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AMP-activated kinase, AMPK, is involved in the maintenance of plasma membrane organization in boar spermatozoa

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ABSTRACT

Spermatozoa undergo energy- and metabolism-dependent processes to successfully fertilize the oocyte. AMP-activated protein kinase, AMPK, is a sensor of cell energy. We recently showed that AMPK controls spermatozoa motility. Our aims are i) to investigate the intracellular localization of AMPK in boar spermatozoa by immunofluorescence, ii) to study whether AMPK plays a role in other relevant processes of spermatozoa: mitochondrial membrane potential ($\Delta\Psi m$), plasma membrane lipid disorganization, outward phosphatidylserine (PS) exposure, acrosome integrity and induced-acrosome reaction by flow cytometry and iii) to investigate intracellular AMPK pathways by western blot. Spermatozoa were incubated under different conditions in the presence or absence of compound C (CC, 30 μM), an AMPK inhibitor and/or cAMP analog 8Br-cAMP. AMPKα protein is expressed at the entire acrosome and at the midpiece of spermatozoa flagellum, whereas phospho-Thr^{172}-AMPK is specifically localized at the apical part of acrosome and at flagellum midpiece. CC treatment rapidly confers head-to-head aggregation-promoting property to spermatozoa. Long term AMPK inhibition in spermatozoa incubated in TCM significantly reduces high $\Delta\Psi m$. Moreover, AMPK inhibition significantly induces plasma membrane lipid disorganization and simultaneously reduces outward PS translocation at plasma membrane in a time-dependent manner. Acrosomal integrity in TCM is significantly enhanced when AMPK is inhibited. However, neither acrosome reaction nor membrane lipid disorganization induced by ionophore A23187 are affected by CC. AMPK phosphorylation is potently stimulated upon PKA activation in spermatozoa. This work suggests that AMPK, lying downstream of PKA, regulates at different levels mammalian spermatozoa membrane function.

1. Introduction

Mammalian ejaculated spermatozoa are not able to fertilize the oocyte and therefore they need to previously undergo relevant cellular processes including motility, capacitation, hyperactivation and acrosome reaction. These processes occur in the female genital tract and allow the spermatozoa to acquire the ability to reach the oocyte, penetrate the cumulus oophorus and to bind to the zona pellucida of the oocyte, which in turn triggers the acrosome reaction and subsequently leads to egg fertilization [1]. Intriguingly, the acquisition of these spermatozoa physiological competences is independent of gene transcription or translation [2,3]. Therefore, the accepted idea is that the acquisition of spermatozoa functionality during capacitation is mainly dependent on post-translational modifications of pre-existing proteins, such as phosphorylation in either tyrosine [4–6] or serine/threonine [7].

In order to successfully achieve the above-mentioned fertilization competent status, spermatozoa needs to undergo several physiological and biochemical modifications including cholesterol loss from the plasma membrane, increased plasma membrane fluidity due to phospholipid scrambling, changes in intracellular ion concentration, hyperpolarization of plasma membrane and increased tyrosine phosphorylation among others [1]. These spermatozoa processes are dependent on the energetic state, determined by the ratio between cellular AMP and ATP [8,9].

The AMP-activated protein kinase AMPK is an evolutionary conserved serine/threonine kinase that acts as a sensor that detects the cell energy state and subsequently regulates metabolism [10]. AMPK is a heterotrimeric protein that has a catalytic α and two regulatory subunits, β and γ. AMPK is extremely sensitive to its allosteric effector AMP, as any increase in the ratio AMP/ATP due to a decrease in cellular energy state, activates AMPK [10,11]. In addition to allosteric activation by AMP, phosphorylation of the Thr^{172} residue, located at the critical activation

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loop of the α subunit, is a requirement for full AMPK activation [12]. When AMP binds to AMPK it causes an inhibition of Thr^{172} dephosphorylation. Activation of AMPK leads to both the stimulation of catabolic pathways that produce ATP and to the inhibition of ATP-consuming anabolic pathways [13], thus the overall metabolic consequence of AMPK activation is the maintenance of cellular energy state under ATP-limiting conditions. However, as AMPK is a ser/thr kinase it might regulate processes outside metabolism [14]. The enzymatic activity of AMPK is also switched on by different types of cellular and metabolic stresses [11,15]. Some of these stimuli, as hyperosmotic stress or an increase in calcium concentration [10,11] do not modify the ratio AMP/ATP, suggesting that other mechanisms, which are likely cell type specific, are involved in AMPK activation.

All AMPK studies had been performed exclusively in somatic cells, until recently when we have demonstrated that AMPK is expressed in mammalian spermatozoa and regulates one of their most important functions: motility [16]. Previously, some studies including knockout mice suggested a crucial role of AMPK-related kinases in spermatozoa function [17,18]. Thus, a new shorter isoform of the AMPK upstream kinase LKB1, called LKB1s, which is expressed predominantly in haploid sperm cells from mammal testes [17], is pointed to play an essential role in spermiogenesis and fertility (motility) in mice. Additionally, the branch of AMPK in the human quinome tree includes the "serine/threonine kinase testis specific" TSSK family. Some members such as TSSK2, TSKS and SSTK have been identified in human spermatozoa [19] and the deletion of TSSK1 and 2 causes male infertility in chimera mice due to haploinsufficiency [18].

The study of AMPK as key kinase in those energy status dependent mechanisms by which spermatozoa regulate their function, including motility [16], is crucial for the understanding of the ability of these germ cells to survive and adapt to external conditions such as the transit through the female reproductive tract. Therefore, the aim of this work is to study the subcellular expression of AMPK in boar spermatozoa, its intracellular pathway and to investigate its possible role(s) in other spermatozoa essential processes different from motility such as the degree of both lipid disorganization and phosphatidylserine exposure at the plasma membrane, mitochondrial membrane potential, integrity of the acrosome membrane and the acrosome reaction, all necessary for successful spermatozoa main function: fertilization.

2. Materials and methods

2.1. Chemicals and sources

Live/dead spermatozoa viability kit including both propidium iodide (PI) and SYBR-14 probes, M540 and YoPro-1 probes were purchased from Molecular Probes (Leiden, The Netherlands); calcium ionophore A23187, 8BrcAMP, compound C (6-[4-(2-Piperidin-1-ylethoxy) phenyl]-3-pyridin-4-ylpyrazolo[1,5-a]pyrimidine) and PNA-FITC were from Sigma-Aldrich® (St Louis, MI, USA); Annexin-V-FITC from Immunostep (Salamanca, Spain); anti-AMPKα and anti-GSK3β antibodies were from Cell Signaling (Beverly, CA); anti-P-Thr^{172}-AMPK antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA); coulter isoton II diluent from Beckman Coulter Inc. (Brea, CA, USA); JC-1 probe, Alexa Fluor 647 goat anti-rabbit IgG and ProLong gold antifade reagent with 4.6-diamidino-2-phenylindole hydrochloride (DAPI) from Life Technologies Ltd. (Grand Island, NY, USA); microscope slides coated with L-lysine from Electron Microscopy Sciences (Hatfield, PA, USA); complete, EDTA-free, protease inhibitor cocktail was purchased from Roche Diagnostics (Penzberg, Germany). Tris/Glycine/SDS buffer (10×) and Tris/Glycine buffer (10×) from Bio-Rad (Richmond, CA). Hyperfilm ECL was from Amersham (Arlington Heights, IL). Enhanced chemiluminescence detection reagents, anti-mouse IgG-horseradish peroxidase conjugated and anti-rabbit IgG-horseradish peroxidase conjugated were from Pierce (Rockford, IL). Nitrocellulose membranes were from Whatman Protran (Dassel, Germany).

