UNIVERSIDAD DE EXTREMADURA

TESIS DOCTORAL

Expresión y función de la proteína quinasa activada por AMP, AMPK, en la célula germinal masculina de cerdo

Ana Hurtado de Llera

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Conformidad de los Directores:

Fdo.: Dña. M. Julia Bragado González Fdo.: Dña. M. Cruz Gil Anaya Fdo.: D. Luís J. García Marín

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LOS RESULTADOS DE LA PRESENTE TESIS DOCTORAL HAN SIDO PUBLICADOS EN LAS SIGUIENTES REVISTAS INTERNACIONALES:

1- AMP-activated kinase AMPK is expressed in boar spermatozoa and regulates motility.

Hurtado de Llera A, Martin-Hidalgo D, Gil MC, Garcia-Marin LJ, Bragado MJ.

PLoS One. 2012;7(6):e38840. doi: 10.1371/journal.pone.0038840. Epub 2012 Jun 14.

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

Hurtado de Llera A, Martin-Hidalgo D, Rodriguez-Gil JE, Gil MC, Garcia-Marin LJ, Bragado MJ.
Biochimica et Biophysica Acta-Biomembranes. 2013 Sep;1828(9):2143-51. doi:10.1016/j.bbamem.2013.05.026. Epub 2013 Jun 4.

3- The calcium/CaMKKα/β and the cAMP/PKA pathways are essential upstream regulators of AMPK activity in boar spermatozoa.

Hurtado de Llera A, Martin-Hidalgo D, Gil MC, Garcia-Marin LJ, Bragado MJ.

Biology of Reproduction. 2014 Jan 3. doi:10.1095/biolreprod.113.112797.

Además, parte de los resultados han permitido elaborar el siguiente manuscrito que se enviará para su publicación:

Up-activation of AMPK down regulates motility and controls essential cellular processes required for boar spermatozoa function.

Hurtado de Llera A, Martin-Hidalgo D, Gil MC, Garcia-Marin LJ, Bragado MJ.

LOS RESULTADOS TAMBIÉN HAN SIDO PUBLICADOS EN LOS SIGUIENTES CONGRESOS CIENTÍFICOS INTERNACIONALES:

Pósters:

- Implicación de la proteína quinasa activada por AMP (AMPK) en la función del espermatozoide de cerdo. María Julia Bragado**,** Ana Hurtado de Llera, David Martín-Hidalgo, María Cruz Gil y Luis Jesús García-Marín. XXXIV Congreso de la Sociedad Española de Bioquímica y Biología Molecular (SEBBM). Barcelona (2011) P-3 pág. 301 www.sebbmbcn2011.com

- Expresión de la proteína quinasa activada por AMP (AMPK) en la célula germinal masculina: Posible función en la regulación de la motilidad del espermatozoide. Ana Hurtado de Llera, David Martín-Hidalgo, María Cruz Gil, Luis Jesús García-Marín y María Julia Bragado. XXXIV Congreso de la Sociedad Española de Bioquímica y Biología Molecular (SEBBM). Barcelona (2011) P-9 pág. 303

- The AMPK activator metformin inhibits one of the main function of boar spermatozoa, motility. Hurtado de Llera A., D. Martín-Hidalgo, M.C. Gil, L. J. García-Marín, M. J. Bragado. C Gil, MJ. 22nd International Union of Biochemistry and Molecular Biology (IUBMB) & 37 th Federation of European Biochemical Societies (FEBS) Congress. Sevilla (2012) P27-26 pág. 545, FEBS Journal 279 (Suppl.1)

- Effects of the AMPK activator metformin in physiological processes of boar spermatozoa necessary to accomplish oocyte fertilization. Hurtado de Llera A., D. Martín-Hidalgo, M.C. Gil, L. J. García-Marín, M. J. Bragado. C Gil, MJ. 22nd International Union of Biochemistry and Molecular Biology (IUBMB) & 37 th Federation of European Biochemical Societies (FEBS) Congress. Sevilla (2012) P27-79 pág. 561, FEBS Journal 279 (Suppl.1)

- La activación de la proteína quinasa activada por AMP (AMPK) regula la velocidad del movimiento del espermatozoide de mamíferos. Ana Hurtado de Llera, David Martín-Hidalgo, M. Cruz Gil, Luis Jesús García-Marín y M. Julia Bragado. XXXVI Congreso de la Sociedad Española de Bioquímica y Biología Molecular (SEBBM). Madrid (2013) P-03-21 www.sebbm.com

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ABREVIATURAS:

- **ACC:** acetil- CoA- carboxilasa
- **AICAR:** 5-aminoimidazol-4-carboxamida ribósido
- **AKAPs:** *A Kinase Anchoring Protein* (proteínas de anclaje de la PKA)
- **ALH:** desplazamiento lateral de la cabeza
- **AMP:** adenosín monofosfato
- **AMPK:** quinasa activada por AMP
- **APS:** persulfato amónico
- **ATP:** adenosín trifosfato
- **BCF:** frecuencia de batido de la cola
- **BSA:** *bovine serum albumin* (albúmina de suero bovino)
- **BTS:** *Beltsville thawing solution* (solución de congelación de Beltsville)
- **C.A.S.A.:** sistema computerizado de análisis seminal
- **CaMKK:** proteína quinasa de la quinasa dependiente de calcio y calmodulina
- **CC:** compuesto C
- **cAMP:** adenosín monofosfato cíclico
- **DAPI:** 4,6- diamino-2-fenilindol clorhidrato
- **DMSO:** dimetilsulfóxido
- **DTT:** dithiothreitol
- **EDTA:** ácido etilendiaminotetraacético
- **EGCG:** galato de epigalocatequina
- **g:** gramos

- **GSK3:** *glycogen sinthase kinase 3* (glucógeno sintasa quinasa 3)
- **h:** horas
- **HRP:** peroxidasa de rábano picante
- **I.S.A.S®:** *integrated semen analysis system* (sistema integrado de análisis de semen)
- **IP:** ioduro de propidio
- **KDa:** kilodaltons
- **LIN:** índice de linealidad del movimiento del espermatozoide
- **mA:** miliamperios
- **Min:** minutos
- **mL:** mililitros
- **mM:** milimolar
- **mV:** milivoltios
- **nm:** nanometros
- **nM:** nanomolar
- **PBS:** *phosphate-buffered saline* (tampón fosfato salino)
- **PC:** fosfatidilcolina
- **PDE:** fosfodiesterasa
- **PE:** fosfatidiletanolamina
- **PFK:** fosfofructoquinasa
- **PI3K:** fosfatidilinositol-3,4,5-trifosfato
- **PKA:** proteína quinasa A
- **PKC**: proteína quinasa C

- **PP:** proteína fosfatasa
- **PS:** fosfatidilserina
- **RT:** *room temperature* (temperatura ambiente)
- **sAC:** adenilato ciclasa soluble
- **SDS:** *sodium dodecil sulphate* (dodecil sulfato sódico)
- **Ser:** serina
- **STR:** índice de rectitud del movimiento del espermatozoide
- **TBM:** *tyrode´s basal medium* (medio basal)
- **TBS:** *tris buffer saline* (tampón salino tris)
- **TCM:** *tyrode´s complete medium* (medio completo con Ca^{2+} y $HCO3^{-}$)
- **Thr:** treonina
- **Tween- 20:** *polysorbate 20* (polisorbato 20)
- **Tyr:** tirosina
- **v/v:** volumen/volumen
- **VAP:** velocidad promedio
- **VCL:** velocidad curvilínea
- **VOCs:** canales de calcio operados por voltaje
- **VSL:** velocidad rectilínea
- **w/v:** peso/volumen
- **WB:** *western blotting*
- **WOB:** índice de oscilación del movimiento del espermatozoide
- **°C:** grados celsius
- **µg:** microgramos

- **µL:** microlitros
- **µM:** micromolar
- **ΨΔm:** potencial de membrana mitocondrial

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Resumen/Summary

El espermatozoide es una célula germinal altamente especializada que debe experimentar unos procesos funcionales característicos, como son la movilidad, hiperactivación, capacitación o la reacción acrosómica, para lograr su función primordial: la fecundación del ovocito. Estos procesos funcionales son por un lado dependientes del estado energético de esta célula germinal, que está determinado, al igual que en células somáticas, por la relación entre los niveles celulares de AMP, ADP y ATP (Ford, 2006; Miki, 2007), y por otro están regulados por mecanismos bioquímicos, entre los que destaca la fosforilación de proteínas. La célula germinal masculina posee una elaborada compartimentación celular que provoca que, en la última fase de su desarrollo, no pueda llevar a cabo ni la transcripción ni la traducción y, consecuentemente, es incapaz de sintetizar proteínas. Por todo ello, las rutas de señalización intracelular que regulan los procesos celulares basadas en las modificaciones postraduccionales de las proteínas pre-existentes, como la fosforilación de proteínas, catalizada por enzimas quinasas, son especialmente relevantes en esta célula germinal.

La proteína quinasa activada por AMP, AMPK, es una Ser/Thr quinasa muy conservada desde el punto de vista evolutivo, que actúa como una molécula sensora de la carga energética celular, y cuya activación provoca una regulación del metabolismo (Hardie y cols., 2006). Estructuralmente es una proteína heterotrimérica que posee una subunidad catalítica α y dos subunidades reguladoras β y γ. Una de las características esenciales de la AMPK es su extremada sensibilidad a su efector alostérico AMP, ya que cualquier aumento en el ratio AMP/ATP (que indica un descenso de la carga energética celular), provoca la activación de la AMPK (Hardie y cols., 2006; Long y Zierath, 2006). De forma adicional a su activación alostérica por AMP, la fosforilación en el residuo de Thr172 localizado en el bucle de activación de su subunidad catalítica α, es necesaria para lograr la actividad máxima de la AMPK (Carling, 2004). Se han descrito varias quinasas que fosforilan dicho residuo en la AMPK: i) LKB1, que está codificada por un gen supresor de tumores y responsable del síndrome *Peutz-Jerhers*, ii) dos isoformas, α y β, de CaMKK, que es la quinasa de la quinasa dependiente de calcio y

calmodulina, iii) TAK1, que es la proteína quinasa activada por TGFβ y iv) la quinasa KSR2. Diversos tipos de estrés celular y metabólico también provocan la activación de AMPK (Hardie y cols., 2006; Long y Zierath, 2006). En cualquier caso, la actividad de AMPK regula el metabolismo al provocar la estimulación de vías catabólicas que producen ATP y la inhibición de las rutas anabólicas que consumen ATP, de tal forma que la consecuencia metabólica es el mantenimiento de los niveles de ATP en condiciones en las que éstos se encuentran comprometidos.

Hasta el momento de plantear esta Tesis Doctoral, todas las investigaciones sobre AMPK se habían realizado en células somáticas, aunque sí existían dos trabajos que indicaban que quinasas relacionadas con la AMPK desempeñaban una función en los espermatozoides de mamíferos. Así, en 2008 se había descrito que una nueva isoforma (corta) de la quinasa que fosforila AMPK, LKB1s, y se expresa predominantemente en células espermátidas de mamíferos (Towler y cols., 2008), tiene un papel esencial en la espermiogénesis y en la capacidad fecundante en ratones. Paralelamente, se demostró que la deleción de TSSK1 y TSSK2, miembros de la familia TSSK de Ser/Thr quinasas específicas de testículos, y que pertenecen a la rama de la AMPK en el árbol del quinoma humano, provoca infertilidad en ratones (Xu y cols., 2008).

Por todo ello, en la presente Tesis Doctoral nos planteamos la hipótesis de que la AMPK podría desempeñar un papel clave en la célula germinal masculina de mamíferos, probablemente en aquellos procesos celulares muy dependientes del estado energético celular, que a su vez son esenciales para que el espermatozoide consiga fecundar al óvulo. Según esta hipótesis, nos planteamos los siguientes objetivos en la presente tesis Doctoral: 1- Estudiar la expresión y localización celular de la proteína AMPK y de su forma activa en la célula germinal masculina de cerdo. 2- Estudiar, en el espermatozoide de cerdo, la regulación de la actividad de la AMPK en diferentes condiciones extracelulares y las vías de señalización intracelular implicadas. 3- Estudiar el posible papel de la proteína AMPK en diferentes parámetros y procesos funcionales del espermatozoide que lo capacitan para llevar

a cabo la fecundación: movilidad, viabilidad, organización lipídica de la membrana plasmática, integridad de la membrana externa del acrosoma, así como el potencial de membrana mitocondrial. Los resultados de la presente Tesis Doctoral nos permiten elaborar las siguientes conclusiones: 1- La proteína quinasa activada por AMP, AMPK, se expresa en el espermatozoide de cerdo y se encuentra enzimáticamente activa en condiciones fisiológicas. 2- La proteína AMPK se localiza principalmente en todo el acrosoma y en la pieza intermedia del flagelo del espermatozoide de cerdo. Cuando está activa, su localización en la pieza intermedia del flagelo se mantiene, sin embargo, en la cabeza del espermatozoide su localización está restringida a la parte más apical del acrosoma. 3- La ruta de señalización que provoca la activación de AMPK por fosforilación en el espermatozoide de cerdo está regulada por mensajeros intracelulares como el calcio y el cAMP, así como por la proteína quinasa A (PKA), proteína quinasa C (PKC) y las proteínas quinasas α y/o β de la quinasa dependiente de calcio y calmodulina (CaMKKα/β). 4- La proteína AMPK está involucrada en la regulación de la movilidad del espermatozoide de cerdo. Es importante mencionar que, de forma paralela a la publicación de los resultados que sustentan esta conclusión, Tartarin y cols., (2012) han demostrado en ratones *knockout* para AMPKα1 que la AMPK está implicada en la movilidad de la célula germinal masculina. Por ello, esta cuarta conclusión se ve apoyada por otro estudio, utilizando una aproximación experimental diferente en otra especie de mamífero, y contribuye a reforzar la idea de que la AMPK es esencial para una correcta movilidad. 5- La proteína AMPK desempeña una importante función en las membranas del espermatozoide de cerdo. Por un lado, está implicada en el mantenimiento de la integridad de la membrana externa del acrosoma y, por otro, contribuye tanto al mantenimiento de la organización lipídica de la membrana plasmática, como al control de la translocación de la fosfatidilserina hacia su cara externa. 6- La proteína quinasa AMPK está implicada en la regulación del potencial de membrana mitocondrial del espermatozoide de cerdo. 7- La proteína quinasa AMPK se activa por fosforilación en el espermatozoide de cerdo en repuesta a determinados tipos de estrés celular. En resumen,

consideramos que los resultados que aporta esta Tesis Doctoral permiten proponer a la AMPK como una quinasa esencial que regula la capacidad de la célula germinal masculina para adaptarse a las fluctuantes condiciones externas, como ocurre durante el tránsito a través del tracto reproductor femenino.

Spermatozoon is a very specialized germ cell that undergoes characteristic functional processes such as motility, hyperactivation, capacitation and acrosome reaction, prior accomplishing its essential function: fertilization of the oocyte. These processes depend on the energy charge of this germ cell, which is determined by the ratio between cell levels of AMP, ADP and ATP, as in somatic cells (Ford, 2006; Miki, 2007). Moreover, these functional processes are also controlled by biochemical mechanisms, such as protein phosphorylation. Because mature spermatozoon possesses elaborated cell compartments that cause the lack of transcription and translation, this germ cell is unable to synthesize proteins. Therefore, intracellular signalling pathways that regulate male germ cell processes based on postranslational modifications of pre-existing proteins, such as protein phosphorylation catalyzed by enzymes kinases, are especially important in spermatozoa function.

AMP-activated kinase AMPK is a evolutivelly well conserved Ser/Thr protein kinase that detects cell energy state and upon activation regulates metabolism (Hardie et al., 2006). AMPK is a heterotrimeric protein that posseses a catalitic subunit α and two regulatory subunits β and γ. One of the essential characteristics of AMPK is its high sensibility to its alosteric effector AMP, as any increase in the ratio AMP/ATP (which indicates a decrease in cell energy charge), leads to activation of AMPK (Hardie et al., 2006; Long and Zierath, 2006). Additionally to allosteric activation by AMP, phosphorylation at Thr172 located in the activation loop of catalytic subunit α, is necessary to achieve maximal activation of AMPK (Carling, 2004). Several kinases have been described to phosphorylate AMPK at Thr172: i) LKB1, encoded by the tumour suppressor gen responsible for the syndrome *Peutz-Jerhers*, ii) two isoforms, α and β, of CaMKK, calcium-calmodulin kinase kinase, iii) TAK1, TGFβ-activated kinase and iv) KSR2 kinase. Several types of cell and metabolic stresses also cause AMPK activation (Hardie et al., 2006; Long and Zierath, 2006). Overall, AMPK activity regulates metabolism by leading to both stimulation of ATP-producing catabolic pathways and inhibition of ATP-consuming

anabolic pathways; therefore, the main metabolic consequence is the maintenance of cellular ATP levels when those are compromised.

At the beginning of this Doctoral Thesis, all research studies about AMPK had been performed in somatic cells, although two works in 2008 indicated that Kinases related to AMPK play a function in mammalian spermatozoa. Thus, a new short isoform of the upstream kinase that phosphorylates AMPK, LKB1s was showed to be mainly expressed in mice spermatozoa (Towler et al., 2008), where it plays an essential role in spermiogenesis and in the fertilizing ability. In parallel, deletions of TSSK1 and TSSK2, members of testis specific TSSK family of Ser/Thr kinases that belongs to AMPK branch in the human kinome tree, cause infertility in mice (Xu et al., 2008).

