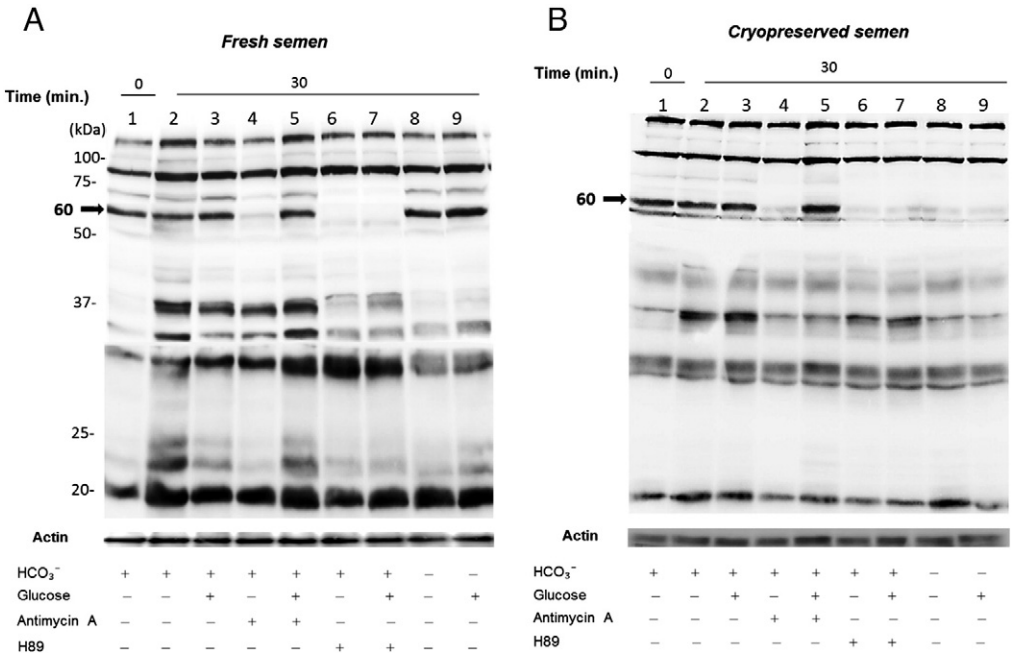

Fig. 8. PKA-mediated protein phosphorylation. Sperm cells were incubated for 30 min in standard or HCO^--deficient mTALP with or without H89 (50 μM), Antimycin A (1 μM), or glucose (10 mM). Samples were removed at the beginning of incubation and after 30 min of capacitation. Proteins were extracted and analyzed by western blot using anti-phospho-(Ser/Thr)PKA–substrate antibody and anti-actin antibody (loading control). The blots shown are from one experiment representative of three.

penetration of glycolytic ATP was not sufficient to induce phosphorylation of the 60 kDa protein in cryopreserved sperm incubated in bicarbonate-deficient medium (Fig. 8). In order to solve this enigma we assumed that fresh sperm contain enough bicarbonate to activate the mitochondrial-sAC; however, this is not the case in cryopreserved sperm, in which the absence of bicarbonate prevents the activation of the mitochondrial sAC despite the ATP supply from glycolytic activity.

To determine the role of the sAC in sperm motility, we used a specific inhibitor of sAC, 2-hydroxyestradiol (2-CE) [21]. In bicarbonate containing medium, 2-CE caused 15% or 33% inhibition of progressive motility in fresh or cryopreserved sperm, respectively (Fig. 10). However, in bicarbonate deficient medium, 2-CE caused 68% inhibition in progressive motility in fresh sperm with no effect on cryopreserved sperm. Under these conditions, the motility of cryopreserved sperm is already very low, possibly because of low sAC activity, so no further significant inhibition by 2-CE was evident.