2.2. Spermatozoa incubation media

Tyrode's basal medium (TBM) was prepared as follows: 96 mmol/l NaCl, 4.7 mmol/l KCl, 0.4 mmol/l $MgSO_4$, 0.3 mmol/l NaH_2PO_4, 5.5 mmol/l glucose, 1 mmol/l sodium pyruvate, 21.6 mmol/l sodium lactate, 20 mmol/l HEPES (pH 7.45), 5 mmol/l EGTA and 3 mg/ml BSA. A free-EGTA variant of TBM medium, which includes direct activators of spermatozoa soluble adenylyl cyclase, sAC, was made by adding 1 mmol/l $CaCl_2$ and 15 mmol/l $NaHCO_3$ and equilibrated with 95% O_2 and 5% CO_2 and termed Tyrode's complete medium (TCM). All Tyrode's mediums were made on the day of use and maintained at pH 7.45 with an osmolarity of 290–310 mOsm kg^{-1}.

2.3. Collection of semen and preparation of spermatozoa samples under different treatments

Sperm samples from Duroc boars (2–4 years old) were commercially obtained from a Regional Porcine Company (Tecnogenext, S.L., Mérida, Spain), without any requirement of approval from the animal research review board of the University of Extremadura. All boars were housed in individual pens in an environmentally controlled building (15–25 °C) according to Regional Government and European regulations, and received the same diet. Artificial insemination using preserved liquid semen from boars demonstrated their fertility. Fresh ejaculates were collected with the gloved hand technique and stored at 17 °C before use and, in order to minimize individual boar variations, samples from up to 3 animals were pooled using semen from no less than 12 boars in different combinations. Only semen pools with at least 80% morphologically normal spermatozoa were used. Semen was centrifuged at 2000 *g* for 4 min, washed with PBS and placed in TBM or TCM medium. Samples of 1.5 ml containing 120×10^6 spermatozoa/ml were incubated at 38.5 °C in a CO_2 incubator for different times for western blotting analysis and lower volume (0.5 ml) was used in samples prepared for evaluation by flow cytometry. When required, a pre-incubation of spermatozoa with compound C was performed for 1 h at RT. In order to minimize possible experimental variations, every condition/treatment studied was performed in the same semen pool. When necessary, a control with the final concentration of the solvent (DMSO 0.1%) was included.

2.4. Immunolocalization of AMPKα in boar spermatozoa by immunofluorescence

After spermatozoa treatments, aliquots of 40 μl of 4% paraformaldehyde-fixed sperm samples were spread onto poly-L-lysine coated microscope slides and were then left to air-dry. Following three washings (5 min in PBS), spermatozoa were permeabilized by incubation for 10 min at room temperature (RT) in a standard phosphate-buffered solution (PBS; pH 7.4) containing 0.25% (vol/vol) Triton X-100. Then, samples were washed three times with PBS and blocked through incubation with PBS including 0.1% (vol/vol) Tween-20 and 1% (wt/vol) BSA for 30 min at RT. Incubation with primary antibodies, AMPKα (1:100) or phospho-Thr172 AMPKα (1:50), diluted in blocking buffer was carried out overnight at 4 °C. Following the binding of specific antibody, samples were washed thoroughly with PBS and incubated with Alexa Fluor 647 goat anti-rabbit IgG (1:200). As negative controls, samples incubated with secondary antibody and without primary antibody were run in parallel. Slides were gently washed with PBS and then incubated with 5 μl of a commercial solution of 4.6-diamidino-2-phenylindole hydrochloride (DAPI) 125 ng/ml as both a nuclear stain and an antifading mounting solution. Any excess of liquid was eliminated and cover slips were finally sealed with colorless nail polish and stored at 4 °C in the dark until microscope observation [20]. Fluorescent images were obtained with a Leica TCS 4D confocal scanning microscope (Leica Lasertechnik; Vertrieb, Germany) adapted to an inverted Leitz DMIRBE

microscope and a 63× (NA 1.4 oil) Leitz Plan-Apo lens (Leitz; Stuttgart, Germany). The light source was an argon/krypton laser.

2.5. Flow cytometry analysis

Flow cytometry analysis was performed using a Coulter EPICS XL-MCL flow cytometer (Beckman Coulter Ltd.) The fluorophores were excited by a 200 mV argon ion laser operating at 488 nm. A total of 10,000 gated events (based on the forward scatter and side scatter of the sperm population recorded in the linear mode) were collected per sample with sample running rates of approximately 500 events/s. Fluorescence data were collected in the logarithmic mode. The fluorescence values of probes PNA-FITC, Annexin-V-FITC, YoPro-1, SYBR-14 and JC-1 were collected in the FL1 sensor using a 525 nm band pass filter. Propidium iodide (PI) fluorescence was collected in the FL3 sensor using a 620 nm BP filter, and M540 and JC-1 fluorescence was collected in the FL2 sensor using a 575 nm BP filter. Flow cytometry data were analyzed using a FacStation computer and EXPO™ 32 ADC software (Beckman Coulter, Inc.).

2.6. Evaluation of the acrosome integrity and acrosome-reacted spermatozoa by flow cytometry

The population of acrosome-reacted or -damaged spermatozoa was assessed after staining these germ cells with phycoerythrin PNA-FITC as a specific marker for acrosomal status and PI as a marker for cell death [21,22]. Aliquots of 100 μl of each semen sample (35×10^6 cells/ml) were incubated at RT in the darkness for 5 min with 1 μg/ml of PNA-FITC and 6 μmol/l of PI. Then, 400 μl of isotonic buffered diluent were added to each sample and mixed before flow cytometry analysis. Results are expressed as the average percentage of PNA-positive and PI-negative spermatozoa ± SEM.

2.7. Analysis of spermatozoa mitochondrial membrane potential (ΔΨm) by flow cytometry

Mitochondrial membrane potential variations, ΔΨm, were evaluated using the specific probe JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′ tetraethylbenzymidazolyl carbocyanine iodide) [21]. This lipophilic cationic fluorochrome JC-1 is present as protomeric aggregates in mitochondria with high membrane potential that emit in orange (590 nm), whereas in mitochondria with low membrane potential, JC-1 is present as monomers that emit in green (525 nm) when excited at 488 nm. Briefly, an aliquot of 100 μl from each spermatozoa sample (35×10^6 cells/ml) was diluted in 400 μl of isotonic buffer containing 0.15 mmol/l of JC-1 and then mixed and incubated at 38.5 °C for 30 min. The samples were mixed again before flow cytometry analysis. The percentage of orange stained cells was recorded and considered the population of spermatozoa with a high mitochondrial membrane potential. Results are expressed as the average of the percentage of orange stained spermatozoa ± SEM.