Based on all above mentioned, in the current Doctoral Thesis we hypothesize that AMPK would play a key regulatory role in male germ cell, likely in those cell processes highly dependent on the energetic state, which in turn are essential for spermatozoa to accomplish oocyte fertilization. According to this hypothesis, we have planed the following objectives: 1- To study the expression and cell localization of AMPK protein and its active form in the boar male germ cell. 2- To study the regulation of AMPK activity in boar spermatozoa under different extracellular conditions, as well as the intracellular signalling pathways involved. 3- To study the possible role of AMPK protein in different spermatozoa functional parameters and processes that are essential for achieving fertilization: motility, viability, maintenance of the organization and integrity of acrosome and plasma membrane, as well as in the mitochondrial membrane potential. Results of the Thesis allow us to establish the following conclusions: 1- AMP-activated protein kinase AMPK is expressed in boar spermatozoa and is active under physiological conditions. 2- AMPK protein is mainly localized at the whole acrosome and in the middle piece of boar spermatozoa flagellum. When active, p-Thr172-AMPK remains localized at the middle piece of flagellum, while in spermatozoa head its localization is restricted to the most apical part of the acrosome. 3- Intracellular mechanisms

leading to AMPK activation by phosphorylation in boar spermatozoa involved intracellular messengers such as calcium and cAMP, as well as signalling pathways of the protein kinase A (PKA), protein kinase C (PKC), protein kinase α and/or β of calcium-calmodulin dependent kinase (CaMKKα/β). 4- AMPK protein Kinase is involved in the regulation of motility in boar spermatozoa. It is important to mention that in parallel to results that support this conclusion obtained during this Thesis, Tartarin et al., (2012) have demonstrated in AMPKα1 knockout mice that AMPK is crucially involved in motility of male germ cells. Therefore, this fourth conclusion of the Thesis is now also supported by this study using a different experimental approach in other mammalian specie, and contributes to reinforce the idea that AMPK is essential for a proper motility in mammalian spermatozoa. 5- AMPK protein plays a relevant function in boar spermatozoa membranes. In one hand, AMPK is involved in the maintenance of the outer acrosome membrane integrity, and on the other hand, it contributes to the maintenance of both the lipid organization of plasma membrane, and the phosphatidylserine translocation to its outer face. 6- AMPK protein kinase is involved in the control of mitochondrial membrane potential in boar spermatozoa.7- AMPK protein kinase becomes activated by phosphorylation in boar spermatozoa in response to several germ cell stresses. In summary, we consider that results elaborated in this Thesis allows our proposal that AMPK acts as an essential kinase in the regulation of male germ cells ability for adapting to the changing conditions of the extracellular medium, as it occurs during their transit though the female reproductive tract for fertilization.

Introducción

A. EL ESPERMATOZOIDE DE MAMÍFEROS

El espermatozoide (del griego *esperma,* semilla y *zoon*, animal) es una célula haploide altamente especializada cuya función es transportar el material genético hasta el ovocito. Al ser la única célula de los mamíferos diseñada para funcionar fuera del organismo en que se produce, muchos de sus rasgos físicos y bioquímicos han evolucionado para garantizar el paso a través del tracto reproductivo masculino y femenino. Los espermatozoides recién eyaculados no tienen capacidad fecundante, por ello deben experimentar una serie de cambios celulares, tanto biofísicos como bioquímicos, que se engloban en un proceso denominado "capacitación". Además, para lograr con éxito la fecundación, la célula germinal masculina debe poder desempeñar unos procesos funcionales característicos como son la movilidad, la hiperactivación y la reacción acrosómica.

A1) ESTRUCTURA DEL ESPERMATOZOIDE

Morfológicamente el espermatozoide de mamíferos consta de dos partes bien diferenciadas, la cabeza y la cola. Estas dos estructuras se encuentran rodeadas por la membrana plasmática, la cual tiene una importante función en los procesos fisiológicos que tienen lugar en el espermatozoide.

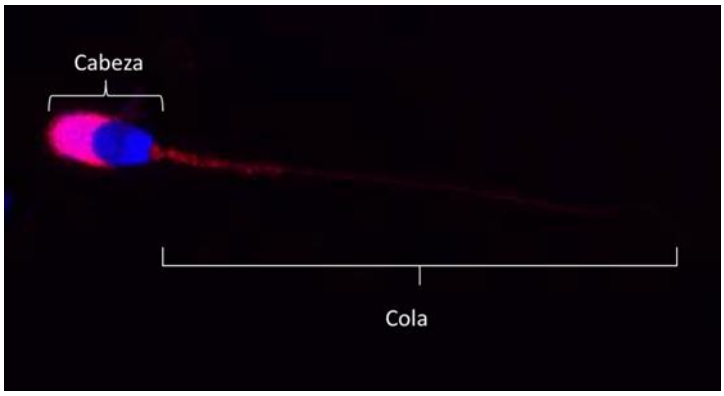


Figura 1: *Estructura del espermatozoide.*

Membrana plasmática

Posee una estructura de mosaico fluido formada por una doble capa lipídica donde la mayoría de sus moléculas son capaces de moverse en el plano de la membrana. Sin embargo, la membrana plasmática del espermatozoide se diferencia de la membrana plasmática de las células somáticas tanto en la composición como en las características biofísicas. Una peculiaridad de estas células germinales es que la membrana plasmática se subdivide en regiones o "dominios" que difieren tanto en composición como en estructura. Estos dominios garantizan la rigidez de la membrana necesaria para evitar capacitaciones prematuras. La cantidad relativa de colesterol en relación a los fosfolípidos determina la fluidez de la membrana y pequeños cambios en ella pueden dar lugar a su reorganización, de tal forma que los dominios pueden alterarse (Schroeder y cols., 1991). Algunos estudios han demostrado que la relación colesterol/fosfolípidos no es constante en los espermatozoides de todas las especies, siendo para espermatozoides de cerdo 0,20 (Green y Watson, 2001), para espermatozoides de caballo 0,36 (Green y Watson, 2001), para espermatozoides de toro 0,40 (Parks y cols., 1987) y para espermatozoides humanos 0,83 (Mack y cols., 1986).

El papel de la membrana plasmática en la comunicación entre el espermatozoide y el medio externo es de vital importancia y engloba el transporte de iones a través de la bicapa lipídica, la unión de diferentes factores, hormonas o neurotransmisores a receptores específicos, lo que a su vez conlleva el mantenimiento del potencial de membrana (Gatti y cols., 1993; Guthrie y Welch, 2005a; Harrison, 1997; Murase y cols., 2004; Petrunkina y cols., 2005).

Los componentes más importantes de la membrana plasmática del espermatozoide se describen a continuación:

- ✓ Glicocálix: una serie de moléculas ricas en carbohidratos forman parte de la superficie de los espermatozoides de mamíferos. El glicocálix parece estar relacionado con la maduración espermática y la inmunoprotección del

espermatozoide en el tracto genital femenino, así como con la unión del espermatozoide a la zona pelúcida del ovocito y posterior fecundación (Schroter y cols., 1999).

- ✓ Lípidos: como se ha mencionado con anterioridad, entre los espermatozoides de las distintas especies existen diferencias en la composición lipídica de la membrana. En general, la membrana plasmática de estas células germinales está formada por aproximadamente un 70% de fosfolípidos, un 25% de lípidos neutros y un 5% de glicolípidos (Gadella y Harrison, 2000).
    - Fosfolípidos: los principales fosfolípidos son la fosfatidilcolina (PC), la fosfatidiletanolamina (PE) y esfingomielina. En menor proporción se encuentran en la membrana del espermatozoide la fosfatidilserina (PS), fosfatidilinositol, ácido fosfatídico, fosfatidilglicerol y lisofosfolípidos. La distribución de los fosfolípidos se mantiene por la actividad combinada de varias enzimas fosfolípidotransferasas (Bevers y cols., 1998), como las aminofosfolípidotransferasas (flipasas) que transportan PS y PE desde la capa externa a la interna, fosfolipasatransferasas (flopasas) que transportan los fosfolípidos desde la capa interna a la externa, y enzimas que lo hacen en ambas direcciones a través de la bicapa lipídica (escramblasas).
    - Lípidos *raft*: son solubles en detergentes no iónicos (Brown y London, 2000; Simons y Vaz, 2004). Se ha sugerido que estos lípidos facilitan la activación de señales y regulan el movimiento de las moléculas en la membrana (Simons y Toomre, 2000).
    - Lípidos neutros: en la membrana plasmática de los espermatozoides la composición de los lípidos neutros difiere entre especies, entre machos de la misma especie e incluso entre eyaculados de un mismo macho. El que más varía es el colesterol.
- ✓ Proteínas: pueden actuar como proteínas estructurales o bien como moléculas de unión para otras proteínas periféricas. Un ejemplo es la proteína transmembrana CatSper1 que participa en la regulación de la entrada de

calcio y se localiza en la membrana del flagelo del espermatozoide (Carlson y cols., 2003).

Cabeza del espermatozoide

La forma de la cabeza del espermatozoide se encuentra condicionada por la forma del núcleo, la cual varía en función de la especie. La cabeza del espermatozoide está formada por el acrosoma, el núcleo y el citoplasma.

- ✓ Acrosoma: el acrosoma es una estructura vesicular, con forma de capuchón, localizada en la parte anterior de la cabeza, entre la membrana plasmática más apical y el núcleo. Se encuentra delimitado por una membrana denominada membrana acrosómica o acrosomal. La forma y tamaño del acrosoma varía ampliamente entre las especies. Contiene numerosas enzimas hidrolíticas, aunque desde el punto de vista funcional, las principales enzimas son la acrosina y la hialuronidasa (Knobil y Neill, 2006). Estas enzimas se liberan mediante exocitosis en un proceso denominado reacción acrosómica con el fin de digerir la matriz extracelular del ovocito para lograr la fecundación.
- ✓ Núcleo: el núcleo de los espermatozoides posee menor volumen que el de las células somáticas. Su cromatina se encuentra altamente condensada y es único tanto en la cantidad de ADN como en la composición de sus nucleoproteínas. Debido a las dos divisiones meióticas que ocurren durante la espermatogénesis, el núcleo de los espermatozoides posee un genoma haploide, con un solo miembro de cada par de cromosomas. Contiene también ARN aunque su función a día de hoy no se encuentra esclarecida.
- ✓ Citoplasma: el citoplasma de los espermatozoides maduros se encuentra muy reducido y carece de ribosomas. Por lo tanto, los espermatozoides maduros han perdido la capacidad de expresar proteínas y transportar vesículas (Miller y Ostermeier, 2006). Debido a que estas células germinales son inactivas desde el punto de vista de la transcripción y de la traducción, deben alcanzar

y fecundar al ovocito con las proteínas sintetizadas durante la espermatogénesis.

Cola del espermatozoide

Proporciona al espermatozoide de mamíferos la capacidad de movimiento. Estructuralmente se diferencian cuatro segmentos: el cuello, la pieza intermedia, la pieza principal y la pieza terminal (Figura 2).

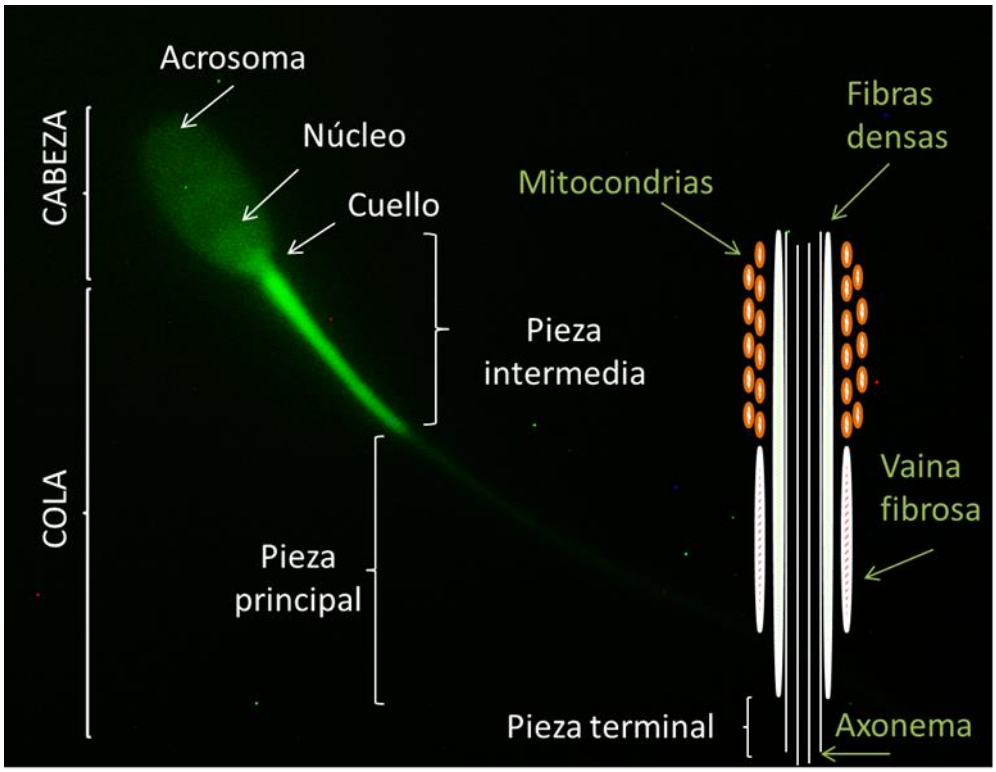


Figura 2: *Estructura de la cabeza y la cola del espermatozoide.*

- ✓ Cuello: es la zona de unión entre la cabeza y la cola y es la base de la que parten las estructuras que conformarán el flagelo o la cola.
- ✓ Pieza intermedia: contiene de forma exclusiva gran cantidad de mitocondrias dispuestas en forma de hélice, formando la denominada vaina mitocondrial. Esta vaina recubre a nueve fibras densas asociadas a los nueve pares de

microtúbulos periféricos internos. Las mitocondrias producen ATP, necesario para el movimiento del flagelo.

- ✓ Pieza principal: compuesta por las fibras densas que rodean al axonema y una vaina fibrosa.
- ✓ Pieza terminal: es la porción final del flagelo. No contiene ni vaina fibrosa ni fibras densas.

El axonema se encuentra anclado a la base de la cabeza del espermatozoide y se extiende a lo largo de los cuatro segmentos de la cola. Posee unas 250 proteínas de distintos tipos: motoras, de citoesqueleto, chaperonas, quinasas, fosfatasas… (Inaba, 2003). Esta estructura está formada por un conjunto de microtúbulos constituidos por dímeros de las proteínas tubulina α y tubulina β. La organización estructural del axonema consiste en dos microtúbulos centrales rodeados por nueve pares de microtúbulos periféricos ("9+2"). Los microtúbulos de cada doblete periférico se denominan A y B, siendo el microtúbulo B más corto que el A. El microtúbulo A se encuentra en posición más interna que el B y posee una serie de proyecciones externas e internas denominadas brazos, que están dirigidas hacia el microtúbulo B del siguiente doblete (Figura 3).

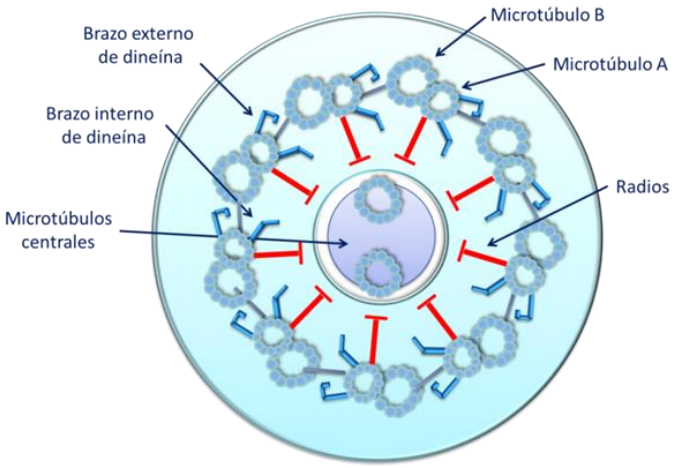


Figura 3: *Axonema: esquema de la distribución de los dobletes de microtúbulos y su conexión mediante los brazos externos e internos de dineína en la cola del espermatozoide. Adaptado de Inaba (Inaba, 2003).*

Los brazos están formados por la proteína dineína que tiene actividad ATPasa, es decir, hidroliza ATP liberando la energía necesaria para el movimiento del flagelo. Los dos microtúbulos centrales se denominan C1 y C2 y están unidos a los microtúbulos A periféricos por unas estructuras denominadas radios. (Figura 3).

A2) PRINCIPALES PROCESOS FUNCIONALES DEL ESPERMATOZOIDE

Como se ha mencionado anteriormente, para lograr con éxito su función primordial, la fecundación del ovocito, el espermatozoide debe ser capaz de desempeñar diferentes procesos fisiológicos que son esenciales y específicos de estas células germinales: movilidad, capacitación y reacción acrosómica.

MOVILIDAD

Los espermatozoides son inmóviles tras la espermatogénesis y a su paso por el epidídimo adquieren la capacidad de movimiento. La mayoría de los espermatozoides de mamíferos muestran dos tipos de movimiento, dependiendo de las condiciones del medio en el que se encuentren. En los eyaculados frescos se produce una activación de la movilidad caracterizada por un movimiento simétrico del flagelo de baja amplitud de onda (Figura 4) y que desencadena un desplazamiento del espermatozoide en línea recta, y de manera consecuente, un movimiento más progresivo. Sin embargo, una vez completado el proceso de capacitación espermática se produce una hiperactivación del movimiento (Figura 4), por el que la cola del espermatozoide genera un movimiento asimétrico y de alta amplitud (Alvarez y Storey, 1984; Turner, 2003), que provoca que el desplazamiento del espermatozoide no sea en línea recta, originando así un movimiento menos progresivo. Esto sugiere que el espermatozoide sufre primero una activación en la movilidad para poder atravesar, con un movimiento progresivo, el tracto genital de la hembra, y posteriormente, una hiperactivación caracterizada por un desplazamiento menos progresivo que esencialmente le permitirá llegar al sitio de fecundación y penetrar la zona pelúcida del ovocito (Turner, 2003).