Finally, we attempted to identify the 60 kDa PKA-substrate detected in the sperm. To this end, the 60 kDa band was excised from Coomassie brilliant-stained gels, and Ser/Thr phosphorylation was analyzed by LC–MS/MS on LTQ-Orbitrap (Thermo); sites were identified by Discoverer software against the bovine Uniprot database, and a decoy database. A total of four phosphoproteins were found, while only two proteins were identified with a high level of confidence (Table 2): Tektin 3 — a filament forming protein, predominantly associated with the surface of the mitochondria and outer dense fibers in the middle piece [39], whose deficiency diminishes progressive motility in mouse sperm [40], and glucose-6-phosphate isomerase (phosphoglucose isomerase (PGI) or phosphohexose isomerase) — the

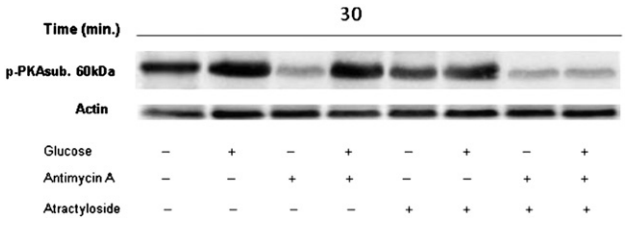

Fig. 9. PKA-dependent phosphorylation of the 60 kDa protein. Sperm cells were incubated for 30 min in mTALP with or without Atractyloside (100 μM), Antimycin A (1 μM), or glucose (10 mM). Proteins were extracted and analyzed by immunoblotting using anti-phospho-(Ser/Thr) PKA substrate. The blots shown are from one experiment representative of three.

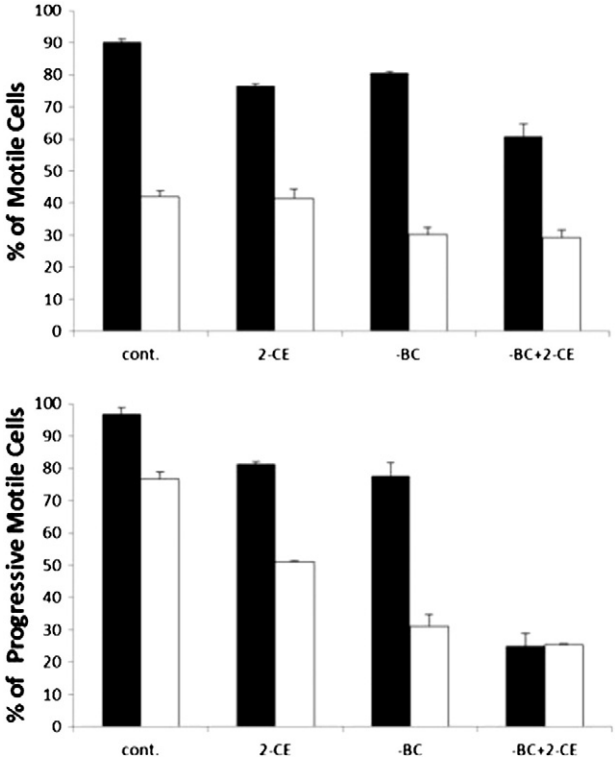

Fig. 10. Effect of sAC inhibition by 2-CE on total and progressive motility. Fresh and cryopreserved sperm were incubated for 30 min in standard mTALP (cont.) or mTALP devoid of HCO^- (−BC), with and without 2-CE (20 μM). Data were analyzed using CASA-Ivos. Black columns — fresh semen; open columns — cryopreserved semen. The values represent the mean ± SD of six experiments.

Table 2
Ser/Thr phosphorylated proteins with molecular weight of 60 kDa recognized by the α-pPKAs antibody and identified by LC–MS/MS.

Accession	Description	Peptides	#PSMs	MW (kDa)
F1MX07	Tektin 3 OS = *Bos taurus* GN = TEKT3 PE = 4 SV = 1-[F1MX07_BOVIN]	40	147	56.6
Q3ZBD7	Glucose-6-phosphate isomerase OS = *Bos taurus* GN = GPI PE = 2 SV = 4-[G6PVIN]	33	211	62.8

second enzyme of glycolysis, whose phosphorylation is shown to regulate its enzymatic activity in cancer cells [41]. Thus we suggest that these two mitochondrial proteins are phosphorylated by mitochondrial PKA.