2.8. Evaluation of the degree of plasma membrane lipid organization of spermatozoa

As described previously [21], fluorescent staining using the membrane probes merocyanine M540, as a lipid fluidity marker, and YoPro-1, as a marker of changes in plasma membrane permeability commonly associated to cell death, was performed to assess changes either in the lipid architecture of spermatozoa plasma membrane. Briefly, aliquots of 100 μl of each semen sample (35×10^6 cells/ml) were diluted in 400 μl of isotonic buffer containing 75 nmol/l of YoPro-1, mixed and incubated at 38.5 °C for 15 min. Then, M540 was added to each sample to a final concentration of 2 μmol/l, incubated for 2 min and remixed before flow cytometry analysis. The spermatozoa were categorized by labeling as follows: (1) viable cells with low plasma membrane scrambling (YoPro-1^-/M540); (2) viable cells with high plasma membrane scrambling (YoPro-1^-/M540^+); or (3) non-viable cells (YoPro-1^+). Results referred to membrane scrambling are expressed as the percentage of viable cells with high plasma membrane fluidity as the average percentage ± SEM.

2.9. Evaluation of the phosphatidylserine externalization at the plasma membrane of spermatozoa

The study of phosphatidylserine (PS) externalization in plasma membrane spermatozoa was performed using Annexin-V-FITC to specifically detect PS translocation from the inner to the outer leaflet of the sperm plasma membrane. Briefly, 60×10^6 sperm cells were pretreated with or without 30 μM CC either in TBM or TCM, and after incubation at 38.5 °C for different times (4–24 h), spermatozoa were diluted at a final concentration of 12×10^6 cells/ml in the following buffer: 96 mmol/l NaCl, 4.7 mmol/l KCl, 0.4 mmol/l $MgSO_4$, 0.3 mmol/l NaH_2PO_4, 5.5 mmol/l glucose, 1 mmol/l sodium pyruvate, 21.6 mmol/l sodium lactate, 20 mmol/l HEPES (pH 7.45), and 2.5 mmol/l $CaCl_2$. Then, a 100 μl aliquot (2.4×10^6 sperm cells) was transferred to 5 ml tubes and stained with 5 μl of Annexin V-FITC and 4 μl propidium iodide (PI) by incubation for 15 min in the dark at room temperature. Finally, 400 μl of above mentioned buffer were added to each sample and mixed before flow cytometry analysis. For statistical analysis, the cell population exhibiting Annexin V-$FITC^+$ and PI^- was expressed as the percentage of viable cells presenting PS externalization. Each experiment was repeated at least six times.

2.10. Western blotting

Spermatozoa under different treatments were centrifuged 20 s at 7000 *g*, washed with phosphate buffered saline (PBS) supplemented with 0.2 mM Na_3VO_4 and then lysated in a lysis buffer consisting in 50 mmol/l Tris/HCl, pH 7.5, 150 mmol/l NaCl, 1% Triton X-100, 1% deoxycholate, 1 mmol/l EGTA, 0.4 mmol/l EDTA, protease inhibitors cocktail (Complete, EDTA-free), 0.2 mmol/l Na_3VO_4, and 1 mmol/l PMSF by sonication for 5 s at 4 °C. After 20 min at 4 °C samples were centrifuged at 10,000 *g* (15 min, 4 °C) and the supernatant (lysate) was used for analysis of protein concentration. Proteins from porcine spermatozoa lysates were resolved by 10% SDS-PAGE and electrotransferred to nitrocellulose membranes. Western blotting was performed as previously described [16] using anti AMPKα (1:1000), anti phospho-Thr^{172}-AMPKα (1:500), anti GSK3β (1:2000) polyclonal antibodies as primary antibodies.

2.11. Statistical analysis

The mean and standard error of the mean were calculated for descriptive statistics. The effect of treatment on the spermatozoa variables was assessed with an analysis of variance (ANOVA) followed by the Scheffe test for comparisons between treatments. All analyses were performed using SPSS v11.0 for MacOs X software (SPSS Inc. Chicago, IL). The level of significance was set at $p < 0.05$.

3. Results

3.1. AMP-activated kinase, AMPK, is localized at the acrosome and in the midpiece of flagellum in boar fresh spermatozoa

The subcellular expression of AMPK protein in boar spermatozoa was investigated by indirect immunofluorescence using antibody against the catalytic α subunit of AMPK as primary antibody. Results show that AMPK protein is highly expressed in boar fresh spermatozoa, at physiological temperature (38.5 °C), and is mainly localized at the entire acrosome of the spermatozoa head and at the midpiece of the flagellum with less intensity, as seen in Fig. 1.

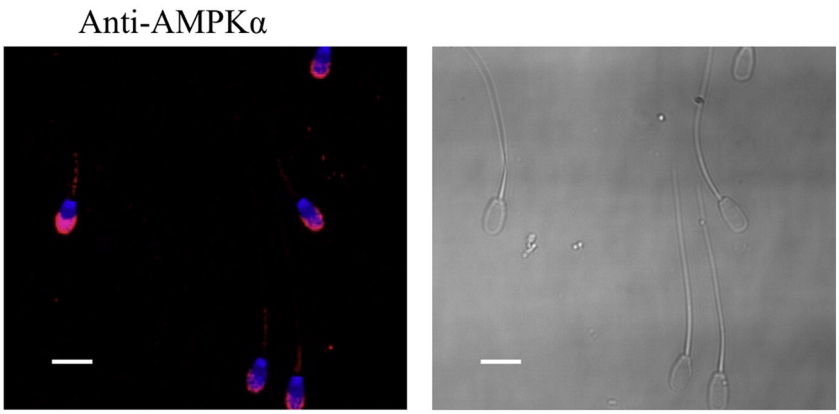

Fig. 1. Immunolocalization of AMP-activated kinase protein, AMPK, in boar spermatozoa. Fresh boar spermatozoa maintained at physiological temperature 38.5 °C were pooled and fixed in 4% paraformaldehyde and immunostaining was performed using antibody against the catalytic subunit of AMPKα. The immunofluorescence was visualized in a confocal microscope and a representative image is shown at the left panel, whereas Normaski optics is shown at the right panel. Immunolocalization of AMPKα is visualized in red while spermatozoa nucleus is visualized in blue after DAPI staining (left side). Scale bar is 10 μm.

3.2. AMPK is phosphorylated (active) under physiological conditions in boar spermatozoa and is localized at the apical region of acrosome and midpiece of flagellum

The level of phosphorylation in Thr^{172} of AMPK was analyzed at physiological temperature of boar spermatozoa (38.5 °C) as an assessment of its enzymatic activity. As shown in Fig. 2, a clear pattern of immunofluorescence is detected using antibody against phospho-Thr^{172}-AMPKα in spermatozoa incubated at 38.5 °C either in TBM (Fig. 2A) or in a medium with physiological stimulus of spermatozoa such as calcium and bicarbonate, TCM (Fig. 2B). Immunofluorescence results show that AMPK phosphorylated at Thr^{172} (active) is localized at specific subcellular locations of the spermatozoa with relative higher intensity in the most apical region of the acrosome and in the equatorial subsegment of spermatozoa head. Phosphorylated AMPK is also found in the midpiece of the flagellum.