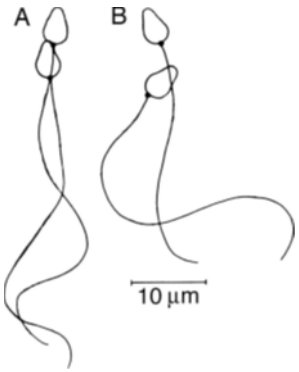


Figura 4: *Patrones flagelares de actividad (A) e hiperactividad (B) espermática (Morales y cols., 1988).*

Como se ha comentado anteriormente, desde un punto de vista estructural el axonema que forma el flagelo se encuentra anclado a la base de la cabeza y está formado por un conjunto de microtúbulos y numerosas proteínas que son fundamentales para el movimiento del espermatozoide. A través de la hidrólisis del ATP, catalizada por la actividad ATPasa de la dineína, se libera la energía necesaria para que los brazos de dineína interactúen con el par de microtúbulos siguiente dando lugar a un movimiento ondulante en la cola. La desfosforilación de la dineína por una fosfatasa dependiente de calmodulina, permite la liberación de los microtúbulos unidos, de tal forma que el axonema vuelve a su situación de partida para poder iniciar un nuevo ciclo de fosforilación/desfosforilación de proteínas y así continuar el movimiento del flagelo del espermatozoide (Turner, 2006).

Como el resto de células, los espermatozoides maduros requieren un aporte de energía para poder realizar los mencionados procesos fisiológicos que los capacitarán para fecundar el ovocito (Garrett y cols., 2008; Miki, 2007). En los últimos años se han publicado diferentes artículos científicos en los que se aborda el estudio de cómo el espermatozoide obtiene, regula y utiliza la energía en diferentes procesos celulares que incluyen la supervivencia, movilidad, capacitación, hiperactivación y reacción acrosómica, en los que la célula germinal debe necesariamente ser capaz de mantener un balance energético apropiado durante el desarrollo de estas funciones celulares. Los mecanismos moleculares que permiten el movimiento del espermatozoide necesitan ATP y, por tanto, la

concentración intracelular de ATP debe mantenerse a unos niveles relativamente altos para lograr una correcta movilidad del espermatozoide. Se ha sugerido que dicho ATP puede proceder tanto de la oxidación mitocondrial como del metabolismo de la glucosa, existiendo variaciones en función de la especie. En este sentido se ha demostrado que los espermatozoides maduros de cerdo pueden utilizar varios tipos de sustratos, tanto extracelulares como intracelulares (Jones y Bubb, 2000; Kamp y cols., 2003), para activar al axonema y provocar el movimiento flagelar. En concreto, el espermatozoide porcino metaboliza con alta eficiencia los azúcares presentes en el plasma seminal como la glucosa (Jones y Connor, 2000; Marin y cols., 2003) o la fructosa (Jones y Connor, 2000). Otras fuentes de energía independientes de los monosacáridos son el piruvato (Jones, 1997), el glicerol o el glicerol 3-fosfato (Jones y cols., 1992) y, siempre y cuando los espermatozoides se encuentren en una atmósfera aerobia (Jones, 1997; Kamp y cols., 2003), a través de la oxidación del lactato a anhídrido carbónico y agua. También se ha descrito el uso de acetato y de propionato para la obtención de energía (Jones y Bubb, 2000). Algunos autores afirman que los espermatozoides tienen la habilidad de utilizar un amplio abanico de sustratos y que el metabolismo varía en función de si el movimiento del flagelo es mantenido o no a lo largo del tiempo (Bohnensack y Halangk, 1986; Storey y Kayne, 1975).

El estudio de los mecanismos por los que el espermatozoide obtiene la energía necesaria es de gran importancia para entender la habilidad de estas células para sobrevivir y adaptarse a las fluctuantes condiciones externas derivadas, por ejemplo, de su paso por el tracto genital femenino. En la especie porcina unos pocos miles de espermatozoides alcanzarán el istmo oviductal y tan sólo un reducido número de ellos llegarán al lugar de la fecundación (Hunter, 1991), disminuyendo de este modo la posibilidad de fecundaciones poliespérmicas y asegurando un número adecuado de espermatozoides en el momento y lugar de la unión al ovocito.

Durante el recorrido a través de los tractos reproductores masculino y femenino, los espermatozoides están sometidos a una serie de fluctuaciones en el medio extracelular, que incluyen cambios en el pH, osmolaridad, concentración de iones, factores de crecimiento, hormonas, neurotransmisores y factores paracrinos, entre otros. El conocimiento de las rutas intracelulares que se activan y regulan la movilidad del espermatozoide durante estas cambiantes condiciones del medio extracelular es fundamental para profundizar en la base científica de la principal función de estas células germinales: la fecundación. Como ya se ha mencionado, el espermatozoide es transcripcionalmente inactivo, por lo que los mecanismos intracelulares de regulación postraduccional de proteínas adquieren una especial importancia. Entre estos últimos se encuentra la fosforilación/desfosforilación de proteínas que está controlada por sistemas de señalización intracelular que incluyen proteínas quinasas y fosfatasas y ocurre tanto en residuos de serina/treonina (Ser/Thr) como de tirosina (Tyr) (Urner y Sakkas, 2003).

En particular, las vías intracelulares que controlan la movilidad del espermatozoide han sido estudiadas en numerosos trabajos (Morgan y cols., 2008; Signorelli y cols., 2012; Visconti, 2009; Yanagimachi, 1994) y, aunque no se conocen en detalle y pueden presentar variaciones según la especie estudiada, se ha demostrado el papel desencadenante que, como se describe con posterioridad, ejercen los iones HCO_3^- y Ca^{2+}, ambos presentes a concentraciones altas en el fluido seminal (Visconti, 2009). Asimismo, está ampliamente aceptado en los espermatozoides de mamíferos, el papel esencial de mensajeros intracelulares como el ión Ca^{2+} y el AMP cíclico (cAMP), este último a su vez generado por la activación de la adenilato ciclasa soluble (sAC), característica de estas células germinales (Figura 5). En el espermatozoide de cerdo, especie objeto de estudio de esta Tesis, se ha demostrado que estos mensajeros provocan la activación de varias vías intracelulares que incluyen a la proteína quinasa A (PKA) (Holt y Harrison, 2002), la fosfatidilinositol 3 quinasa (PI3K) (Aparicio y cols., 2005; Aparicio y cols., 2007a), la proteína quinasa C (PKC) (Bragado y cols., 2010), y consecuentemente, de la fosforilación de otras proteínas que pueden estar incluidas en otra cascada de

señalización, como la glucógeno sintasa quinasa 3-A (GSK3A) (Aparicio y cols., 2007b; Bragado y cols., 2010).

Entre los estímulos desencadenantes de la activación de la movilidad del espermatozoide se encuentra el calcio extracelular que activa directamente la adenilato ciclasa soluble sAC (Figura 5) y regula tanto la activación como la hiperactivación del movimiento de los espermatozoides de diversas especies de mamíferos (Ho y cols., 2002; Lindemann y Goltz, 1988; Suarez y cols., 1987; Tash y Means, 1987; White y Aitken, 1989; Yanagimachi, 1994).

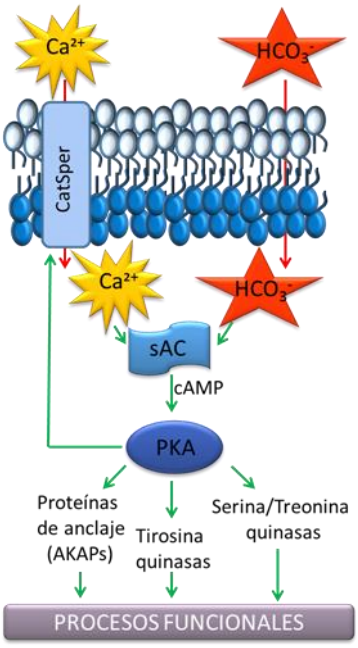


Figura 5: *Mecanismos de activación de la adenilato ciclasa soluble (sAC) en espermatozoides de mamíferos. Adaptado de Signorelli y cols. (Signorelli y cols., 2012)*.

Al igual que el Ca^{2+}, el aumento en la concentración de HCO_3^- también activa la adenilato ciclasa soluble del espermatozoide de cerdo (Garty y Salomon, 1987; Okamura y cols., 1985). Además, el HCO_3^- es el responsable de un aumento en el pH intracelular y de la activación de las escramblasas, enzimas anteriormente mencionadas, que son las encargadas de translocar fosfolípidos de la membrana como la fosfatidilserina y la fosfatidiletanolamina (Gadella y Harrison, 2000), aumentando así la disponibilidad del colesterol por aceptores externos (Salicioni y

cols., 2007; Visconti, 2009). Algunos autores han observado que el efecto inducido por el HCO_3^- puede ocurrir en ausencia completa de Ca^{2+} extracelular (Harrison y Gadella, 2005).

La activación de la adenilato ciclasa soluble produce un aumento del cAMP y éste, a su vez, activará la proteína quinasa A (PKA) al unirse de forma alostérica a su subunidad reguladora. Existen en la bibliografía numerosos trabajos acerca de la función clave de la PKA en la movilidad de los espermatozoides de diferentes especies. La PKA fosforila un gran número de proteínas en residuos de Ser y Thr, activando tanto, directa como indirectamente, numerosas proteinas quinasas y/o inhibiendo proteinas fosfatasas, que en última instancia producen un aumento en los residuos de Tyr (Bajpai y cols., 2003; Holt y Harrison, 2002; Luconi y cols., 2005; Okamura y cols., 1985; Visconti y cols., 2011). En la vaina fibrosa del flagelo existen proteínas de anclaje para la proteína quinasa A llamadas AKAPs las cuales están involucradas en el movimiento del espermatozoide (Muratori y cols., 2011). Así, se ha descrito que la PKA juega un papel importante en la iniciación y mantenimiento de la movilidad, al fosforilar, entre otras, a la dineína del axonema (Tash y Bracho, 1994; Tash y Means, 1982; Tash y Means, 1983). Estudios que han utilizado H89, inhibidor de la PKA, han demostrado una disminución de la movilidad de los espermatozoides humanos (Bajpai y cols., 2003; Luconi y cols., 2005), de cerdo (Holt y Harrison, 2002), y de hámster (Si y Okuno, 1999), reforzando la importancia de esta vía en el control de la movilidad. Por otro lado, la activación de la PKA modula la respuesta de los canales de calcio, como los CatSper, que pueden producir cambios en el potencial de membrana y aumentar la concentración intracelular de Ca^{2+} (Wennemuth y cols., 2003). En este sentido, se ha descrito que la concentración citoplasmática de calcio es mayor en espermatozoides mótiles que en los inmótiles (Suarez y cols., 1993). Experimentos en los que se provocó una entrada de calcio al interior celular, añadiendo el ionóforo A23187, demostraron un aumento en la movilidad de los espermatozoides de ratón (Turner, 2006). Por otro lado, al igual que en células somáticas, los espermatozoides poseen depósitos intracelulares de calcio, y al provocar un aumento de este ión en el citosol mediante

el tratamiento con tapsigargina, Ho y Suarez observaron un aumento en la activación de la movilidad de los espermatozoides (Ho y Suarez, 2001). Uno de los mecanismos intracelulares inducido por el Ca^{2+}, además de la activación de la sAC, es a través de su unión a la calmodulina, proteína que en el espermatozoide se ha localizado en el acrosoma y en la pieza principal (Schlingmann y cols., 2007). Se ha demostrado que el efecto del calcio en la adenilato ciclasa soluble es independiente de la calcio-calmodulina (Jaiswal y Conti, 2003; Litvin y cols., 2003) y, en este sentido, han propuesto que el calcio regula la movilidad de los espermatozoides a través de varias rutas y que tan sólo algunas de éstas requerirían calcio-calmodulina.

Las referencias bibliográficas acerca del papel de las fosfatasas en la movilidad de los espermatozoides son escasas. Se ha detectado actividad tirosina fosfatasa en espermatozoides humanos (Smith y cols., 1996), de hámster (Devi y cols., 1999), de toro (Hoskins y cols., 1983) y de ratón (Krapf y cols., 2010; Tomes y cols., 2004) y actividad serina/treonina fosfatasa en espermatozoides humanos (Fardilha y cols., 2011; Korrodi-Gregorio y cols., 2013). Algunas de estas proteínas fosfatasas estarían implicadas en la maduración de los espermatozoides en el epidídimo (Chakrabarti y cols., 2007; Mishra y cols., 2003; Vijayaraghavan y cols., 1996), lo cual los habilita para iniciar el movimiento. Además, se ha demostrado la implicación de las fosfatasas en la adquisición del movimiento hiperactivo del espermatozoide (Fardilha y cols., 2011; Hoskins y cols., 1983; Krapf y cols., 2010; Smith y cols., 1996).

CAPACITACIÓN

Los procesos bioquímicos y fisiológicos por los cuales el espermatozoide adquiere la capacidad para fecundar al ovocito se engloban bajo el término *capacitación* (Yanagimachi, 1994), que fue descubierto simultáneamente en espermatozoides de rata (AUSTIN, 1951) y de conejo (CHANG, 1951). Como se ha mencionado anteriormente, los espermatozoides de mamíferos recién eyaculados son morfológicamente maduros, pero inmaduros desde un punto de vista funcional.

Una vez depositados en el tracto genital de la hembra, la población de espermatozoides sufre de manera secuencial (Dobrinski y cols., 1997) una serie de cambios que afectan principalmente a la composición y estructura de la membrana plasmática y que le confieren la capacidad fecundante. La capacitación implica, por un lado, sucesos rápidos y tempranos que dan lugar a la activación del movimiento asimétrico y vigoroso del flagelo, y que tiene lugar tan pronto como el espermatozoide abandona el epidídimo y por otro lado, también implica sucesos lentos y tardíos que conducen a cambios en los patrones del movimiento (hiperactivación) y que habilitan a los espermatozoides para llevar a cabo la reacción acrosómica (Salicioni y cols., 2007; Visconti, 2009).

El momento en el cual se inicia la capacitación *in vivo*, así como el tiempo requerido para completarla depende del lugar de deposición del semen en el momento de la cubrición. En el cerdo, la deposición del semen es uterina y los espermatozoides necesitan de 5 a 6 horas en el interior del tracto genital femenino para adquirir la capacidad fecundante (Polge, 1978). La capacitación también puede ser provocada *in vitro* utilizando medios de incubación que incluyen tampones fisiológicos isosmóticos suplementados con una fuente de energía como glucosa, piruvato y lactato, un aceptor de colesterol como la albúmina, e iones como HCO_3^-, Ca^{2+} y K^+ (Choi y Toyoda, 1998; Flesch y cols., 1999; Flesch y cols., 2001; Go y Wolf, 1985; Tardif y cols., 2004).

La capacitación incluye un proceso de desestabilización de la membrana plasmática que da lugar a un aumento en la entrada de Ca^{2+} al interior del espermatozoide (Yanagimachi, 1994), lo que provoca la hiperactivación del movimiento, que a su vez facilita su desplazamiento por el fluido viscoso del oviducto (Katz y cols., 1978; Suarez, 2008; Suarez y Ho, 2003; Yanagimachi, 1994). Durante la capacitación se produce asimismo una pérdida del colesterol de la membrana plasmática y, por tanto, un aumento de su fluidez (Choi y Toyoda, 1998; Cross, 1998; Shadan y cols., 2004; Travis y Kopf, 2002). También se producen cambios en la distribución de las proteínas de la bicapa lipídica, en la carga de

superficie y en la localización de los antígenos de la membrana del espermatozoide (Baker y cols., 2004b). Además, en el transcurso de la capacitación aumenta el pH, se generan especies reactivas de oxígeno (Rivlin y cols., 2004) y se incrementa la actividad quinasa (Arcelay y cols., 2008; Baldi y cols., 2000; Visconti, 2009) y por tanto la fosforilación de proteínas (Harayama y cols., 2012; Visconti y cols., 1995; Visconti, 2009). En referencia a este último punto, algunos autores han relacionado el grado de fosforilación de proteínas en tirosina con el estado de capacitación del espermatozoide de cerdo (Bravo y cols., 2005; Kaneto y cols., 2002; Tardif y cols., 2001). Por otro lado, Harayama y Nakamura demostraron en 2008 que un extenso número de proteínas también se fosforilan en los residuos de serina y treonina durante la capacitación de los espermatozoides de cerdo (Harayama y Nakamura, 2008). Se ha descrito también que para provocar la fusión de la membrana acrosomal externa con la cara interna de la membrana plasmática, durante el paso siguiente a la capacitación, la reacción acrosómica, es necesario que previamente se produzca la despolimerización de la F-actina (Breitbart y cols., 2005; Brener y cols., 2003).

La regulación de los procesos celulares requiere una acción coordinada entre las proteínas quinasas y fosfatasas. No es bien conocida la implicación de las proteínas fosfatasas en la capacitación de los espermatozoides de cerdo, aunque los resultados de algunos estudios sugieren que la proteína fosfatasa 1 (PP1), presente en la región post-acrosomal, participaría en la desfosforilación de las proteínas de esta región, independientemente del tiempo de capacitación de los espermatozoides (Adachi y cols., 2008; Harayama, 2003; Harayama y Nakamura, 2008). Para alcanzar la reacción acrosómica los espermatozoides de cerdo necesitan sufrir un proceso de desfosforilación y en este sentido se les ha atribuido a las fosfoproteínas de la región post-acrosomal un papel en la inhibición de la reacción acrosómica prematura tras la eyaculación (Signorelli y cols., 2012).

En definitiva, todos estos cambios intracelulares en el espermatozoide dan lugar a la aparición de áreas muy inestables, altamente fusogénicas y permeables,

que se han propuesto como zonas de fusión entre la membrana plasmática y la membrana acrosomal externa que permiten que ocurra la reacción acrosómica.