4. Discussion

We showed here that both total motility and progressive motility of fresh or cryopreserved sperm samples are dependent on HCO^-. In fresh sperm, the bicarbonate is supplied by the Krebs cycle, however, in cryopreserved sperm exogenous supply of bicarbonate is mandatory, since complete inhibition of motility was seen when these cells are incubated in bicarbonate-deficient medium (Fig. 1). Intracellular alkalization by addition of NH_4Cl did not restore the motility of cryopreserved sperm incubated in HCO^--deficient capacitation media (Fig. 2), leading us to conclude that the low motility of cryopreserved sperm in bicarbonate-deficient medium is not due to modulation of intracellular pH, but rather due to some other regulatory mechanism.

In mammalian sperm, bicarbonate stimulates sAC to produce cAMP, which plays a pivotal role in governing the activation of sperm motility [42–45]. However, addition of exogenous 8Br-cAMP only partially restored total or progressive motility loss found in bicarbonate-deficient media (Fig. 3). The presence of mitochondrial sAC as a source of mitochondrial cAMP [37], as well as the inability of cAMP to diffuse far from its source, and to thereby enter the mitochondria [46–48], led us to assume that the inability of 8Br-cAMP to restore full motility might be due to relatively low concentrations of 8Br-cAMP that can enter the mitochondria.

Oxidative phosphorylation (OXPHOS) and glycolysis are the primary biochemical pathways for the supply of ATP needed for sperm motility [9]. It is known that glucose abrogates bovine sperm capacitation [49], therefore under capacitation conditions there is no glucose in the incubation medium and the motility depends on mitochondrial ATP supply.

Blockage of sperm mitochondrial ATP production using Antimycin A in the absence of glucose (Table 1), severely diminished both total and progressive motility (Fig. 4); therefore, sperm motility indirectly reflects the ability of mitochondrial activity to propel the sperm cell, and represents an indirect measure of ATP production.

The ability of glucose to restore the loss of motility or ATP production induced by HCO^- restriction or by inhibiting mitochondrial respiration (Fig. 4 and Table 1) indicates that the cryopreserved cells exhibit insufficient mitochondrial activity for ATP production. These findings indicate that bicarbonate deficiency in the media impacts mitochondrial function, resulting in motility loss. The ability of bicarbonate to restore motility and ATP production suggests its ability to enhance mitochondrial ATP production.

Cryopreservation has been reported to cause damage to sperm mitochondria [50] and the proportion of fully functional sperm that retain intact mitochondrial activity after freeze–thawing is low [51]. However, transmission electron microscopic observation of sperm mitochondria showed no morphological changes in cryopreserved sperm mitochondria compared to fresh sperm (not shown). The diameter of both fresh and cryopreserved semen mitochondria varied from 154 to 174 nm, which falls into a normal size range, with no signs of stress-induced increase of volume.

Despite the lack of mechanical damage to the mitochondria, cryopreservation may result in functional defects. Mitochondrial dysfunction without detectable morphological abnormalities has been detected in neuronal cells affected with Parkinson's disease [52] as well as in oocytes after preservation [53]. Therefore, we examined the mitochondrial function by measuring the mitochondrial membrane potential (MMP, $\Delta\Psi m$) which is necessary for ATP production. We showed here that cryopreserved semen incubated in HCO^--deficient capacitation media have much lower MMP and ATP than cryopreserved sperm incubated with bicarbonate, or fresh semen incubated in both standard medium, and medium devoid of HCO^- (Fig. 5 and Table 1). In fact, the MMP and ATP levels in cryopreserved sperm incubated in bicarbonate-deficient medium was similar to the MMP and ATP levels in Antimycin A treated cells, in which the respiration is blocked and no apparent MMP is created. Dysfunction of the Krebs cycle would result in a lack of electron donors, and consequent retardation of electron transfer and relaxation of MMP. Moreover, this dysfunction would result in a lack of carbon dioxide/bicarbonate generation within the mitochondria. Thus, we suggest that bicarbonate regulates mitochondrial activity in sperm cells, and the motility loss in cryopreserved semen incubated in HCO^--deficient capacitation media is due to possible alternation of Krebs cycle function. Full restoration of MMP, ATP levels and motility by bicarbonate in cryopreserved sperm indicates that the respiratory chain, as well as the H^+-ATPase, was not damaged after freezing and thawing of the sperm. Moreover, inhibition of PKA by Ht31 caused complete reduction of the MMP, similar to the inhibition shown by Antimycin A or by incubation in HCO_3^--deficient media (Fig. 5) indicating that mitochondrial-PKA activity mediates the creation of MMP in sperm mitochondria. These data support the importance of mitochondrial bicarbonate in creating MMP, suggesting that HCO_3^-/sAC/cAMP/PKA signaling is tightly linked to mitochondrial activity. Interestingly, under hypoxic conditions, there is greater involvement of PKA in phosphorylation of COX subunits [54], suggesting a role for PKA under stress conditions. This might be the case in cryopreserved sperm, conditions in which the relatively high activation of mitochondrial sAC/PKA is needed in order to efficiently operate the OXPHOS to synthesize enough ATP for motility.