3.3. Effect of the AMPK inhibitor, compound C, in the mitochondrial membrane potential of boar spermatozoa

A widely used inhibitor of the AMPK activity in somatic cells is the compound C (CC), a cell-permeable pyrrazolopyrimidine compound that acts as a potent, reversible, and ATP-competitive inhibitor of AMPK (K_i = 109 nM in the presence of 5 μM ATP and the absence of AMP). Thus, we initially confirmed that CC (30 μM) effectively blocks the Thr^{172} phosphorylation (activation) of AMPK in boar spermatozoa at physiological temperature [16].

The effect of AMPK inhibition by CC in the spermatozoa mitochondrial membrane potential, ΔΨm, was evaluated after incubation of these male germ cells in TBM or TCM in the presence or absence of 30 μM CC for different times at boar physiological temperature 38.5 °C (Fig. 3). In addition, we have analyzed this parameter ΔΨm after semen preservation temperature 17 °C in parallel samples. Our data show that mitochondrial membrane potential is sensitive to temperature at which boar spermatozoa are maintained. Thus, the percentage of spermatozoa presenting high ΔΨm measured at semen preservation temperature (17 °C) is always higher than that when maintained at physiological temperature (38.5 °C) for 4 or 24 h (Fig. 3). Our results show that short time exposure (4 h) to AMPK inhibitor compound C does not significantly affect the population of spermatozoa presenting high ΔΨm in any medium (Fig. 3). However, longer time of CC treatment (24 h) leads to a slight but significant decrease in the percentage of spermatozoa with high ΔΨm (from 55% in absence to 43% in presence of CC) when these germ cells are incubated in TCM, whereas no effect of AMPK inhibitor is observed in TBM (Fig. 3).

3.4. Effect of the AMPK inhibition in the degree of lipid organization of spermatozoa plasma membrane

A well-known parameter that contributes to the function of spermatozoa is the degree of lipid organization of their plasma membrane. Therefore, we next evaluated the effect of AMPK inhibition in plasma membrane lipid disorganization after incubation of spermatozoa in TBM or TCM in the presence or absence of 30 μM CC at physiological temperature for different times or at semen preservation temperature 17 °C. Our data show that the degree of lipid organization of spermatozoa plasma membrane, evaluated by flow cytometry using merocyanine M540, varies with both temperature and medium of incubation (Fig. 4). Results from $M540^+$ viable spermatozoa confirm that TCM, that includes Ca^{2+} and bicarbonate, markedly increases the percentage of spermatozoa with plasma membrane lipid disorganization at 4 h (3-fold compared with TBM, Fig. 4). Short-term exposure (4 h) to CC at physiological temperature does not affect the percentage of $M540^+$ live spermatozoa incubated in TBM in a significant manner, although values indicative of plasma membrane lipid disorganization are higher after CC treatment (Fig. 4). However, short term of AMPK inhibition in spermatozoa incubated in TCM induces a marked increase in the percentage of $M540^+$ live spermatozoa (1.8 fold) (Fig. 4). Longer incubation (24 h) of germ cells with AMPK inhibitor at 38.5 °C induces a marked and significant increase in the population of live spermatozoa with plasma membrane lipid disorganization either in TBM (2.3 fold) or in TCM (1.7 fold), as seen in Fig. 4.

It is interesting to mention that treatment of spermatozoa with CC confers aggregation-promoting properties to these male germ cells, as seen in microscopy images in Fig. 5. This head-to-head sperm agglutinating action of CC is rapid as it can be observed after 2 h of spermatozoa incubation and is clearly more visible in the presence of Ca^{2+} and bicarbonate (TCM, lower images in Fig. 5) than in their absence (TBM, upper images in Fig. 5).

3.5. Effect of the AMPK inhibition in phospatidylserine externalization in spermatozoa plasma membrane

We further studied the involvement of AMPK in the plasma membrane scrambling of boar spermatozoa by analyzing the effect of AMPK inhibition by CC in the phosphatidylserine externalization, process that indicates plasma membrane scrambling, which occurs in relevant spermatozoa functions. As seen in Fig. 6, the level of phosphatidylserine externalization in spermatozoa plasma membrane is very low at semen preservation temperature (17 °C), independently of the incubation media TBM or TCM. However, the incubation of germ cells at 38.5 °C, independently of the time or the medium, causes externalization of

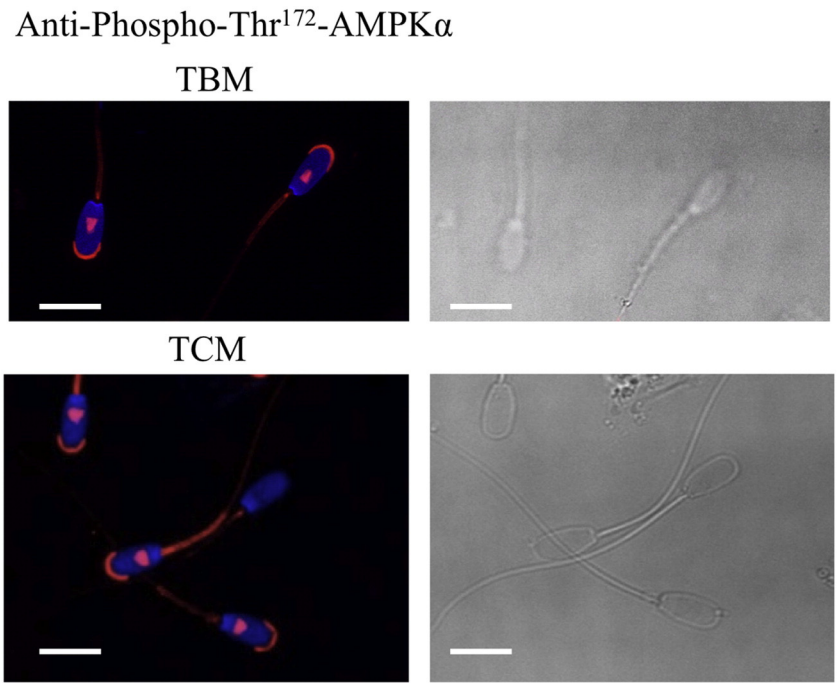

Fig. 2. Immunolocalization of the Thr^{172} phosphorylated (active) form of AMP-activated kinase, AMPK, in boar spermatozoa. After 2 h incubation in TBM (upper image) or TCM (lower image) at 38.5 °C, boar spermatozoa were fixed in 4% paraformaldehyde and immunostaining was performed using anti phospho-Thr^{172}-AMPKα antibody. The immunofluorescence was visualized in a confocal microscope and representative images are shown at the left, whereas Normaski optics images are shown at the right hand. Active phospho-Thr^{172}-AMPKα immunolocalization is visualized in red while spermatozoa nucleus is visualized in blue after DAPI staining. Scale bar is 10 μm.

phosphatidylserine at the plasma membrane, indicating that a spermatozoa scramblase(s) activity is active at the plasma membrane under these conditions (Fig. 6). The treatment of germ cells with CC for 4 h causes a significant decrease (more than 50%) in the percentage of spermatozoa with phosphatidylserine externalization either in TBM or TCM. Although the degree of phosphatidylserine externalization at 24 h remains higher than at 17 °C, however this inhibitory CC effect in membrane scrambling is not observed.