REACCIÓN ACROSÓMICA

La reacción acrosómica es el proceso de exocitosis mediante el cual las enzimas hidrolíticas del acrosoma del espermatozoide capacitado son liberadas hacia el exterior donde degradan la zona pelúcida del ovocito (Breitbart, 2003; Florman y cols., 2008; Roldan y Shi, 2007; Yanagimachi, 1994). Este tipo de exocitosis ocurre mediante la fusión de la membrana acrosomal externa con la cara interna de la membrana plasmática y permite así la inyección del contenido del material genético del espermatozoide hacia el interior del ovocito.

La cascada de eventos intracelulares que ocurre durante la reacción acrosómica en espermatozoides de mamíferos no es del todo conocida, aunque está descrita la implicación de ciertas proteína quinasas como la PKA (Spungin y Breitbart, 1996), la PKC (Naor y Breitbart, 1997) y la PI3K (Etkovitz y cols., 2007; Fisher y cols., 1998; Jungnickel y cols., 2007). Una de las consecuencias más importantes de la pérdida de colesterol de la membrana plasmática durante la capacitación es la entrada masiva de calcio extracelular, que es considerado como un requisito indispensable para que se produzca la reacción acrosómica (Flesch y Gadella, 2000). Evidencias recientes indican que la entrada de Ca^{2+} al interior del espermatozoide de ratón durante la reacción acrosómica estaría mediada principalmente por canales de calcio dependientes de voltaje (VOCCs), los cuales se activan mediante la hiperpolarización de la membrana durante la capacitación (De La Vega-Beltran y cols., 2012). También se ha postulado que en espermatozoides de ratón ciertas glicoproteínas específicas de la zona pelúcida del ovocito, como las ZP3 (Litscher y cols., 2009) y la ZP2 (Gahlay y cols., 2010), inducen de forma fisiológica la reacción acrosómica y facilitan la unión al ovocito. Sin embargo, otros estudios aseguran que la reacción acrosómica de los espermatozoides de ratón se produce antes del contacto con la zona pelúcida (Jin y cols., 2011). Entre los inductores fisiológicos de la reacción acrosómica se encuentra la progesterona, hormona que

induce la entrada de calcio extracelular en espermatozoides humanos (Lishko y cols., 2011).

La reacción acrosómica se puede inducir *in vitro* en espermatozoides previamente capacitados mediante la incubación de los mismos con determinadas sustancias inductoras como la progesterona, factores de crecimiento como el epidérmico (EGF) ó ionóforo de calcio, entre otros (Breitbart y cols., 1997). En este sentido, se ha demostrado que en espermatozoides de cerdo, en los que se induce la reacción acrosómica con progesterona, se observan cambios específicos en la expresión y localización acrosomal de proteínas fosforiladas en residuos de serina (Ramio-Lluch y cols., 2012)

B. PROTEÍNA QUINASA ACTIVADA POR AMP (AMPK)

En los años 70, se identificó la proteína quinasa activada por AMP, también llamada AMPK por sus siglas en inglés, *AMP activated kinase* (Beg y cols., 1973; Carlson y Kim, 1973). Esta proteína, que presenta ortólogos en todas las células eucariotas (Hardie, 2004), está muy conservada desde el punto de vista evolutivo (Hardie y cols., 2006; Sanz, 2008; Witczak y cols., 2008) y actúa como un sensor que detecta y regula el estado energético celular y del organismo (Carling, 2004; Hardie y cols., 2011; Hardie y cols., 2012).

B1) ESTRUCTURA Y EXPRESIÓN DE LA AMPK

AMPK es una proteína heterotrimérica con actividad serina/treonina quinasa que posee una subunidad catalítica α (63 KDa) y dos reguladoras β y γ (Hardie y cols., 2003). La subunidades α y β están codificadas por 2 genes (α1, α2 y β1, β2), mientras que la subunidad γ está codificada por 3 genes (γ1, γ2 y γ3), originando en total 12 combinaciones posibles de la holoenzima, con diferente distribución en los tejidos y localización subcelular. Los complejos AMPK que presentan la isoforma α2 predominan en el corazón, hígado y músculo esquelético, mientras que AMPK α1 se expresa de forma ubicua (Woods y cols., 2000). Además, las isoformas α1 y α2

presentan diferente especificidad por sus proteínas diana. Schimmack y colaboradores sugirieron en 2006 que, debido a estas diferencias entre la localización subcelular y la especificidad por sus sustratos, α1 y α2 podrían desarrollar diferentes funciones (Schimmack y cols., 2006).

La subunidad catalítica α está constituida por dos dominios, uno con actividad quinasa y otro autorregulador; además, esta subunidad posee un residuo de treonina en la posición 172 (Figura 6) cuya fosforilación es imprescindible para la completa actividad de la enzima (Hawley y cols., 1996; Hawley y cols., 2003). La subunidad α se une a las subunidades β y γ por su extremo C-terminal (Crute y cols., 1998). La subunidad β contiene una región de unión a carbohidratos y se ha sugerido que actúa como un detector de las reservas energéticas celulares en forma de glucógeno (McBride y cols., 2009). Finalmente, la subunidad γ está formada por dos motivos *Bateman* (Figura 6), constituidos a su vez por cuatro dominios CBS (cistationina beta sintasa) repetidos en tándem que proporcionan el sitio de unión de los nucleótidos de adenina AMP y ATP (Scott y cols., 2004), lo que sugiere que es realmente la subunidad γ la que detecta el contenido celular de AMP y ATP.

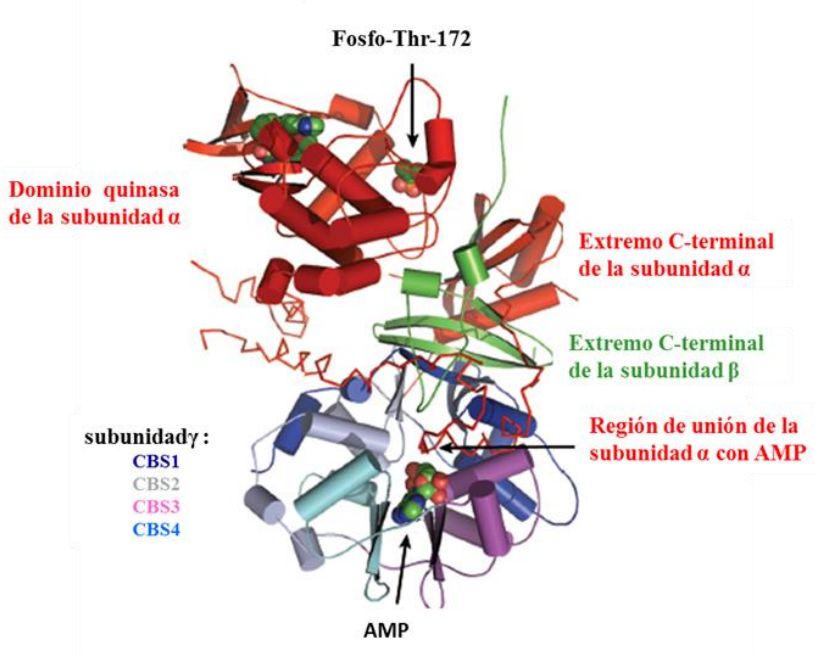


Figura 6: *Estructura de la AMPK. Adaptado de Hardie y Alessi (Hardie y Alessi, 2013).*

La función concreta de cada heterotrímero aún no ha sido definida, pero se ha demostrado que los ratones *knockout* para AMPK α2 desarrollan obesidad y diabetes tipo II (Viollet y cols., 2003), mientras que mutaciones en la subunidad γ dan lugar a enfermedades cardíacas (Scott y cols., 2004).

B2) REGULACIÓN DE LA ACTIVIDAD DE LA AMPK

El ATP es una molécula clave en el metabolismo de los seres vivos, considerada como "la moneda energética celular", ya que libera gran cantidad de energía (7,3 Kcal/mol) cuando se hidrolizan cada uno de sus enlaces fosfoanhidro. Una de las características esenciales de la AMPK como quinasa que detecta el estado energético es su extremada sensibilidad, ya que cualquier descenso en la carga energética celular se acompaña de un aumento en el cociente AMP/ATP de la célula, lo que activa la AMPK (Hardie y Alessi, 2013; Long y Zierath, 2006). La unión de AMP a la subunidad γ activa la AMPK de forma alostérica y también favorece un incremento en su fosforilación en el residuo Thr^{172}, situado en el dominio de activación de la subunidad catalítica α (Figura 7). La combinación de la activación alostérica y el incremento en la fosforilación da lugar a un aumento de la actividad enzimática de más de cien veces (Gowans y cols., 2013; Suter y cols., 2006).

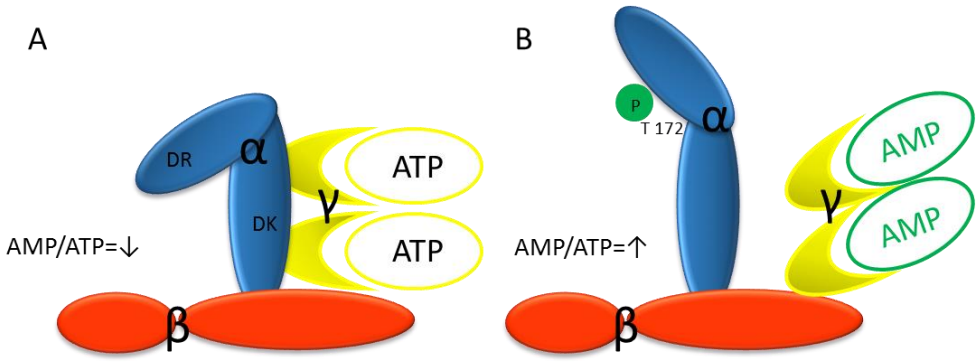


Figura 7: *Activación de la AMPK*. A: El dominio catalítico (DK) de la subunidad α permanece bloqueado cuando los niveles de ATP son normales. B: Cuando éstos disminuyen la subunidad γ sufre una modificación conformacional debida a la unión alostérica de AMP, lo que facilita la fosforilación en el dominio catalítico. Adaptado de Fragoso y Coello (Fragoso Iñiguez y Coello Coutiño, 2008).

Adicionalmente, la unión de AMP a la AMPK inhibe la desfosforilación de la Thr^{172} por las proteínas fosfatasas PP2A y PP2C (Davies y cols., 1995; Gowans y cols., 2013). El hecho de que el AMP active a la AMPK por varios mecanismos (Figura 8) enfatiza la extremada sensibilidad de la quinasa a pequeños cambios en el AMP celular (Hardie y cols., 2003; Hardie y cols., 2006; Long y Zierath, 2006). Estos efectos activadores del AMP son antagonizados por altas concentraciones de ATP que compite con el AMP por la unión a los dominios *Bateman* de la subunidad γ (Hardie y cols., 2006; Witczak y cols., 2008). Estos mecanismos de regulación resaltan el potencial para desarrollar compuestos que activen a la AMPK antagonizando los efectos de los dominios de autoinhibición (Pang y cols., 2008).

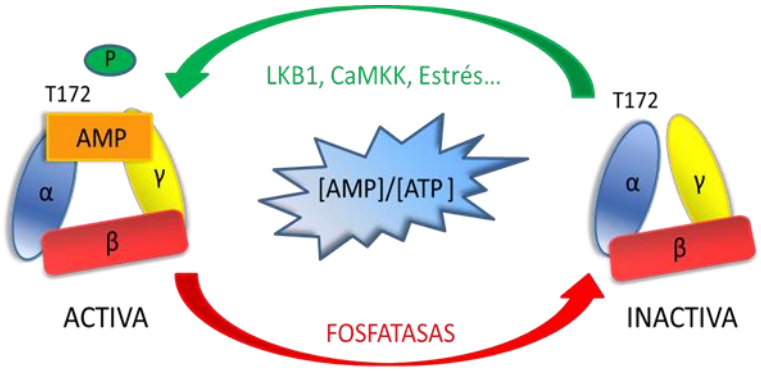


***Figura 8**:* Esquema de la *regulación de la actividad de la AMPK.*

Por otra parte, la actividad enzimática de la AMPK también se induce por varios tipos de estímulos que suponen estrés celular (Figura 8), incluyendo hipoxia, isquemia, estrés oxidativo o estrés hiperosmótico, además del estrés metabólico causado por la ausencia de glucosa (Long y Zierath, 2006). Otros estímulos que activan a la AMPK son ejercicio, drogas antidiabéticas como la metformina (Zhou y cols., 2001), salicilatos (Steinberg y cols., 2013), determinados ligandos extracelulares como factores de crecimiento, citoquinas y hormonas, compuestos naturales (berberina, resveratrol, genisteína y EGCG) y diversos inhibidores metabólicos (que actúan como venenos) entre los que se encuentran, rotenona, cianuro, oligomicina y dinitrofenol.

Hasta el momento se han descrito varias quinasas responsables de la activación de la AMPK por fosforilación en la Thr^{172}. La primera que se describió fue LKB1, proteína de expresión ubicua codificada por un gen supresor de tumores (Hawley y cols., 2003; Shaw y cols., 2004; Woods y cols., 2003), cuyas mutaciones pueden dar lugar a un cáncer hereditario, el síndrome de *Peutz- Jerhers* (Hemminki, 1999). Otras de las enzimas que activan la AMPK por fosforilación son las isoformas α y β de la proteína CaMKK, quinasa de la quinasa dependiente de calcio y de calmodulina (Hardie y cols., 2006; Long y Zierath, 2006). Quinasas como la activada por TGFB, TAK1 (Xie y cols., 2006) o KSR2 (Costanzo-Garvey y cols., 2009) también fosforilan a la AMPK. LKB1 se expresa de forma ubicua, mientras que las CaMKKs se expresan principalmente en el tejido nervioso, y su expresión es menor en otros tejidos como hígado y musculo esquelético (Long y Zierath, 2006), sugiriendo que la ruta de la AMPK estaría regulada por múltiples mecanismos que podrían ser específicos de cada tejido (Birnbaum, 2005).

B3) FUNCIONES METABÓLICAS DE LA AMPK

La consecuencia metabólica general de la activación de AMPK (Figura 9) es el mantenimiento de los almacenes celulares de energía en aquellas condiciones en que los niveles celulares de ATP están comprometidos. En concreto, la forma activa de la AMPK fosforila proteínas que estimulan rutas catabólicas que producen ATP, como la glicólisis y la oxidación de ácidos grasos, mientras que simultáneamente fosforila proteínas implicadas en la inhibición de las rutas anabólicas que consumen energía (Hardie y cols., 2011; Hardie y cols., 2012; Kahn y cols., 2005).

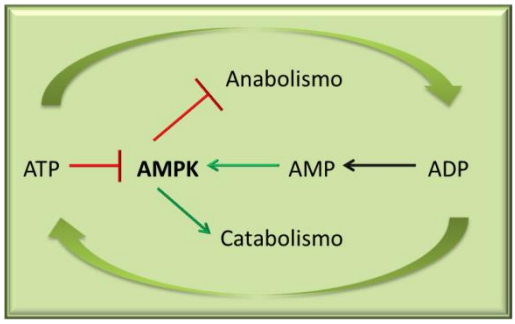


Figura 9: *Esquema general de la regulación del metabolismo por AMPK. En verde se representa activación y en rojo inhibición.*

Todos estos efectos metabólicos llevados a cabo por la AMPK tienen como fin último reducir el gasto de ATP en la célula para de esta forma restablecer los niveles energéticos y favorecer la supervivencia celular. Por este motivo, también se le ha atribuido a la AMPK un papel fundamental en el balance energético del organismo, regulando la ingesta de alimentos a través de circuitos de señalización entre tejidos periféricos y el hipotálamo (Kahn y cols., 2005).

Como se ha mencionado, la activación de la AMPK provoca la inhibición de la síntesis de la mayoría de las macromoléculas presentes en la célula (Figura 10), incluyendo los ácidos grasos, triglicéridos, colesterol, glucógeno, ARN ribosómico y proteínas (Hardie y cols., 2012; Hardie y Alessi, 2013). Los sustratos metabólicos mejor conocidos de la AMPK incluyen la acetil-CoA carboxilasa (Carlson y Kim, 1973) y la hidroximetilglutaril CoA reductasa (Ingebritsen y cols., 1978), que son las enzimas más reguladas de las rutas bioquímicas de la síntesis de ácidos grasos y del colesterol, respectivamente. La AMPK fosforila directamente la acetil-CoA carboxilasa (ACC), tanto la isoforma 1 como la 2, en los residuos de Ser79 y Ser212, respectivamente (Hardie y Pan, 2002). La ACC es la enzima que cataliza la reacción de formación del malonil-CoA a partir de acetil-CoA. El malonil-CoA es un inhibidor alostérico de la carnitina palmitoil transferasa CPT1b (Mills y cols., 1983), la proteína responsable de la entrada de ácidos grasos a la mitocondria para que se produzca la β-oxidación. La fosforilación de la ACC por la AMPK conlleva su inactivación (Hardie y Pan, 2002), que se traduce en una inhibición de la síntesis de ácidos grasos y, por tanto, del malonil-CoA. A su vez, el descenso de los niveles de malonil-CoA provoca que la CPT1b no esté inhibida, lo que conlleva a que se facilite el flujo de ácidos grasos hacia la mitocondria, donde se catabolizarán mediante la β-oxidación.