It was shown that mitochondrial energy metabolism is controlled by the cAMP/PKA pathway in yeast, and basal Ca^{2+}/cAMP/PKA signaling is tightly linked to mitochondrial ATP production in heart cells [55]. Additionally, PKA regulates ATP production by phosphorylation of mitochondrial proteins, including subunits of cytochrome *c* oxidase [37]. This regulation is sensitive to physiologically relevant intra-mitochondrial bicarbonate concentrations, metabolically generated CO_2, and carbonic anhydrase inhibitors [37].

We show here that PKA modulates mitochondrial-dependent sperm motility (Fig. 6). H89 did not alter motility supported by glucose when mitochondrial activity was blocked with Antimycin A, but inhibits motility in the absence of glucose; this indicates that mitochondrial-dependent motility but not glycolytic motility is mediated by PKA. Additionally, the inability of H89 to inhibit motility in Antimycin A treated cells in the presence of glucose indicates that sperm motility is not mediated by cytosolic PKA, but rather by mitochondrial localized PKA as was found (Fig. 7). It was shown in other studies that bovine sperm motility [56] or hyper-activated motility [57] are PKA-independent processes. Also, the ablation of the sperm-specific catalytic subunit of PKA (Cα2) in mice resulted in cauda epididymal sperm that could swim spontaneously *in vitro*, suggesting that the initiation of motility in the epididymis occurs through a non-PKA-dependent pathway [58]. We assume that in these three studies, motility was supported by glycolitic activity which is independent of PKA activity as we suggested above.

Using anti-phospho-PKA-substrate antibodies, we demonstrate complete inhibition by H89 of the phosphorylation of a 60 kDa protein band in the presence or absence of glucose, whereas Antimycin A caused complete inhibition of phosphorylation in the absence of glucose, but had no effect in the presence of glucose (Fig. 8). We also show (Table 1), that there is insufficient ATP in Antimycin A treated cells, and glucose supplementation can overcome this deficiency by supplying glycolytic-synthesized ATP directly to the motility apparatus. Moreover, glycolytic-synthesized ATP can lead to PKA-dependent phosphorylation of the 60 kDa protein (Fig. 8). However, in cryo-preserved sperm incubated in bicarbonate-deficient medium, glucose could restore motility but could not promote phosphorylation of the 60 kDa proteins (Fig. 8).