3.6. Effects of the AMPK inhibition in both the acrosomal integrity and the induced acrosome reaction

We analyzed the effect of AMPK inhibition in the integrity of acrosome membrane of boar spermatozoa in TBM or TCM at 38.5 °C without any external stimulus — non-stimulated, which can also be considered as a marker for acrosomal integrity, as spermatozoa labeling with PNA might also reflect acrosome damaged. Acrosomal integrity is generally maintained in boar spermatozoa under different experimental conditions (90% of live spermatozoa are PNA$^-$) unless these germ cells are incubated for long time at 38.5 °C in TCM (Fig. 7), as described previously. Treatment with compound C in TBM does not affect the integrity of sperm acrosome at either short term (4 h) or long term (24 h, Fig. 7). However, CC treatment in TCM, which includes bicarbonate and Ca^{2+} that is necessary for an exocytosis-based process, induces a significant increase in the percentage of live spermatozoa PNA$^+$/PI$^-$, which indicates higher population of spermatozoa with reacted or damaged acrosomes at 4 h and 24 h (Fig. 7).

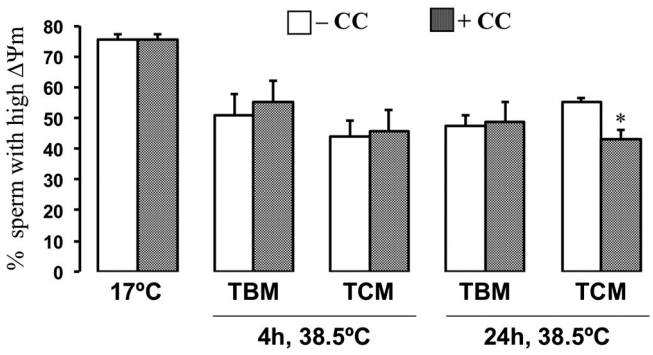

Fig. 3. Effect of the AMPK inhibition in the mitochondrial membrane potential of boar spermatozoa. Spermatozoa were incubated in TBM or TCM at 17 °C or in a CO_2 incubator at 38.5 °C in the presence or absence of the AMPK inhibitor CC (30 μM, filled histograms) for indicated times. Mitochondrial membrane potential was measured by flow cytometry as described in the Materials and methods section using JC-1 as a probe. Spermatozoa population exhibiting high ΔΨm is expressed as percentage of total spermatozoa. Each experiment was performed at least 4 times and results express the mean ± standard error of the mean. Statistical differences are showed with one asterisk when $p < 0.05$.

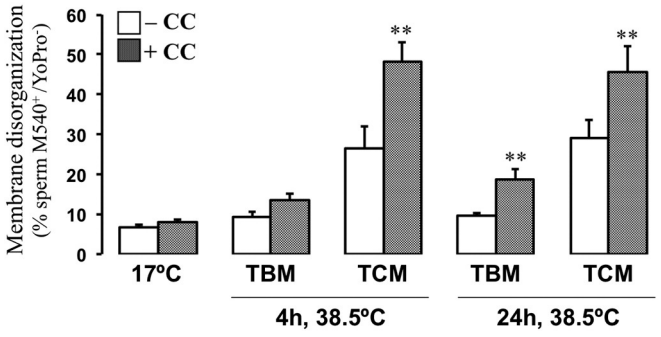

Fig. 4. Effect of the AMPK inhibition in the degree of lipid organization of plasma membrane in boar spermatozoa. Spermatozoa were incubated in TBM or TCM at 17 °C or in a CO_2 incubator at 38.5 °C in the presence or absence of the AMPK inhibitor CC (30 μM, filled histograms) for indicated times. The level of lipid disorganization of spermatozoa plasma membrane was measured by flow cytometry as described using merocyanine M540 as a probe. Spermatozoa population exhibiting plasma membrane lipid disorganization (high M540$^+$) is expressed as percentage of the total live spermatozoa (YoPro-1$^-$). Each experiment was performed at least 4 times and the results express the mean ± standard error of the mean. Statistical differences are showed with two asterisks when $p < 0.01$.

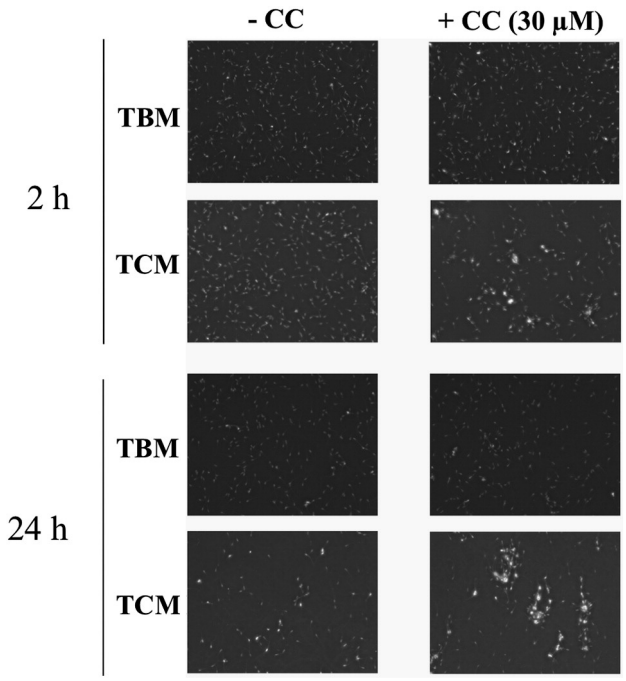

Fig. 5. The AMPK inhibitor, compound C rapidly confers spermatozoa-aggregation ability. Spermatozoa from several boars were pooled, incubated in TBM (upper images) or TCM (lower images) in the presence (right images) or absence (left images) of AMPK inhibitor, compound C (CC 30 μM) in a CO_2 incubator at 38.5 °C for 2 h and 24 h. Representative microscope images of boar spermatozoa untreated and treated with CC are shown.