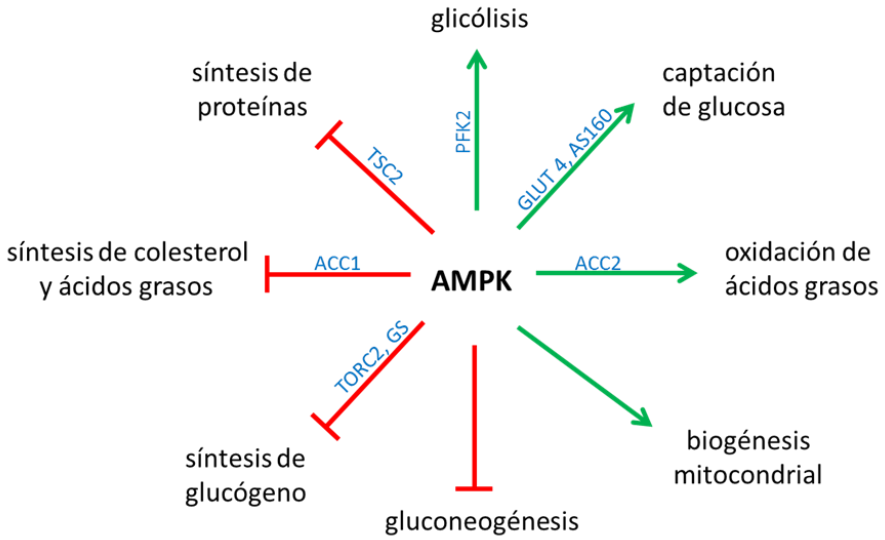


Figura 10: *Rutas metabólicas reguladas por AMPK. En verde se representan los procesos activados y en rojo los inhibidos Adaptado de Hardie (Hardie y cols., 2006).*

Por otro lado, en relación al metabolismo de carbohidratos, la activación de la AMPK conlleva la fosforilación de la glucógeno sintasa en el residuo Ser7 que inhibe su actividad y, por consiguiente, disminuye la síntesis de glucógeno (Carling y Hardie, 1989). Otras proteínas que son sustratos de la AMPK son las fosfofructoquinasa 2 y 3 (PFK2/3), enzimas claves en el metabolismo de glúcidos (Hardie y cols., 2003; Hardie y cols., 2006; Long y Zierath, 2006), cuya fosforilación causa la estimulación de la glicólisis. A su vez, la actividad de la AMPK permite la entrada de la glucosa a la célula a través de su efecto en la familia de transportadores GLUT (Hardie y Alessi, 2013). En resumen, de forma general, la actividad de la AMPK, al estimular la actividad glicolítica y la β-oxidación e inhibir la síntesis de ácidos grasos y de glucógeno, contribuye a aumentar los niveles celulares de ATP, necesarios para los requerimientos energéticos de los diferentes procesos funcionales de la célula.

Aunque los efectos más conocidos de la AMPK, como ya se ha mencionado, se ejercen en la regulación del metabolismo, recientemente se están descubriendo nuevas funciones de esta proteína, como la regulación de la biogénesis mitocondrial (Gowans y cols., 2013).

Debido a la regulación metabólica inducida por la AMPK (Figura 10), esta quinasa ha sido considerada como una diana para el posible tratamiento de algunas enfermedades como son la obesidad, la diabetes mellitus tipo 2 y la esteatosis hepática (Miranda y cols., 2007). En relación a la obesidad, recientemente se ha identificado a la proteína Cidea como posible regulador de la estabilidad de la AMPK en el tejido adiposo (Gong y cols., 2009). En ratones carentes de esta proteína, la cantidad y actividad de AMPK se encuentra aumentada y tienen menos grasa que los ratones controles (Qi y cols., 2008). Otros autores sugieren que la AMPK, al actuar como un punto de control metabólico, sería la molécula que podría de algún modo determinar si las fuentes de energía son suficientes para continuar con el proceso de división celular (Jones y cols., 2005).

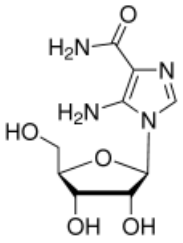
B4) ACTIVADORES E INHIBIDORES DE LA AMPK

Las principales herramientas farmacológicas para estudiar la función de la AMPK incluyen los siguientes activadores e inhibidores de su actividad:

ACTIVADORES

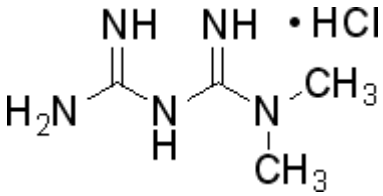
- **AICAR (5-aminoimidazol-4-carboxamida 1-b-D-ribofuranósido)**

  Es transportado al interior celular por el transportador de adenosina y una vez en el citosol es transformado por la adenosina quinasa en ZMP (Witczak y cols., 2008), que es un análogo estructural no metabolizable del AMP y se une al sitio alostérico de la AMPK, provocando así su activación enzimática. AICAR activa a la AMPK a tiempos cortos, ya que a tiempos largos se acumula ZTP que es similar estructuralmente al ATP y, por tanto, como ya se ha mencionado, inhibe a la AMPK. Su fórmula química es la siguiente:



- **METFORMINA**

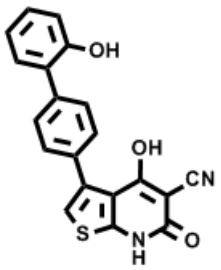
Fármaco que se utiliza desde hace varias décadas en el tratamiento y prevención de la diabetes *mellitus* tipo 2, también conocida como diabetes no dependiente de insulina. La metformina reduce los niveles de glucosa en sangre, principalmente por inhibición de la gluconeogénesis hepática (Hundal y cols., 2002). Esta biguanida no activa a la AMPK por fosforilación directa de la treonina 172 (Hawley y cols., 2002). Su mecanismo de acción no está claro; por un lado, algunos trabajos indican que actúa mediante un aumento en la relación AMP/ATP por la inhibición del complejo I mitocondrial (El-Mir y cols., 2000; Owen y cols., 2000). Por otro lado, otros trabajos indican que la metformina no modifica la relación AMP/ATP y que tampoco se ven afectadas las actividades de LKB1, fosfatasas, ni los niveles de Ca^{2+} intracelular (Witczak y cols., 2008). En este sentido, se ha postulado que la activación de AMPK por metformina podría depender de la presencia de especies reactivas de nitrógeno (RNS), dado que la sobre-expresión de superóxido dismutasa y la inhibición de óxido nítrico sintasa endotelial (eNOS) evitan la activación de AMPK por metformina (Sanz, 2008). Su fórmula química es la siguiente:



- **A769662**

Este compuesto es un potente y selectivo activador de la AMPK (Cool y cols., 2006). La activación de la AMPK inducida por A769662 no está mediada por un aumento en el cociente AMP/ATP, ni tampoco por unión a la subunidad γ, ya que en 2007 Sanders y colaboradores (Sanders y cols., 2007a), demostraron que en ratones mutados en AMPKγ el A769662 continuaba activando a la AMPK. Este compuesto activa alostéricamente la AMPK, a la

vez que inhibe su desfosforilación en la treonina 172, siendo su efecto similar al del AMP (Goransson y cols., 2007). Su fórmula química es la siguiente:

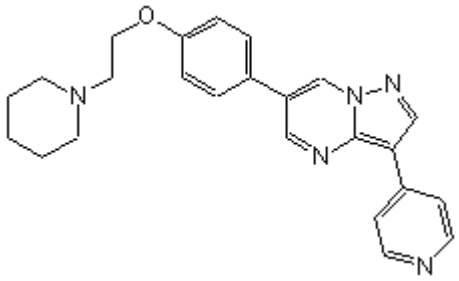


Entre otros activadores de la proteína AMPK se encuentran productos naturales derivados de plantas como la berberina (alcaloide utilizado en la medicina tradicional china), el resveratrol (presente en las uvas y el vino tinto), el galato de epigalocatequina, EGCG (procedente del té verde), la genisteína (polifenol derivado de la soja), la curcumina, etc. Muchos de estos compuestos presentan actividad antidiabética y anticancerígena, aunque su mecanismo de acción no se encuentra esclarecido y sus efectos *in vivo* continúan siendo estudiados en la actualidad.

INHIBIDOR DE LA AMPK

- **6-[4-(2-piperidin-1-il-etoxi)-fenil)]-3-piridin-4-il-pirazol[1,5-α]-pirimidina ó Compuesto C**

  También llamado dorsomorfina, es el inhibidor farmacológico de la AMPK más utilizado. Es una pirrazolopirimidina que actúa como un potente, selectivo y reversible inhibidor de la AMPK que compite con el ATP (K_i =109 nM en presencia de 5 µM de ATP y en ausencia de AMP). No afecta las actividades de las siguientes quinasas: ZAPK, Syk, PKCθ, PKA, o JAK3 (Zhou y cols., 2001). Se ha demostrado que bloquea las actividades celulares inducidas por AICAR o metformina (Fryer y cols., 2002). Su fórmula química es la siguiente:



C. AMPK Y ESPERMATOZOIDES

Los estudios realizados acerca de la AMPK se han centrado en células somáticas, no existiendo en el momento de plantear esta Tesis Doctoral ningún trabajo que estudiara su posible función en la célula germinal masculina de mamíferos. Entre 2007 y 2008 se publican tres trabajos científicos sobre quinasas relacionadas con la AMPK y su posible papel en espermatozoides. Así, en 2008 se describió que una nueva isoforma más corta de LKB1, quinasa que fosforila y activa AMPK, denominada LKB1s, se expresa de forma predominante en las células espermátidas haploides de los testículos de mamíferos (Towler y cols., 2008). Estos autores describieron que los ratones *knockout* de LKB1s resultan estériles al presentar una dramática reducción en el número de espermatozoides maduros en el epidídimo, y los pocos que se producen, además de no ser móviles, presentan una morfología anómala de la cabeza. Estos resultados sugieren que esta variante más corta de la quinasa que activa AMPK, LKB1s, tiene un papel crucial en la espermiogénesis y en la fertilidad en ratones. Por otro lado, se ha descrito en espermatozoides humanos la expresión de TSSK2, TSKS y SSTK, miembros de la familia de serina/treonina quinasa específicas de testículos (TSSK) que en el árbol del quinoma humano pertenece a la rama de la AMPK (Xu y cols., 2007). Se ha mostrado que la deleción de TSSK1 y 2 causa infertilidad masculina en ratones quimera debido a una haploinsuficiencia (Xu y cols., 2008).

Como ya se ha mencionado, la proteína AMPK se activa en células somáticas en respuesta a cambios en la carga energética (AMP/ATP) y también en la concentración intracelular de Ca^{2+}. Es interesante mencionar que estas dos vías de

regulación de la AMPK son esenciales en los espermatozoides de mamíferos y se han relacionado entre ellas en espermatozoides humanos y de ratón, ya que Baker y colaboradores han demostrado que el calcio extracelular provoca un descenso de los niveles intracelulares de ATP a corto plazo. Estos autores proponen un modelo en el que los niveles de ATP tienen un papel regulador clave de la señalización que acompaña a los procesos fisiológicos que ocurren *in vivo* en espermatozoides humanos (Baker y cols., 2004a).

En 2012, durante la última fase de realización de esta Tesis Doctoral, cuando una parte de los resultados de esta Tesis ya estaban publicados, Tartarin y colaboradores demostraron que los espermatozoides de ratones *knockout* para la subunidad α1 de AMPK presentan más morfoanomalías y son menos mótiles. Además, estas alteraciones se relacionan con un descenso del 50% en la actividad mitocondrial y una disminución del 60% en el consumo basal de oxígeno. Sin embargo, no observaron diferencias en la morfología testicular, ni en la producción espermática de estos ratones.

Todas las funciones del espermatozoide (supervivencia, capacitación, reacción acrosómica, movilidad) están reguladas por la interacción de los espermatozoides con los factores presentes en el medio, por la carga energética celular y por la activación de vías de señalización intracelular. La hipótesis de la presente Tesis Doctoral plantea que entre esas vías que regulan la función del espermatozoide y son dependientes del nivel de energía, estaría la AMPK. Es importante señalar el hecho de que el espermatozoide sufre diferentes tipos de estrés, como el oxidativo y el osmótico, así como incrementos en la concentración intracelular de calcio, que preceden a la fecundación del ovocito. Estos procesos acontecen durante el paso del espermatozoide por el tracto genital femenino, o también durante el proceso de conservación de estas células germinales, mediante refrigeración o congelación, previa a su uso en la inseminación artificial (Gogol y Pieszka, 2008; Rath y cols., 2009).

Objetivos

Basándonos en los antecedentes bibliográficos anteriormente expuestos en esta memoria, nos propusimos los siguientes objetivos en la presente Tesis Doctoral:

1. Estudiar la expresión y localización celular de la proteína AMPK y de su forma activa en la célula germinal masculina de cerdo.

2. Estudiar, en el espermatozoide de cerdo, la regulación de la actividad de la AMPK en diferentes condiciones extracelulares y las vías de señalización intracelular implicadas.

3. Estudiar el posible papel de la proteína AMPK en diferentes parámetros y procesos funcionales del espermatozoide que le capacitan para llevar a cabo la fecundación: movilidad, viabilidad, organización lipídica de la membrana plasmática, integridad de la membrana externa del acrosoma, así como el potencial de membrana mitocondrial.

Materiales y Métodos

MATERIALES

- 1,1-Dimetil biguanida hidrocloruro (Metformina). Sigma-Aldrich.
- 2-Mercaptoetanol. Merck Millipore.
- 6-[4-(2-piperidin-1-il-etoxi)-fenil)]-3-piridin-4-il-pirazolo[1,5-α]-pirimidina ó Compuesto C. Sigma-Aldrich.
- 8 Br-cAMP. Sigma-Aldrich.
- A769662. Tocris.
- Ácido desoxicólico. Sigma-Aldrich.
- Ácido etilen diamino tetraacético ACS (EDTA). Sigma-Aldrich.
- Ácido etilen glicol-bis diamino tetraacético (EGTA). Sigma-Aldrich.
- Acrilamila/bisacrilamida 29:1. Bio-Rad.
- Albúmina sérica bovina (BSA). Sigma-Aldrich.
- Alexa Fluor 647 y Alexa Fluor 488. Life Technologies.
- Aminoimidazol carboxamida ribonucleótido. (AICAR). Sigma-Aldrich.
- AnexinaV-FICT. Immunostep.
- Anticuerpo anti- AMPKα. Cell Signaling.
- Anticuerpo anti-fosfo- Thr 172 AMPK. Santa Cruz Biotechnology.
- Anticuerpo anti- GSK3β. Cell Signaling.
- Anticuerpo secundario anti-conejo. Pierce.
- Azul de bromofenol. Bio-Rad.
- BAPTA-AM. Sigma-Aldrich.
- DAPI. Life Technologies.
- Dimetil sulfóxido (DMSO). Sigma-Aldrich.
- Dodecil sulfato sódico (SDS). Bio-Rad.
- Glicerol. Sigma-Aldrich.
- H89. Sigma-Aldrich.
- IBMX. Sigma-Aldrich.
- Ioduro de propidio. Molecular Probes.
- Ionóforo. A23187. Sigma-Aldrich.

- JC-1. Life Technologies.
- Líquidos de revelado: Revelador y Fijador Kodak. Sigma-Aldrich.
- LY29400. Cayman Chemical Company.
- Marcadores de peso molecular de proteínas. Invitrogen.
- Membranas de nitrocelulosa 45 µm. Whatman Protran.
- Merocianina M540. Molecular Probes.
- Mezcla de inhibidores de proteasas. Roche.
- Película fotosensible. Amersham.
- Persulfato amónico (APS). Bio-Rad.
- PMA. Sigma-Aldrich.
- PNA-FICT. Sigma-Aldrich.
- Ro-32-0432. Calbiochem.
- Soluciones de quimioluminiscencia: sustrato de la peroxidasa y solución amplificadora de la señal. Thermo Scientific.
- STO-609. Sigma-Aldrich.
- SYBR-14. Molecular Probes.
- TEMED. Bio-Rad.
- Tris/Glicina (10 veces concentrado). Bio-Rad.
- Tris/Glicina /SDS (10 veces concentrado). Bio-Rad.
- Triton X-100. Sigma-Aldrich.
- Tween 20. Bio-Rad.
- YoPro-1. Molecular Probes.

OBTENCIÓN DE MUESTRAS

Todos los experimentos incluidos en esta Tesis se realizaron utilizando eyaculados de cerdos de raza Duroc, procedentes de la empresa Tecnogenext S.L., Arroyo de San Serván (Badajoz, España). Los animales, de edades comprendidas entre los 2 y 4 años y de fertilidad probada, se mantuvieron bajo condiciones controladas de temperatura, luz y humedad, según la normativa regional relativa al bienestar animal.

La extracción del semen se realizó mediante la técnica de la mano enguantada. Sólo aquellos eyaculados con al menos un 80% de espermatozoides con morfología normal, un 70 % de espermatozoides mótiles y un número de espermatozoides totales superior a 1.000 x 10^6 fueron utilizados para la realización de los ensayos experimentales. Los eyaculados, diluidos 1:1 en un volumen adecuado de diluyente comercial (BTS), se recogieron a primera hora de la mañana y se trasportaron a la Facultad de Veterinaria de Cáceres (45 min. aprox.) en un contenedor para dosis (Climate box, IMV Technologies, Humeco, Huesca, España) dotado de un sistema de refrigeración controlado digitalmente por un termostato a una temperatura de 22°C y en condiciones de oscuridad.

Una vez en el laboratorio, las muestras se mantuvieron a una temperatura de 17°C y en oscuridad durante un tiempo máximo de 48 horas.

Para la realización de esta Tesis se han utilizado eyaculados procedentes de 19 animales.

MÉTODOS

1) TRATAMIENTO DE LAS MUESTRAS

La variabilidad interracial fue obviada, al utilizar siempre muestras procedentes de cerdos de raza Duroc. Sin embargo, para minimizar la variabilidad existente entre el semen de los diferentes animales (variabilidad interindividual) se mezclaron eyaculados de tres machos distintos para cada experimento. Una vez realizada la mezcla, se procedió al lavado de las muestras. Para ello, se centrifugaron dos veces a 2.130 *g* durante 4 minutos en tampón fosfato salino (PBS) atemperado a temperatura ambiente (composición Tabla 1). Tras cada centrifugación, se desechó el sobrenadante y el precipitado se resuspendió en PBS.