Thus it remained enigmatic why glucose cannot stimulate the phosphorylation of the 60 kDa protein in the absence of bicarbonate. To address this issue, we assumed that the 60 kDa protein might be a mitochondrial protein whose phosphorylation requires mitochondrial ATP as well as the mitochondrial sAC/PKA system. Thus, glycolytic-synthesized ATP might be transported into the mitochondria *via* the ADP/ATP exchanger of the inner mitochondrial membrane. During normal mitochondrial function, the ADP/ATP exchanger exports the ATP from mitochondria in exchange for external ADP. However, at low electrochemical potential, F_0F_1-ATPase induces ATP hydrolysis in an attempt to maintain the mitochondrial membrane potential, and the ADP/ATP exchanger functions in a "reverse mode", transporting ATP to the matrix [59]. The ability of glucose to reverse the Antimycin A inhibition of the 60 kDa phosphorylation can be explained by such "reverse mode" function of the ADP/ATP exchanger and transportation of glycolytic-synthesized ATP into the mitochondrial matrix, further activating mitochondrial PKA to phosphorylate the 60 kDa substrate. However, glucose cannot affect phosphorylation in the absence of bicarbonate, since the sAC/PKA system is not activated. The use of Atractyloside, an inhibitor of the ADP/ATP exchanger, completely blocked the effect of glucose on 60 kDa phosphorylation (Fig. 9), suggesting that glycolytic-synthesized ATP penetrates into the mitochondria and activates the mitochondrial sAC/PKA system leading to phosphorylation of the 60 kDa protein. The fact that glucose cannot restore phosphorylation of the 60 kDa protein in cryo-preserved sperm (Fig. 8) indicates that although glycolytic-ATP can penetrate into the mitochondrial matrix, the lack of bicarbonate in cryo-preserved sperm is the limiting factor for sAC/PKA activation. Since inhibition of PKA does not affect glycolytic-dependent motility, whereas the 60 kDa phosphorylation is completely inhibited, we conclude that mitochondrial PKA mediates OXPHOS activity, ATP synthesis, and mitochondrial-dependent motility, whereas glycolytic-dependent motility is independent of cytosolic PKA activity in bovine sperm. Thus, bull sperm motility is regulated by mitochondrial sAC in response to Krebs cycle generated CO_2, which is further converted to HCO_3^- by carbonic-anhydrase.

Two phosphor-proteins were identified in the 60 kDa band: Tektin and glucose-6-phosphate isomerase (Table 2). Mammalian sperm flagella express filament-forming Tektin proteins (Tektin 1–5) reported to be involved in the stability and structural complexity of flagella. Several functional studies demonstrated that tektins are critical for sperm motility [60–62]. Immunofluorescence microscopy and pre-embedding immunoelectron microscopy revealed that Tektin 3 is predominantly associated with the surface of mitochondria and outer dense fibers in the middle piece [39]. Significantly, male mice deficient in Tektin 3 produce sperm with diminished progressive motility, although sperm counts and other aspects of reproductive physiology remain unperturbed [40].

Glucose-6-phosphate isomerase (PGI) is a ubiquitous cytosolic enzyme that plays a critical role in both Embden–Meyerhof glycolytic and glucogenetic pathways, catalyzing the reversible interconversion between glucose-6-phosphate and fructose-6-phosphate [63]. And indeed it was shown elsewhere that mitochondria are involved in the regulation of glycolysis. Hexokinase 1 (the first enzyme of glycolysis) was found to bind to porin (also known as voltage-dependent anion channels; VDACs) on the outer mitochondrial membrane in sperm cells [64–66], presumably giving Hexokinase 1 preferential access to ATP produced by oxidative phosphorylation [67,68]. It was shown elsewhere that phosphorylation of PGI regulates hexokinase activity in cancer cells [41], however it is not clear how PGI phosphorylation affects enzymatic activity in sperm cells.

In conclusion, our results demonstrate that sperm mitochondrial activity but not glycolytic activity depends on bicarbonate, which activates the mitochondrial sAC/PKA system leading to OXPHOS activation and ATP production. Therefore, mitochondrial but not glycolytic-dependent motility depends on PKA activity.

It is well known that sperm motility can be driven by ATP produced in the mitochondria or by the glycolytic pathway. The bicarbonate-dependent motility described here, is in fact mitochondrial driven motility. The data in Table 1 reveal that the cellular levels of ATP produced by the mitochondria or by glycolysis are similar, thus each system can provide enough ATP to support full scale motility. We assume that the two alternative ATP-produced systems are important to insure motile sperm and fertilization under possible physiological conditions by which one of the systems is not fully active. Low mitochondrial activity can be seen if there is not enough oxygen or bicarbonate and low glycolytic activity when there is not enough glucose in the female reproductive tract.

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