Based on the fact that AMPK inhibition in spermatozoa incubated in presence of Ca^{2+} and bicarbonate causes a loss of acrosomal integrity, we next investigated the effect of CC in the acrosome reaction induced by calcium ionophore A23187 (Fig. 8). To perform this experiment we incubated spermatozoa during 4 h at 38.5 °C in TCM to induce a capacitated state and then added 10 μM A23187 for 1 h to trigger the acrosome reaction (Fig. 8). In addition to analyze the population of acrosome-reacted live spermatozoa PNA^+/PI^- (Fig. 8A), we have evaluated in parallel the level of lipid disorganization of the plasma membrane associated to this spermatozoa functional process (spermatozoa population $M540^+$/Yo-Pro^- in Fig. 8B). As stated before, the percentage of spermatozoa with basal acrosome reacted or damaged measured at 4 h in TCM just before the induction with A23187 is significantly higher in the presence of CC (Fig. 8A), which correlates with a significant increase in the percentage

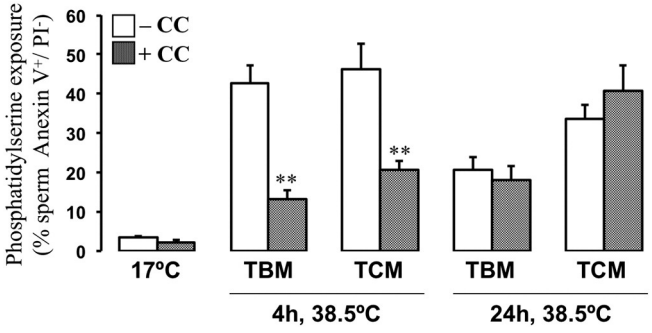

Fig. 6. Effect of the AMPK inhibition in the phosphatidylserine externalization at the plasma membrane of boar spermatozoa. Spermatozoa were incubated in TBM or TCM at 17 °C or in a CO_2 incubator at 38.5 °C in the presence or the AMPK inhibitor CC (30 μM, filled histograms) for indicated times. The level of phosphatidylserine externalization at the spermatozoa plasma membrane was measured by flow cytometry as described using Annexin V-FITC as probe. Spermatozoa population exhibiting outward translocation of phosphatidylserine at the plasma membrane (Annexin V^+) is expressed as percentage of total live spermatozoa (PI^-) analyzed. Each experiment was performed at least 4 times and the results express the mean ± standard error of the mean. Statistical differences are showed with two asterisks when $p < 0.01$.

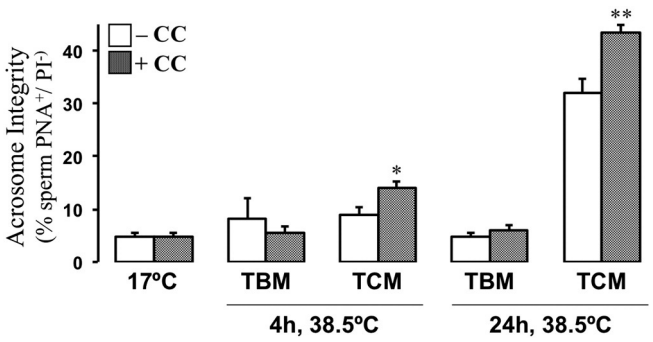

Fig. 7. Effect of the AMPK inhibition in the acrosomal integrity of boar spermatozoa. Spermatozoa were incubated in TBM or TCM at 17 °C or in a CO_2 incubator at 38.5 °C in the presence or absence of the AMPK inhibitor CC (30 μM, filled histograms) for indicated times. Acrosomal integrity was measured by flow cytometry as described in the Materials and methods section using PNA-FITC as a probe. Spermatozoa population exhibiting PNA^+ is expressed as percentage of total live (PI^-) spermatozoa analyzed. Each experiment was performed at least 4 times and the results express the mean ± standard error of the mean. Statistical differences are showed with one asterisk when $p < 0.05$ and two asterisks when $p < 0.01$.

of spermatozoa presenting plasma membrane lipid disorganization (Fig. 4). However, the inhibition of AMPK by CC does not affect neither the percentage of spermatozoa that undergo A23187-induced acrosome reaction (Fig. 8A) nor the lipid disorganization degree of plasma membrane induced by this calcium ionophore (Fig. 8B).

To investigate whether this lack of effect of CC was dependent of the stimulus triggering acrosome reaction in boar spermatozoa, we additionally performed another model of experimentally induced acrosome reaction by incubating these germ cells at 38.5 °C in TCM including 1 mM of 8Br-cAMP. Confirming previous results with A23187 as inductor of acrosome reaction, AMPK inhibition by compound C does not significantly affect the 8Br-cAMP-induced acrosome-reaction in boar spermatozoa (data not shown).

3.7. The AMPK pathway is stimulated by the activation of protein kinase A in boar spermatozoa and inhibited by compound C

As PKA is the key intracellular pathway that regulates spermatozoa function, we hypothesized that AMPK might lie downstream of PKA in these germ cells. Thus, PKA activity was directly stimulated by the incubation of boar spermatozoa at physiological temperature with a non-hydrolysable cAMP analog, 0.1 mM of 8Br-cAMP for different times and the activity of AMPK was analyzed by western blot. As observed in Fig. 9A, AMPK activity measured as the level of AMPK phosphorylated at Thr^{172}, is rapid and potently stimulated by 8Br-cAMP in a time-dependent manner. The phosphorylation of AMPK induced by 8Br-cAMP in boar spermatozoa is greatly inhibited by CC treatment (Fig. 9B, film overexposed to visualize unstimulated phospho-Thr^{172}-AMPK levels).

4. Discussion

Mammalian spermatozoa require a fine regulation of energy levels to maintain cellular structure, stability and function of their membranes and intracellular ions composition during changing extracellular conditions, such as those leading to oocyte fertilization within the female reproductive tract. In mammalian cells the AMP-activated protein kinase AMPK controls cellular metabolism [10,12,14,23] by switching on metabolic pathways that produce ATP and simultaneously switching off anabolic pathways that consume ATP [13]. In mammalian germ cells, we have recently demonstrated that AMPK is expressed a relative high level and that is involved in the regulation of one of spermatozoa essential functions, motility [16]. The present study shows for the first time that the metabolic sensor kinase AMPK is localized at the entire acrosome at relatively high level and in the midpiece of flagellum in boar

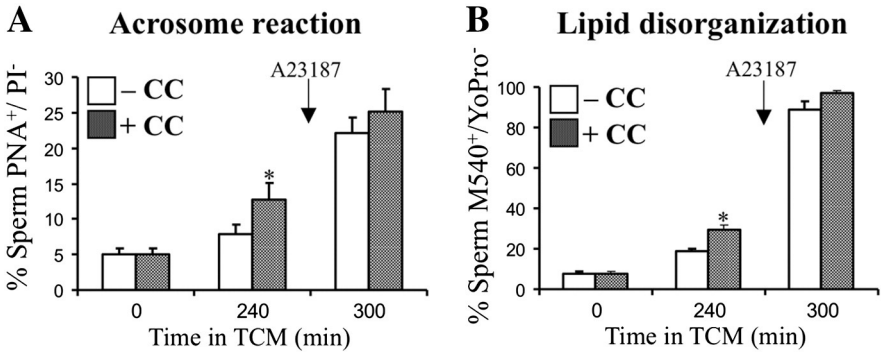

Fig. 8. Effect of the AMPK inhibition in the acrosome reaction and the plasma membrane lipid disorganization induced by calcium ionophore A23187. Spermatozoa were induced to capacitate by 4 h incubation in TCM in a CO_2 incubator at 38.5 °C in the presence or absence of the AMPK inhibitor CC (30 μM, filled histograms) and then acrosome reaction was triggered by adding A23187 (10 μM) for 1 h more. Those spermatozoa samples evaluated at 17 °C were considered as time 0. The population of acrosome reacted-spermatozoa PNA^+ (A) and the degree of plasma membrane lipid disorganization (B) were analyzed at 0 h, 4 h and 5 h by flow cytometry using PNA-FITC/PI and M540/YoPro1 as probes, respectively. Spermatozoa populations PNA^+/PI^- (A) and high $M540^+$/Yo-Pro1$^-$ (B) are expressed as percentage of total live spermatozoa. This experiment was performed at least 6 times and the results express the mean ± standard error of the mean. Statistical differences were considered when $p < 0.05$ are showed with an asterisk.

spermatozoa. Additionally, this work shows that the enzymatically active form of AMPK, phosporylated at Thr^{172}, is specifically localized at the most apical part of the acrosome, subequatorial segment and midpiece of flagellum at physiological conditions.