Reactivos	Molaridad
NaCl	136,9 mM
KCl	2,70 mM
Na_2HPO_4	5,62 mM
NaH_2PO_4	1,09 mM
KH_2PO_4	1,47 mM

Tabla 1: *Tampón Fosfato Salino (PBS). El pH se ajustó a 7,4*

Posteriormente se evaluó la concentración de espermatozoides en la muestra mediante el sistema computerizado CASA (ISAS®, Proiser R+D, Paterna, Valencia, España) y se realizaron los correspondientes cálculos para obtener una concentración final de 120 x 10^6 espermatozoides/mL, en el medio de elección de cada experimento, que consistió en un medio basal (TBM) que no incluye ni calcio ni bicarbonato, o en un medio con estímulos capacitantes (TCM) (composición en Tablas 3 y 4). Ambos medios de incubación (TBM y TCM) fueron preparados en el día de su uso con una osmolaridad de 290-310 mOsm kg^{-1} y ajustados a pH 7,4.

Reactivos	Molaridad
HEPES	20 mM
NaH_2PO_4	0,3 mM
NaCl	96 mM
KCl	4,7 mM
$MgSO_4$	0,4 mM
Glucosa	5,5 mM
Piruvato.Na	1 mM
Lactato.Na	21,5 mM

Tabla 2: *Tyrode's Medium (TM)*

Reactivos	Molaridad
Tyrode's Medium	
NaCl	10 mM
EGTA	5 mM
BSA	0,3% w/v

Tabla 3: *Medio TBM (Tyrode´s Basal Medium)*

Reactivos	Molaridad
Tyrode's Medium	
$NaHCO_3$	15mM
$CaCl_2$	1 mM
BSA	0,3% w/v

Tabla 4: *Medio TCM (Tyrode´s Complete Medium)*

Para estudiar la función de la AMPK en la regulación de la fisiología de los espermatozoides de cerdo se utilizaron inicialmente varios activadores comerciales de la misma, en concreto, A769662, AICAR y metformina y un inhibidor, el compuesto C (Dorsomorfina).

Para investigar las rutas de señalización intracelular involucradas en la regulación de la AMPK, se utilizaron los siguientes compuestos:

- IBMX: Inhibidor de las fosfodiesterasas.
- PMA: Activador de la proteína quinasa C (PKC).
- RO-32-0432: Inhibidor de la PKC.
- STO-609: Inhibidor de las CaMKKα/β.
- LY29400: Inhibidor de la PI3K.
- 8Br-cAMP: Análogo no hidrolizable del cAMP y por tanto, activador de la proteína quinasa A (PKA).
- H89: Inhibidor de la PKA.
- BAPTA-AM: Quelante de calcio intracelular unido a un residuo acetometil para poder atravesar la membrana plasmática.

- $CaCl_2$: En aquellos experimentos en los que se estudió el efecto del calcio 5mM en la regulación de la AMPK, se empleó un TBM sin EGTA.
- Determinados compuestos inductores de estrés celular como rotenona y cianuro para inhibir la actividad mitocondrial, y sorbitol para provocar estrés hiperosmótico.

Los espermatozoides se incubaron en las distintas condiciones experimentales a 38,5°C con 5% de CO_2 y en oscuridad durante un tiempo máximo de 24 horas en un incubador (Mini Galaxy A, RS Biotech). Las muestras fueron analizadas a los tiempos diseñados experimentalmente, mediante los siguientes equipos o técnicas: el sistema CASA, citometría de flujo, western blotting ó inmunocitoquímica. Paralelamente, se tomaron muestras de los espermatozoides en los mismos medios de incubación a temperatura ambiente ó 17°C para evaluar el estado de los espermatozoides al inicio de experimento y poder conocer el efecto de otra variable, la temperatura.

2) WESTERN BLOTTING

Se empleó esta técnica para identificar la proteína AMPK y su forma activa, fosfo-Thr^{172}-AMPK, en lisados celulares de espermatozoides de cerdo, así como para realizar los correspondientes controles de carga evaluando la proteína GSK3β. Su realización se detalla a continuación:

Obtención de lisados celulares

Tras la incubación de 1,5mL de espermatozoides (120 x 10^6 espermatozoides/mL) en TBM o TCM con los diferentes tratamientos y a los distintos tiempos de acuerdo al diseño experimental, la suspensión celular se centrifugó durante 20 segundos a 7.000*g* en una minicentrífuga (Eppendorf, AG 22331, Hamburgo, Alemania), se retiró el sobrenadante y el precipitado se resuspendió en 1 mL de PBS con ortovanadato sódico 0,2mM a 4°C. A continuación

las muestras fueron nuevamente centrifugadas, se aspiró el sobrenadante y al precipitado celular se le añadió tampón de lisis a 4°C (Tabla 5).

Reactivos	Concentración
Tris/HCl (pH 7,5)	50 mM
NaCl	150 mM
Tritón X-100	1% v/v
Desoxicolato	1% w/v
EGTA	1 mM
EDTA	0,4 mM
Na_3VO_4	0,2 mM
PMSF	1,0 mM
Mezcla de inhibidores de proteasas	

Tabla 5: *Composición del tampón de lisis. El pH se ajustó a 7,4*

Para conseguir una eficaz solubilización de las proteínas del espermatozoide, se utilizaron dos detergentes en el tampón de lisis: un detergente no iónico (Tritón X-100) y un detergente iónico (desoxicolato sódico). Posteriormente, la suspensión celular se sonicó durante 5 segundos y se mantuvo a 4°C y en agitación constante durante un tiempo mínimo de 20 minutos, para facilitar la acción de los detergentes.

Pasado este tiempo, las muestras se centrifugaron a 10.000 *g* durante 15 minutos a 4°C, con la finalidad de separar la fracción celular insoluble de la soluble. La fracción insoluble, compuesta principalmente por el citoesqueleto y proteínas insolubles asociadas a éste, se desechó, mientras que la fracción que contiene las proteínas solubles en el sobrenadante se utilizó en los estudios posteriores.

Determinación de la concentración de proteínas

Para cuantificar la concentración de las proteínas presentes en los lisados celulares se utilizó el ensayo de Bradford, basado en la propiedad del colorante azul brillante de Coomassie G-250 de experimentar un cambio en su máximo de absorción a 595 nm cuando se une a los aminoácidos arginina y lisina de las proteínas. La intensidad de color azul obtenida tras la unión del colorante a estos

aminoácidos es proporcional a la cantidad de proteínas presentes en la muestra, según lo postulado en la ley de Lambert Beer.

La medida de la concentración de proteínas se realizó en un lector de placas (Asys-UVM 340, Biochrom, Cambridge, Inglaterra) a 595 nm y como estándar para realizar la recta patrón se utilizó albúmina sérica bovina (BSA) en el rango 1-10µg.

Electroforesis de proteínas en geles de poliacrilamida SDS PAGE

Se utilizó la electroforesis del tipo SDS-PAGE (electroforesis en gel de poliacrilamida en presencia de dodecil sulfato sódico, SDS) El SDS provoca la desnaturalización de las proteínas al conferirles una carga neta negativa, de tal forma que, en presencia de un campo eléctrico, su movilidad electroforética depende de su peso molecular y no de su carga.

- ✓ Preparación de las muestras:

Se añadió tampón de carga o de Laemmli 4X (Tabla 6) a los lisados celulares y posteriormente se calentaron a 95°C durante 5 minutos. Una vez desnaturalizadas las proteínas por calentamiento, las muestras se centrifugaron a 7.000 g durante 1 minuto.

Reactivos	Concentración
Tris/HCl (pH 6.8)	**0,4 M**
DTT (ditiotreitol)	**0,4 M**
SDS	**8,0% w/v**
Glicerol	**20% v/v**
Azul de bromofenol	**0,02% w/v**
2-mercaptoetanol	**5% v/v**

Tabla 6: *Composición del tampón de carga 4X*

- ✓ Preparación de los geles de poliacrilamida:

Los geles de poliacrilamida se prepararon en el interior de unos soportes comerciales de plástico (casetes de 1,0mm, Invitrogen). En primer lugar se preparó el gel separador con un porcentaje de acrilamida del 10% y pH 8,8, mientras que en

la parte superior, el gel acumulador se preparó con un porcentaje de acrilamida del 4% y pH 6,8 (Tabla 7).

Reactivos	Gel 4%	Gel 10%
Acrilamida/Bisacrilamida	4% v/v	10% v/v
Tris/HCl	375 mM	375 mM
TEMED	0,1% v/v	0,05% v/v
APS (persulfato amónico)	0,05% v/v	0,05% v/v
SDS	0,05% v/v	0,05% v/v

Tabla 7: *Composición de los geles de poliacrilamida*

Una vez polimerizados los geles, los casetes se dispusieron en una cubeta de electroforesis (Xcell II™ MiniCell de Invitrogen) y se añadió el tampón de electroforesis a la cubeta (Tabla 8). A continuación se cargaron las muestras en los pocillos del gel, dejando al menos un carril para el estándar comercial de marcadores de peso molecular de proteínas. Una vez cargadas las muestras en el gel, se les aplicó un campo eléctrico constante de 90 mV en el gel acumulador y de 140 mV en el gel separador utilizando la fuente PowerPac HC Power Supply de Bio-Rad.

Reactivos	Concentración
TrisHCl (pH 8,3)	2,5 mM
Glicina	19,2 mM
SDS	0,01% w/v

Tabla 8*: Composición del tampón de electroforesis*

- ✓ Electrotransferencia en húmedo de proteínas a una membrana:

Mediante la aplicación de un campo eléctrico, las proteínas del gel se transfirieron a una membrana de nitrocelulosa con un tamaño de poro de 45µm embebidas en el tampón de transferencia (transferencia en húmedo). Previamente las membranas se equilibraron en tampón de transferencia (Tabla 9).

Reactivos	Concentración
TrisHCl (pH 8,3)	2,5 mM
Glicina	19,2 mM
Metanol	20% v/v

Tabla 9: *Composición del tampón de transferencia*

Para la preparación del "sándwich" se colocaron, sobre un casete de plástico, una esponja, papel de filtro, el gel de poliacrilamida, la membrana de nitrocelulosa, papel de filtro y, por último, otra esponja según se indica en el siguiente esquema:

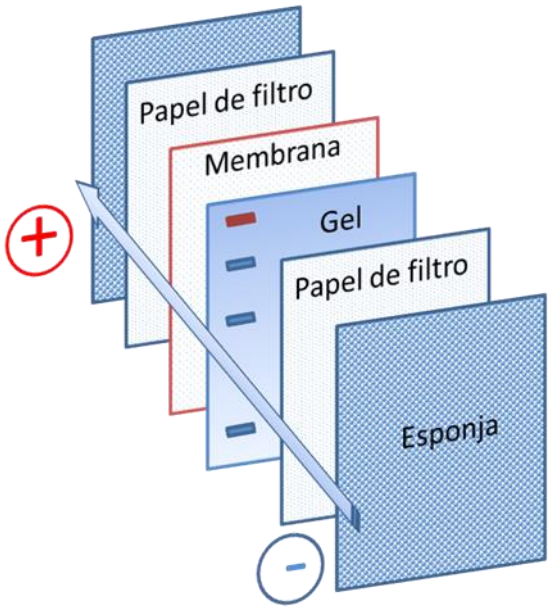


Figura 11: *Disposición de los componentes del sándwich durante la electotransferencia*

Se introdujo el sándwich en la cubeta de transferencia (Migthy Small Transphor, de Hoefer). Se rellenó la cubeta con tampón de transferencia y se colocaron los electrodos de tal manera que el gel quedó orientado hacia el ánodo (+) y la membrana hacia el cátodo (-). La electrotransferencia tuvo lugar durante 2,5 horas a 380 mA, condiciones optimizadas previamente por el grupo de investigación SINTREP para las proteínas de estudio en esta Tesis Doctoral.

✓ Western blotting o Inmunoblotting de proteínas:

Para detectar la proteína AMPK y su forma fosforilada p-Thr^{172}-AMPK en la membrana de nitrocelulosa mediante Western blotting, se realizaron los siguientes pasos:

1. *Bloqueo de la membrana*: Antes de proceder a la incubación con el anticuerpo primario, la membrana de nitrocelulosa se incubó durante 1 hora a temperatura ambiente y en agitación constante con tampón de bloqueo (Tabla 10), el cual contiene una elevada concentración de proteínas y cuya función es impedir las uniones inespecíficas del anticuerpo a la membrana.

Reactivos	Concentración
Tris-HCl (pH 8,0)	**50 mM**
$CaCl_2$	**2 mM**
NaCl	**80 mM**
Tween 20	**0,05% v/v**
Leche desnatada en polvo	**5% w/v**

***Tabla 10**: Composición del tampón de bloqueo*

2. *Incubación de la membrana con el anticuerpo primario:* El anticuerpo reconoce un epítopo de la proteína objeto de estudio, uniéndose a ella de forma específica. En concreto, el anticuerpo utilizado anti-AMPK reconoce una secuencia del extremo amino terminal de la subunidad catalítica α de la AMPK y según la casa comercial, es capaz de interaccionar específicamente con sus dos isoformas descritas α1 y α2, sin unirse a las subunidades reguladoras β y γ. El otro anticuerpo utilizado, reconoce específicamente la forma activa de la AMPK fosforilada en el residuo Thr 172 (anti-p-Thr^{172}-AMPK) y se utilizó para detectar de forma indirecta el grado de actividad enzimática de la AMPK. El anticuerpo primario anti-AMPK α se diluyó (1:1000) en tampón de lavado con BSA (Tabla 11) y se incubó con la membrana y en agitación durante toda la noche a 4°C.

Reactivos	Concentración
Tris-HCl pH 8,0	50 mM
$CaCl_2$	2 mM
NaCl	80 mM
Tween 20	0,05% v/v
BSA	5% w/v

***Tabla 11**: Composición del tampón de dilución del anticuerpo primario.*

Los anticuerpos primarios anti-p-Thr^{172}-AMPK y GSK3β se diluyeron en tampón de bloqueo (1:500 y 1:2000, respectivamente). Para detectar la forma activa de AMPK la incubación se llevó a cabo durante toda la noche a 4°C, mientras que para GSK3β las condiciones óptimas fueron dos horas a temperatura ambiente.

3. *Incubación de la membrana con el anticuerpo secundario*. Antes de proceder a la incubación con el anticuerpo secundario, las membranas se lavaron 3 veces durante 5 minutos y en agitación con tampón de bloqueo con el objetivo de eliminar el exceso de anticuerpo primario. El anticuerpo secundario utilizado fue un anti-conejo conjugado con la peroxidasa de rábano picante (HRP) diluido 1:15000 en tampón de bloqueo y se incubó en agitación durante 45 minutos a temperatura ambiente. Tras la incubación con el anticuerpo secundario, se procedió a lavar las membranas 2 veces con tampón de bloqueo (Tabla 10) y otras 2 con tampón de lavado (Tabla 12) durante 5 minutos.

Reactivos	Concentración
Tris-HCl pH 8,0	50 mM
$CaCl_2$	2 mM
NaCl	80 mM
Tween 20	0,05% (v/v)

***Tabla 12**: Composición del tampón de lavado*

4. *Detección de la señal por quimioluminiscencia (Revelado):* Para llevar a cabo la visualización de la señal, provocada por la unión de los anticuerpos primario y secundario, la membrana se incubó durante 5 minutos con una solución 1:1 formada por el sustrato de la peroxidasa, peróxido de

hidrogeno (Supersignal West Pico Stable Peroxide Solution, Thermo Scientific®, USA) y una solución potenciadora de la señal que incluye luminol (Supersignal West Pico Luminol/Enhancer Solution, Thermo Scientific®, USA). Cuando este sustrato entra en contacto con la enzima peroxidasa HRP, que está unida al anticuerpo secundario, produce una reacción de quimiolumiscencia fácilmente detectable tras exponer la membrana a una película fotosensible (Figura 12). El tiempo de exposición de la película dependió de la señal generada por la unión de cada uno de los anticuerpos primarios utilizados. Una vez expuesta la película se procedió al revelado fotográfico de la misma en una cámara oscura.

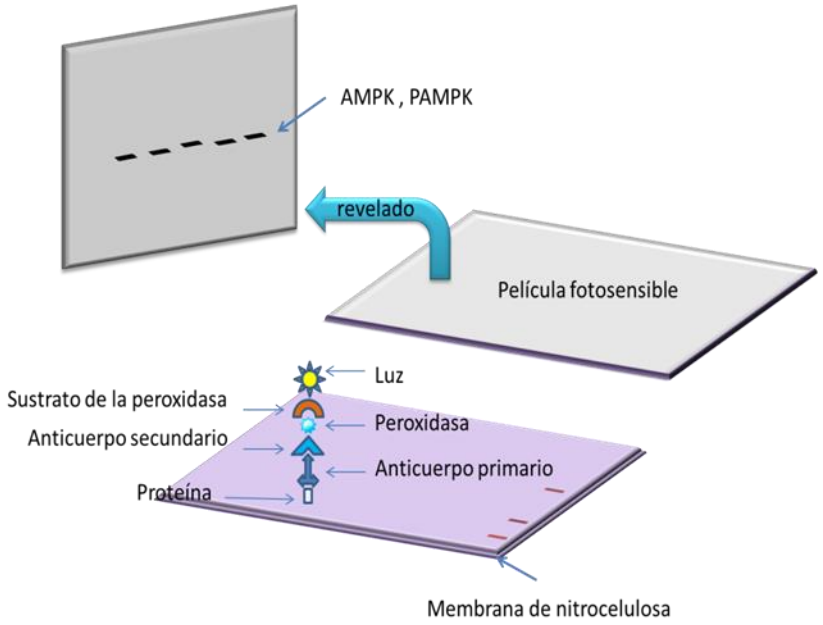


Figura 12: *Esquema de los pasos realizados en la técnica de Western Blotting para el estudio de AMPK y su forma activa p-Thr^{172}-AMPK.*

5. *Análisis de la señal:* una vez escaneada la señal obtenida de la película fotosensible ésta se cuantificó mediante el programa FUJIFILM BAS V2.2 para Macintosh.