The mitochondrial membrane potential, ΔΨm, is generally used as an indicator of mitochondrial status, since this measure of ion transport reflects metabolic activity and integrity of the mitochondrial membrane. Our results, using the AMPK inhibitor CC, suggest that AMPK activity is involved in the long-term maintenance of the spermatozoa mitochondrial membrane potential, ΔΨm, in a Ca^{2+} and/or HCO_3^- dependent manner, as CC treatment for 24 h leads to a slight but significant decrease in the percentage of TCM-incubated spermatozoa presenting high ΔΨm. Supporting this role of AMPK in the spermatozoa mitochondrial activity is the intracellular localization of active phospho-Thr^{172}-AMPK, which is found in the midpiece of the flagellum, where mitochondria are exclusively localized and helically arranged in spermatozoa. A reduction in ΔΨm might occur during the process of cell death, as it has been shown in human spermatozoa [24]. However, our previous data obtained from boar spermatozoa co-labeled with propidium iodide and SYBR-14 indicate that 24 h treatment with CC in either TBM or TCM does not cause spermatozoa death [16]. Therefore, as previously we ruled out any potential side effect of the inhibitor of AMPK, CC, in boar spermatozoa [16], our results suggest that the decrease in the number of boar spermatozoa with high ΔΨm caused by AMPK inhibition is not related to spermatozoa death but to other germ cell processes. As we

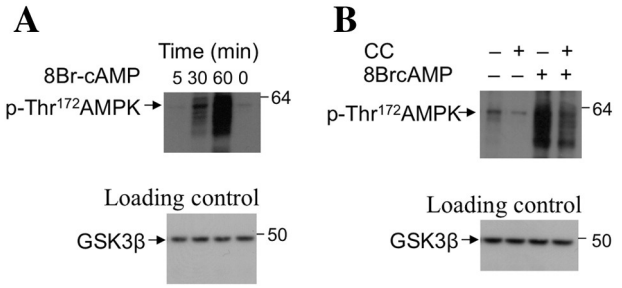

Fig. 9. PKA activation by 8Br-cAMP stimulates the phosphorylation of AMPK, which is inhibited by compound C in boar spermatozoa. Spermatozoa were incubated in TBM at 17 °C (0 min) or in a CO_2 incubator at 38.5 °C in the presence or absence of PKA activator, the cAMP analog, 8Br-cAMP for indicated times (A) and then lyzed. Proteins (20 μg) from sperm lysates were analyzed by western blotting using anti-phospho-Thr^{172}-AMPKα as primary antibody. The arrow indicates the cross-reactive band of phospho-Thr^{172} AMPK. This experiment was performed 6 times and a representative film is shown. B: AMPK phosphorylation at Thr^{172} was evaluated in spermatozoa incubated in TBM in the presence (+) or absence (−) of 8Br-cAMP for 2 h and/or AMPK inhibitor, CC (30 μM) at 38.5 °C. This experiment was performed 5 times and a representative film is shown. Loading controls for each experiment using anti-GSK3β antibody are showed at lower panels.

suggest in this work, AMPK activity is involved in the maintenance of the integrity of spermatozoa membranes, such as plasmalemma or at the acrosome, therefore one possible explanation is that AMPK inhibition might also affect the integrity of mitochondrial membrane, which would effectively lead to a decrease in ΔΨm. Moreover, many mitochondrial functions, including protein import, ATP generation and lipid biogenesis, depend on the maintenance of ΔΨm [25]. It is therefore plausible that the degree to which changes in the mitochondrial membrane potential occurs could be indicative of changing physiological conditions of spermatozoa, including different Ca^{2+} and/or bicarbonate concentrations [26] as mimicked by TCM. Having in mind the energy-regulating role of AMPK in somatic cells, it is conceivable that AMPK might play a role in spermatozoa specific processes that are tightly related to the ATP levels such as mitochondrial membrane potential.

In addition to this effect in mitochondria, AMPK inhibition in boar spermatozoa causes a marked effect in plasma membrane greatly enhancing its lipid disorganization measured by flow cytometry as $M540^+$-live spermatozoa. Merocyanine 540 is a lipophilic dye that is capable of penetration into the exoplasmic leaflet during the increased phospholipids disorganization initiated during different cellular events in somatic and germ cells that are associated with cell recognition and increased fusogenic properties [27]. The CC-induced disorganization of spermatozoa plasma membrane is dependent of the time and the incubation medium at physiological temperature. Interestingly CC-induced lipid disorganization in boar spermatozoa membrane is more rapid in the presence of Ca^{2+} and HCO_3^- in the medium as it significantly occurs in TCM as rapid as 4 h. However, inhibition of AMPK for longer time causes significant lipid disorganization of spermatozoa plasma membrane also in a medium without the addition of Ca^{2+} and HCO_3^-. It has been described that merocyanine 540 is not able to specifically detect capacitation-related membrane modifications in human sperm [24], although in different species of spermatozoa like stallion [28] and boar [29], the cell membrane phospholipid disorganization detected by M540 has been considered a manifestation of capacitation. However, our results indicate that the population of $M540^+$-live boar spermatozoa does not always represent capacitated spermatozoa but it might reflect other boar spermatozoa processes influenced by changing extracellular conditions. In fact, our study shows that an enhancement in lipid disorganization of plasma membrane evaluated with M540 does not unequivocally indicates a spermatozoa capacitating state, indirectly estimated by a higher percentage of spermatozoa PNA^+ that have lost acrosome integrity. It is important to mention that a loss of acrosomal integrity measured as live spermatozoa PNA^+ might be attributed to acrosomes reacted or damaged. Several data from this work sustain this idea: a) by contrast to TCM, the marked membrane lipid disorganization induced by long-term spermatozoa

treatment with CC in TBM does not lead to a loss of acrosomal integrity. A possible explanation, only valid when considering loss of acrosomal integrity as a result of basal acrososome reaction, is that although plasma membrane lipid disorganization caused by AMPK inhibition could effectively accompany the capacitation, the acrosome reaction cannot occur in a Ca^{2+}-free medium (TBM, which contains EGTA), given that the cellular process of exocytosis is totally Ca^{2+}-dependent, and b) the CC-enhanced membrane lipid disorganization is not related to boar spermatozoa capacitation in TCM, as there is no higher population of spermatozoa that undergoes acrosome reaction when it is triggered with calcium ionophore under these conditions of AMPK inhibition.

In male germ cells it is well known that physiological levels of HCO_3^- produce a rapid collapse of the asymmetry of the sperm plasma membrane attributable to the activation of scramblase enzymes that translocate membrane phospholipids, such as phosphatidylethanolamine and phosphatidylserine [30], outward of plasma membrane. Our results show that the asymmetry of the boar spermatozoa plasma membrane is well maintained at semen preservation temperature, 17 °C, as the binding of Annexin V under these conditions remains very low (only 2.3 ± 0.5% of live spermatozoa show outward PS exposure). As previously described [30], the phosphatidylserine externalization in the plasma membrane is triggered by incubation of germ cells in the presence of HCO_3^- at physiological temperature. In boar spermatozoa the loss of membrane asymmetry observed under these conditions is dependent of the incubation time at 38.5 °C, showing a maximum effect at 4 h where more than 40% of live spermatozoa exhibit PS exposure at their plasma membrane. Interestingly, under these conditions the inhibition of AMPK causes a significant inhibition of the outward exposure of phosphatidylserine in spermatozoa plasma membrane, suggesting that inhibition of AMPK, at least at short time, might be leading to a downstream inhibition of scramblase(s) activity. Previously Vucicevic et al. [31] described that CC caused an increase in PS exposure with a concomitant induction of cell cycle arrest and apoptosis in glioma cells. Additionally in erythrocytes [32] from AMPKα1-deficient mice ($ampk^{-/-}$) there is an increase in the outward exposure of PS in the plasma membrane. Differences between our results and above mentioned might be explained by several reasons: i) previous works have been performed in different somatic cell types and ii) in previous studies the CC-induced PS exposure in the plasma membrane accompanies a CC apoptotic cellular effect, whereas in boar germ cells CC does not induced spermatozoa death [16].

The head-to-head aggregation ability of spermatozoa induced by CC treatment is enhanced in the presence of Ca^{2+} and bicarbonate in the incubation medium, in agreement with Harayama [33] and Harrison et al. [34] that described that the presence of Ca^{2+} and bicarbonate in the incubation medium is a potent agglutinating factor for spermatozoa from different species. However, there are not works studying the effect of AMPK inhibition in somatic or germ cell aggregation. The few studies most related to this CC effect found in the literature are referred to different changes in the morphology of somatic cells. Thus, a considerable CC effect has been observed in glioma cells, likely related with apoptosis events [31], and in preadipocytes 3T3-L1 where CC-induced morphological changes accompanied its inhibitory effect of adipocyte differentiation [35]. Furthermore, AMPK mediates morphological alterations of astrocytes in response to energy depletion [36]. As none of these studies describe morphological effects related to cell agglutination and in addition they have been performed in somatic cells, at this moment we cannot establish any solid explanation about the spermatozoa aggregation-promoting property conferred by CC.

A spermatozoa plasma membrane with normal integrity and function is required for successful fertilization [37]. Several and relevant functions of the spermatozoa plasma membrane are related to cell metabolism, spermatozoa motility, capacitation, acrosome reaction and sperm–oocyte interaction. The plasma membrane lipid composition and organization in boar sperm cells are modulated during their epididymal transit [38]. Spermatozoa that undergo capacitation process during the transit trough the female genital tract have to destabilize their plasma membrane i) locally, at the oviductal binding sites and ii) temporary, at the time of ovulation [34] by an increased disordering of the fatty acid chains of the phospholipids and enhanced lateral fluidity, as well as distorted phospholipids asymmetry [30,39]. The plasma membrane phospholipid disorganization is associated with cell recognition or increased fusogenic properties [27] or in somatic cells with other processes related to blood clotting [40] or apoptosis [41,42]. Our results suggest that the regulatory role of AMPK at the spermatozoa plasma membrane must be quantitatively relevant as confirmed by the high percentages of live spermatozoa showing $M540^+$ or annexin-V^+ binding. In addition, this work also suggests that both processes occurring at the spermatozoa plasma membrane, lipid disorganization and PS externalization are not likely related. Thus, when about 50% spermatozoa show lipid disorganization at 4 h in TCM in the presence of CC, less than 20% of spermatozoa exhibit PS exposure at their plasma membrane.

It is well known that spermatozoa plasma membrane regulation is controlled by the cAMP-dependent protein kinase A pathway [30,43]. The activation of sperm specific soluble adenylate cyclase sAC by high levels of bicarbonate and Ca^{2+} catalyzes the formation of cAMP that in turn directly activates PKA which plays a central and important role in the regulation of the any spermatozoa functional process. Our results show that the intracellular pathway by which AMPK is activated in boar spermatozoa includes PKA as an upstream regulator as the direct activation of PKA leads to a rapid and potent effect in the phosphorylation at Thr^{172} of AMPK, which indicates an increase in AMPK activity. This is the first work showing that the metabolic sensor AMPK lies downstream of PKA in male germ cells. Our finding is in accordance with previous studies in different somatic cells that have pointed to PKA as an upstream of AMPK [44,45].

In addition our work demonstrates that the AMPK inhibitor CC is able to effectively block the potent activation of AMPK in boar spermatozoa induced by direct stimulation of PKA.

Effects of AMPK inhibition in spermatozoa plasma membrane including lipid disorganization and the outward exposure of PS are likely occurring in the plasma membrane surrounding the most apical part of the acrosome, where a majority of active phospho-Thr^{172}AMPK is localized and where in fact its inhibition causes a loss of acrosomal integrity. Interestingly, this membrane area is likely involved in the spermatozoa aggregation ability induced by CC as it occurs head-to-head, which suggest that these mentioned CC effects in plasma membrane and in agglutination might be related. Additionally this work suggests that AMPK activity is necessary to maintain the correct physiological plasma membrane lipid organization including a specific and relevant role regulating the outward translocation of phosphatidylserine and the integrity of acrosome membrane at a level adequate to the different extracellular conditions at which boar spermatozoa are physiologically exposed.

5. Conclusions

In summary, the present study demonstrates that AMPK is mainly expressed in boar spermatozoa at the entire acrosome and the midpiece of the flagellum and that when active/phosphorylated at Thr^{172} is localized at the most apical part of the acrosome, remaining also in the midpiece of flagellum. Our findings indicate that the activity of the cell energy sensor molecule, AMPK, is not involved in the calcium-induced acrosome reaction. Moreover, this work suggests that AMPK is clearly involved in the maintenance of i) physiological lipid organization of plasma membrane, ii) outward translocation of phosphatidylserine at the plasma membrane, iii) the mitochondrial membrane potential and iv) the integrity of the acrosome membrane in boar spermatozoa. As these spermatozoa processes, together with spermatozoa motility [16], are required under different environmental conditions of spermatozoa when transiting trough the female reproductive tract, their result is essential for the ultimate function of spermatozoa, oocyte fertilization. Additionally this work describes that the main kinase controlling

spermatozoa function, PKA, is upstream of AMPK in spermatozoa. Therefore, we propose that AMPK protein, which lies downstream of PKA, plays an important regulatory role at different levels of the spermatozoa function.

Acknowledgements

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