3) INMUNOCITOQUÍMICA

Esta técnica nos permitió la identificación y localización subcelular de la AMPK y su forma fosforilada en el espermatozoide. Los pasos realizados fueron los siguientes:

Tras el tratamiento correspondiente, 40µL de cada muestra se fijaron en PBS pH 7,4 con paraformaldehido al 4% durante 20 minutos a temperatura ambiente, sobre un portaobjetos previamente tratado con poli-lisina.

Después de lavar tres veces con PBS durante 5 minutos, se permeabilizaron los espermatozoides con PBS más Triton al 0,25% (v/v) durante 10 minutos. Tras la permeabilización, las células se lavaron tres veces con PBS durante 5 minutos. Posteriormente se bloquearon las uniones inespecíficas 30 minutos con PBS más BSA al 1% (w/v) y 1% Tween 20 (v/v).

La incubación con los anticuerpos primarios (AMPKα 1:100 ó p-Thr^{172}-AMPK 1:50 diluido en tampón de bloqueo) se realizó en cámara húmeda a 4°C toda la noche.

Al día siguiente se lavaron los portaobjetos tres veces con PBST (PBS con Tween 20 al 1% v/v) y se incubaron con el anticuerpo secundario anti-conejo ligado a la sonda fluorescente Alexa Fluor 488 ó Alexa Fluor 647 diluido 1:200 en tampón de bloqueo durante una hora en la oscuridad. A partir de este momento todos los pasos se hicieron en oscuridad para optimizar la fluorescencia del fluorocromo.

Por último, se procedió a lavar de nuevo los portaobjetos 3 veces durante 5 minutos y se añadieron 5µL de medio de montaje con DAPI, fluorocromo que tiñe de azul los núcleos de los espermatozoides y que se encuentra en una solución que previene la pérdida de fluorescencia a corto plazo.

Una vez retirado el exceso de líquido, se colocó un cubreobjetos y se selló por los extremos con laca de uñas sin color. Los portaobjetos se guardaron en la oscuridad a 4°C hasta su observación al microscopio.

En paralelo se realizaron controles negativos, en los que las muestras fueron sometidas a todos los pasos descritos con anterioridad a excepción de la incubación con el anticuerpo primario.

Las imágenes se obtuvieron con un microscopio de fluorescencia confocal (Fluoview FV1000, Olympus, Tokyo, Japan). El programa informático utilizado para el procesado de las imágenes fue Leica Application Suite Advanced Fluorescence (LAS AF) (Leica Microsistemas S.L.U., Barcelona, España).

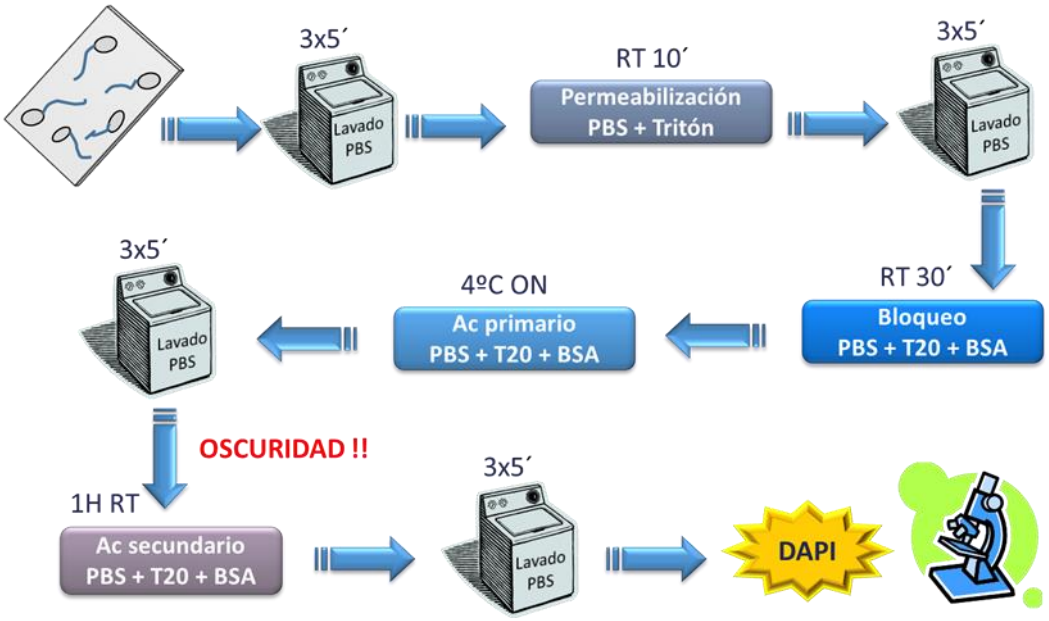


Figura 13: *Esquema de los pasos realizados en la inmunocitoquímica.*

4) ESTUDIO DE LA MOVILIDAD DE LOS ESPERMATOZOIDES

Se utilizó el Sistema Computerizado de Análisis Seminal (C.A.S.A.) para estudiar, de forma objetiva, los diferentes parámetros que caracterizan la movilidad de los espermatozoides de cerdo en cada uno de los diferentes tratamientos experimentales realizados.

El Sistema Computerizado de Análisis Seminal (C.A.S.A.) utilizado fue el *"Integrated Semen Analysis System"* (ISAS®, Proiser R+D S. L. Valencia, España). Este sistema consta de una cámara de vídeo BASLER de alta resolución conectada a un microscopio (Nikon®, Japón) dotado con un objetivo de contraste de fases (10X). La cámara, a su vez, está conectada a un ordenador, el cual tiene instalado el software de análisis seminal. Para el análisis de movilidad, las muestras de semen fueron diluidas de manera homogénea en el correspondiente medio de incubación a una concentración final de unos 50 x 10^6 espermatozoides/mL, concentración optimizada previamente en nuestro grupo de investigación SINTREP.

El análisis de movilidad se realizó en cámaras Leja® (Luzernestraat, Holanda), las cuales se mantuvieron atemperadas a 38,5°C mediante una platina térmica adaptada al microscopio, con el fin de realizar el análisis a la temperatura de incubación de las muestras. Igualmente se atemperaron a 38,5°C las puntas de pipeta utilizadas para tomar las muestras, mediante la platina térmica anexa.

El análisis de movilidad de los espermatozoides se realizó tras capturar al menos cuatro campos en cada uno de los cuales se examinaron 25 imágenes digitalizadas consecutivas, obtenidas a una velocidad de 40 milisegundos cada una. Por cada muestra seminal se analizaron al menos 300 espermatozoides.

El número de elementos incorrectamente identificados como espermatozoides por el programa informático, así como las trayectorias erróneas se corrigieron tras analizar posteriormente cada una de las secuencias de vídeo pregrabadas. Respecto a los diferentes parámetros o patrones de movimiento de

los espermatozoides analizados por el programa informático, se resumen a continuación los más relevantes en nuestro estudio:

PARÁMETRO	UNIDAD	DEFINICIÓN
VELOCIDAD RECTILÍNEA (VSL)	µm/s	Distancia recorrida por el espermatozoide desde el primer punto hasta el último de su trayectoria
VELOCIDAD CURVILÍNEA (VCL)	µm/s	Distancia recorrida por el espermatozoide a lo largo de su trayectoria real en función del tiempo.
VELOCIDAD PROMEDIO (VAP)	µm/s	Distancia recorrida por el espermatozoide a lo largo de su trayectoria media en función del tiempo
ÍNDICE DE LINEALIDAD (LIN)	%	Relación porcentual entre la VSL y la VCL
ÍNDICE DE RECTITUD (STR)	%	Relación porcentual entre la VSL y la VAP
ÍNDICE DE OSCILACIÓN (WOB)	%	Relación porcentual entre la VAP y la VCL
AMPLITUD DEL DESPLAZAMIENTO LATERAL DE LA CABEZA (ALH)	µm	Desplazamiento medio efectuado por la cabeza del espermatozoide en su trayectoria curvilínea de un lado a otro de la trayectoria media o lineal
FRECUENCIA DE BATIDO DE LA COLA (BCF)	Hz	Frecuencia con la cual la trayectoria curvilínea atraviesa la lineal o media en función del tiempo
E. MÓTILES PROGRESIVOS (MP)	%	Porcentaje de espermatozoides con un valor STR>80%
HIPERACTIVIDAD	%	Porcentaje de espermatozoides que cumplen los siguientes parámetros: VCL≥95 µm/s, LIN≤30%, ALH>3,5µm y WOB≤ 70%

El criterio para definir las características de la velocidad espermática analizada mediante el sistema informático fue el siguiente: espermatozoides estáticos (velocidad promedio VAP < 10µm/s), espermatozoides mótiles lentos (VAP entre 10 y 15µm/s), espermatozoides con movilidad media (VAP entre 15 y 80µm/s) y espermatozoides mótiles rápidos (VAP>80µm/s).

A continuación se indican en la Figura 14 los diferentes parámetros de movilidad obtenidos tomando como referencia la distancia recorrida por el espermatozoide a lo largo de su trayectoria real (VCL).

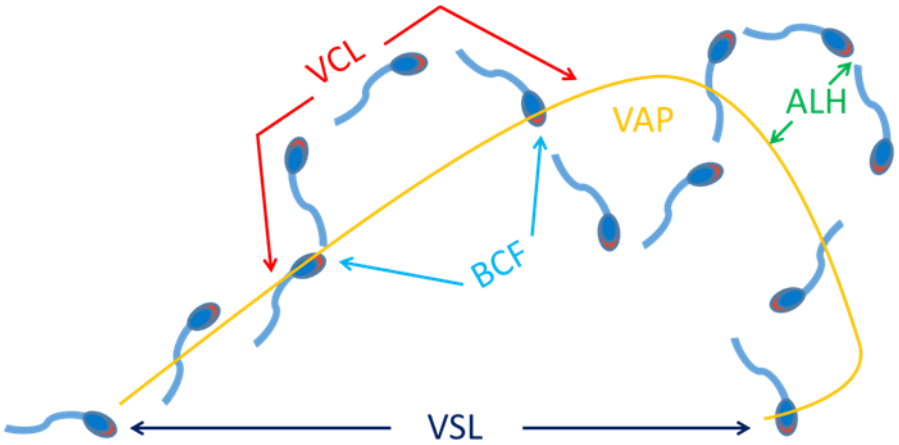


Figura 14: *Esquema que representa los parámetros cinéticos de la movilidad de los espermatozoides. Adaptado de Owen y Katz (Owen y Katz, 1993).*

5) ESTUDIO DE PARÁMETROS FUNCIONALES DE LOS ESPERMATOZOIDES POR CITOMETRÍA DE FLUJO

Esta técnica permite analizar de forma rápida, sensible y objetiva los espermatozoides previamente marcados con fluorocromos. El análisis es individualizado, ya que los espermatozoides deben pasar alineados y de uno en uno por el haz luminoso del citómetro de flujo.

Los elementos que forman el citómetro son: una fuente de luz, un sistema de flujo celular, unos componentes ópticos, unos componentes electrónicos que amplifican y procesan la señal resultante y un ordenador provisto de un software específico para el análisis de la señal.

La dispersión de la luz proporciona información sobre la medida y la rugosidad de la célula. Mediante la denominada "*Forward-Scattered Light*" (FSC) obtenemos información del tamaño celular y ésta nos permite discriminar partículas ajenas al objetivo de nuestro estudio. Por otro lado, la granulosidad y complejidad interna del espermatozoide se evalúan mediante la "*Side-Scattered Light*" (SSC).

Los citómetros de flujo normalmente están provistos de tres fotodetectores (FL): FL1 (detecta longitudes de onda entre 520 y 575nm, color verde), FL2 (detecta longitudes de onda entre 575-620, color naranja) y FL3 (detecta longitudes de onda superiores a 620nm, color rojo). Utilizando las sondas fluorescentes adecuadas excitadas a una determinada longitud de onda, el citómetro permite diferenciar poblaciones celulares dentro de la muestra de espermatozoides analizados. Una gran ventaja es que se pueden utilizar de forma simultánea diferentes fluorocromos, lo que permite evaluar varias características celulares.

En la presente Tesis Doctoral se ha utilizado la citometría de flujo para evaluar el grado de desorganización lipídica de la membrana plasmática, la viabilidad, la integridad del acrosoma, la exposición de la fosfatidilserina en la cara externa de la membrana y el potencial de membrana mitocondrial de los espermatozoides de cerdo. Para ello, las muestras se diluyeron en el tampón correspondiente a una concentración aproximada de 35x 10^6spz/mL. Tras el tratamiento correspondiente, se añadieron 400µL de Fac's Flow, a excepción de la prueba de la anexina en la que se añadieron 400 µL de tampón con calcio. Las muestras fueron analizadas en el citómetro Coulter EPIC XL (Beckman Coulter Ltd.). Los fluorocromos se excitaron con un láser de argón de 200mV a 488nm. En cada muestra se capturaron 10.000 eventos a una velocidad de paso de unos 500 eventos/segundo y posteriormente se analizaron con el programa informático EXPO TM 32 ADC software (Beckman Coulter, Inc.).

Evaluación de la desorganización lipídica de la membrana plasmática

Para determinar el grado de desorden lipídico de la membrana plasmática de los espermatozoides mediante citometría de flujo, se utilizó una combinación de las siguientes sondas fluorescentes (Harrison y cols., 1996):

- Merocianina (M-540, Molecular Probes®, Eugene, Oregon, USA): este fluorocromo muestra afinidad por membranas con alto grado de desestructuración lipídica, indicando el grado de ordenamiento de los lípidos de membrana.

- YOPRO-1 (Molecular Probes, Eugene, Oregon, USA): es un fluorocromo incapaz de penetrar a través de la membrana de los espermatozoides vivos y por tanto se puede unir exclusivamente al ADN de los espermatozoides con la membrana dañada. Emite a 509nm y es un indicador de la viabilidad espermática (ver citogramas representativos en la figura 15).

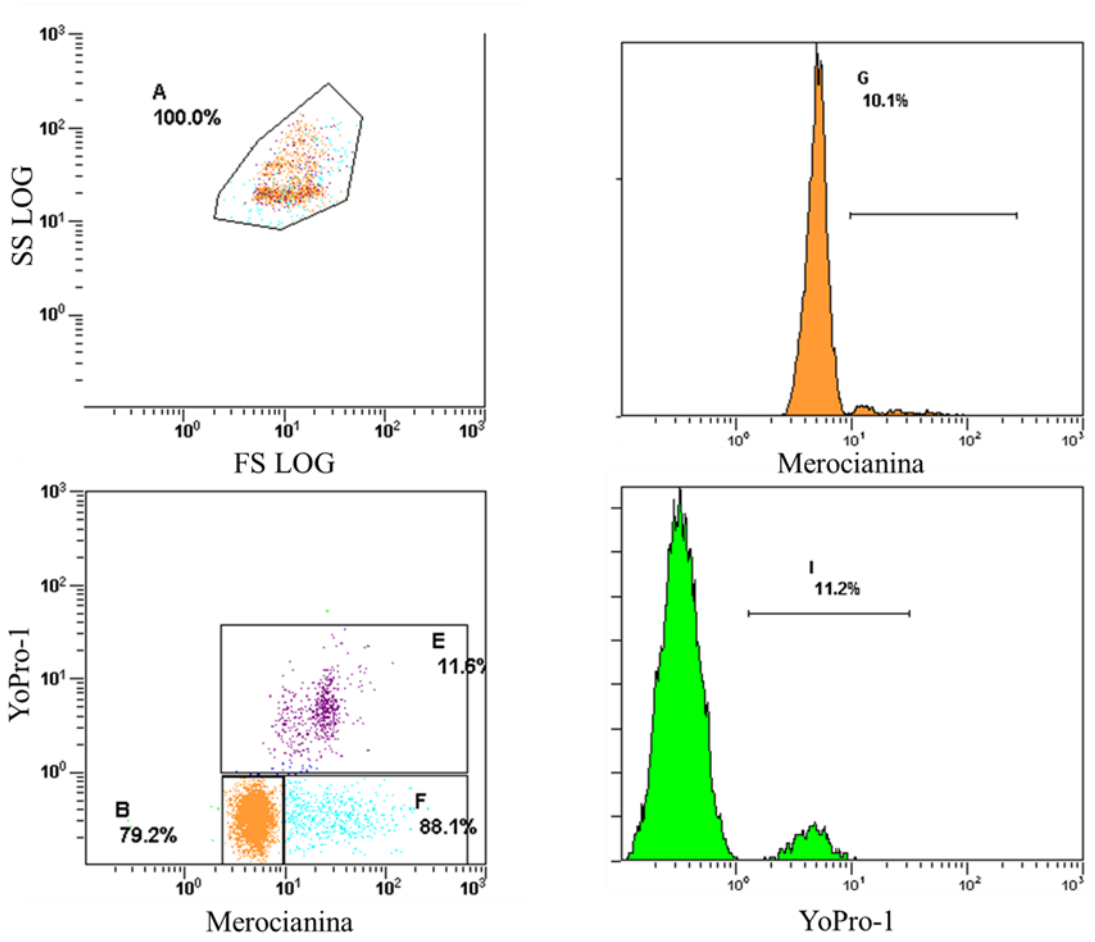


Figura 15: *Citogramas representativos de la población de espermatozoides con desorganización de fosfolípidos de membrana (tinción con M-540 y YoPro-1). En el citograma izquierdo de la parte superior se selecciona la población de espermatozoides en función de los valores de FS y SS. En el citograma izquierdo de la parte inferior se seleccionan los espermatozoides de interés, espermatozoides vivos YoPro -1^{-} con desorganización de los fosfolípidos de la membrana plasmática M540^{+}. El citograma superior de la parte derecha representa el análisis de la fluorescencia de la población F en el eje X, donde se aprecia que el 10,8 % de los espermatozoides vivos presentan desorganización en los lípidos de membrana. El citograma inferior de la parte derecha representa los espermatozoides teñidos con YoPro-1, observándose un 11,2 % de espermatozoides no viables.*

A 100µL de la muestra objeto de estudio se le añadieron 400µL de tampón isotónico, posteriormente los espermatozoides se incubaron durante 15 minutos a 38°C y en oscuridad con 2µL de YoPro-1 a una concentración final de 0,08µM, finalmente se añadieron 2µL de Merocianina a una concentración final 4µM, se homogeneizó la mezcla y se incubó durante 2 minutos antes de su análisis. Los resultados (ver citogramas representativos en la figura 15) se expresaron como el porcentaje de espermatozoides viables con la membrana plasmática inestable ($YoPro\text{-}1^{-}/M\text{-}540^{+}$).

Evaluación de la viabilidad de los espermatozoides

Para la determinación de la viabilidad de la célula germinal masculina mediante citometría de flujo se utilizó el kit comercial Live&Dead® (Molecular Probes, USA) compuesto por dos sondas fluorescentes:

-SYBR-14: es un fluorocromo permeable a la membrana plasmática. Se une al ADN y emite en verde cuando es excitado a 488nm. Esta sonda nos permite diferenciar espermatozoides de otras partículas presentes en la muestra.

-Ioduro de Propidio (IP): este fluorocromo identifica positivamente los espermatozoides no viables o muertos, al penetrar únicamente en células con un daño en la membrana plasmática. Se intercala de forma estequiométrica entre los pares de bases nitrogenadas del DNA o de RNA a razón de una molécula de IP por cada 4-5 pares de bases. Emite en rojo cuando se excita a 536nm.

Tras el tratamiento experimental correspondiente, la muestra de espermatozoides (100 µL) se diluyó en un tampón isotónico (Coulter Isoton II Diluent, Beckman Coulter; Inc. Brea, CA, USA) a una concentración aproximada de 6 x 10^6 células/mL. A una alícuota de 500 µL de la muestra diluida se añadieron 5 µL de SYBR-14 y 10 µL de ioduro de IP a unas concentraciones finales de 2 µM y 5 µM, respectivamente. Antes del análisis en el citómetro de flujo se realizó una incubación durante 20 minutos en la oscuridad y a temperatura ambiente y el

resultado fue expresado como el porcentaje de espermatozoides viables: SYBR-14^{+}/IP^{-} (ver citogramas a continuación).

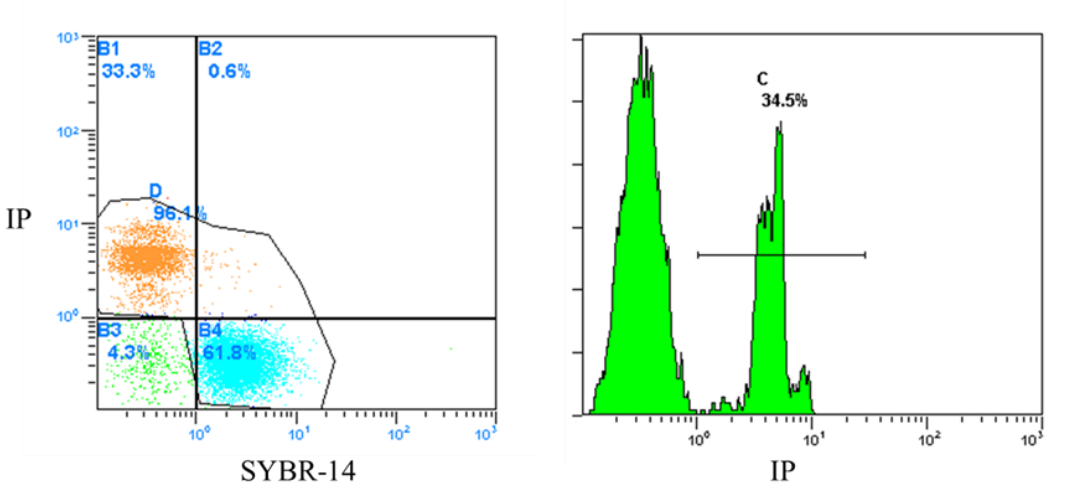


Figura 16: *Citogramas correspondientes a la evaluación de la viabilidad espermática. En el citograma izquierdo se muestra el análisis de la fluorescencia: eje X, FL1: espermatozoides marcados con SYBR-14; eje Y, FL3 espermatozoides marcados con IP. En el citograma derecho se muestra la representación de la fluorescencia de las partículas identificadas como espermatozoides, que se corresponden con la población D del citograma izquierdo, donde observamos un 34,5% de espermatozoides muertos.*

Evaluación de la integridad de la membrana del acrosoma

Para determinar el grado de integridad de la membrana externa del acrosoma de los espermatozoides se utilizó una combinación las siguientes sondas:

-PNA-FITC: Se trata de una lectina procedente del cacahuete unida a un colorante de xanteno que le proporciona fluorescencia (isotiocianato de fluoresceína, FITC). Esta sonda presenta una gran afinidad por los receptores internos del acrosoma, pero es incapaz de atravesar la membrana plasmática, de este modo, solamente aquellas células germinales masculinas con el acrosoma reaccionado o dañado permitirán la unión del PNA a sus receptores y consecuentemente emitirán fluorescencia.

-Ioduro de Propidio (IP): Ya mencionado anteriormente.

Para la realización de esta técnica se tomaron 100µL de la muestra de espermatozoides previamente tratada y se incubaron durante 5 minutos a temperatura ambiente y en oscuridad con 5µL de IP a una concentración final 6µM y 5µL de PNA-FITC procedentes de una solución stock 1 µg/mL en DMSO. Justo antes del análisis en el citómetro se añadieron 400µL de tampón isotónico. El resultado del análisis mediante el citómetro de flujo (espermatozoides PNA^+/IP^-) se expresó como el porcentaje de espermatozoides con el acrosoma dañado (PNA^+) respecto al total de espermatozoides vivos (IP^-) (ver citogramas en el esquema siguiente).

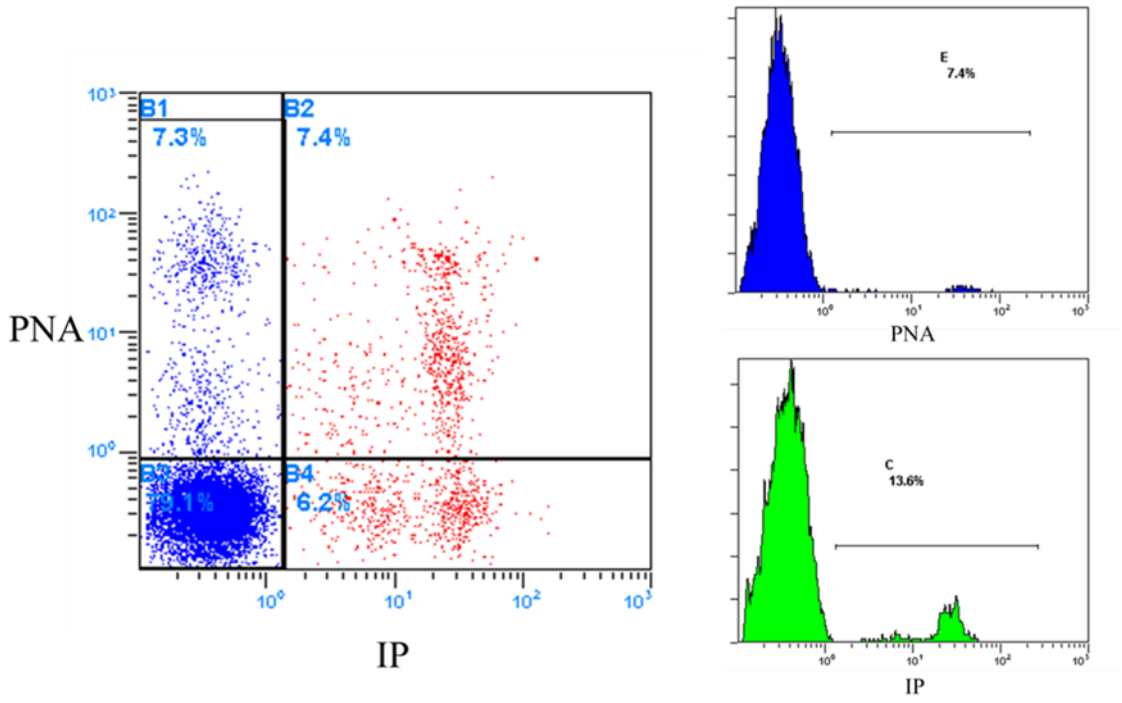


Figura 17: *Citogramas representativos de la población de espermatozoides con acrosoma dañado o que ha reaccionado. En el citograma izquierdo se representa el análisis de la fluorescencia: en azul aparece la población de interés, espermatozoides vivos IP^- y con el acrosoma dañado o reaccionado $PNA\text{-}FITC^+$. Citograma de la derecha: En la parte superior se representan los espermatozoides teñidos con PNA-FITC donde se aprecia un 7,4% de espermatozoides con el acrosoma reaccionado o dañado ($PNA\text{-}FITC^+$). En la parte inferior se representan los espermatozoides muertos respecto al total de la población analizada (13,6%).*

Evaluación del potencial de membrana mitocondrial

Para el estudio del potencial de membrana mitocondrial de la célula germinal masculina mediante citometría de flujo se utilizó una única sonda fluorescente, JC-1, marcador catiónico que se acumula en la membrana mitocondrial situada en la pieza intermedia del flagelo del espermatozoide. Forma agregados denominados J-agregados que emiten en el canal del rojo (FL3), mientras que en estado monomérico emite en el espectro del verde (FL1). Los espermatozoides fluorescen en verde (525 nm) o rojo (595 nm) en función de si el potencial de membrana es bajo o alto, respectivamente.

Para la realización de esta técnica se añadieron 3 µL de JC-1 a una concentración final de 1µM a 500 µL de muestra previamente diluida en tampón isotónico; posteriormente se incubaron a 38°C durante 30 minutos. Los resultados del análisis mediante el citómetro de flujo se expresaron como el porcentaje de espermatozoides con alto y bajo potencial de membrana mitocondrial (ver citogramas a continuación).

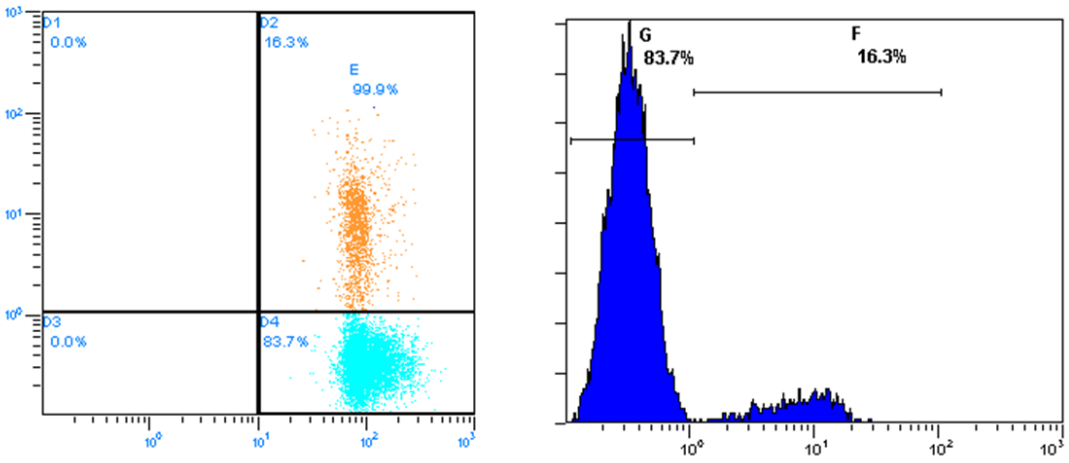


Figura 18: *Citogramas representativos del potencial de membrana mitocondrial de la población de espermatozoides (tinción con JC-1). En el citograma izquierdo se representa el análisis de la fluorescencia: eje X, FL1, espermatozoides marcados con JC-1 en estado monomérico; eje Y, FL3, espermatozoides marcados con JC-1 en estado agregado. El citograma derecho representa la fluorescencia de los espermatozoides con alto y bajo potencial de membrana mitocondrial. Se observa un 16,3% de espermatozoides con alto potencial de membrana mitocondrial.*

Evaluación de la de la translocación de la fosfatidilserina

Para evaluar la externalización de la fosfatidilserina en la cara externa de la membrana del espermatozoide se utilizó:

-Anexina V: es una proteína con alta afinidad por la fosfatidilserina, y para que emita en verde debe estar unida al isotiocianato de fluoresceína (FITC).

-Ioduro de Propidio (IP): diferencia los espermatozoides vivos con externalización de la fosfatidilserina de los muertos, con o sin externalización de la misma.

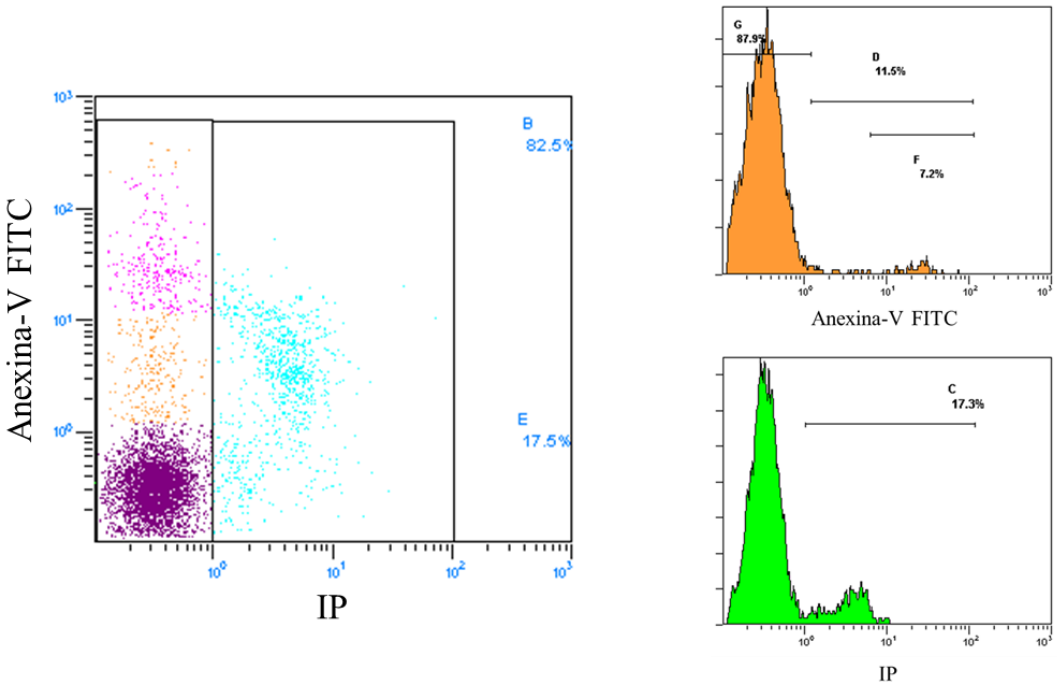


Figura 19: *Citogramas representativos de la población de espermatozoides con externalización de la fosfatidilserina en la cara externa de la membrana plasmática. En el citograma izquierdo se representa el análisis de la fluorescencia: en el eje de las Y del cuadrante izquierdo aparece la población de interés, espermatozoides vivos (IP^-) y con externalización de la fosfatidilserina (Anexina V-$FITC^+$) Citograma de la derecha: en la parte superior se representan los espermatozoides teñidos con Anexina V-FITC donde se aprecia un 11,5% de espermatozoides teñidos y un 7,2 % de espermatozoides teñidos con más intensidad. En la parte inferior se representan los espermatozoides muertos del total de la población analizada (17,3%).*

Tras el tratamiento, la muestra se diluyó a 12 millones de espermatozoides por mililitro en un tampón preparado en el día de uso compuesto por: 106 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 glucosa

1mmol/L piruvato sódico, 21,6mmol/L lactato sódico 20 mmol/L Hepes (pH 7,4), 2,5 mmol/L $CaCl_2$. Posteriormente, se tomó una alícuota de 100µL y se incubó durante 15 minutos a temperatura ambiente y en oscuridad con 4µL de ioduro de propidio y 5 µL de Anexina V-FITC. Justo antes de medir en el citómetro se añadieron a cada tubo 400 µL del tampón anteriormente descrito. Para el análisis estadístico se tomaron los datos de la población positiva a la Anexina V- FITC y negativa al ioduro de propidio. (ver citogramas Figura 19).

6) ANÁLISIS ESTADÍSTICO

Como parámetros estadísticos descriptivos se obtuvieron la media y el error estándar de la media. El análisis multivariante se realizó por medio de un ANOVA seguido del test de Scheffe (comparación entre tratamientos). Para analizar el porcentaje de espermatozoides motiles y rápidos se utilizó la prueba Chi-cuadrado de Pearson. Las diferencias se consideraron estadísticamente significativas cuando $p<0,05$.

El análisis estadístico se realizó con el programa informático SPSS versión 11.0 para Macintosh (SPSS inc. Chicago, IL).

Discusión

EXPRESIÓN E INMUNOLOCALIZACIÓN DE LA AMPK Y SU FORMA ACTIVA EN ESPERMATOZOIDES DE CERDO

Acorde a su papel de molécula que detecta tanto el estado energético celular como los diversos tipos de estrés que comprometen la función celular, la proteína AMPK ayuda a mantener los niveles celulares de energía, determinados mediante su carga energética (AMP/ATP). De esta forma, la AMPK regula el metabolismo (Carling y cols., 2008; Hardie, 2004) mediante la activación de rutas catabólicas que producen ATP, a la vez que provoca la inhibición de aquellas rutas anabólicas que consumen ATP (Kahn y cols., 2005; Solaz-Fuster y cols., 2008). Al igual que se ha descrito en células somáticas, los espermatozoides de mamíferos necesitan una correcta regulación de sus niveles de energía para poder desempeñar los procesos fisiológicos característicos de esta célula germinal basados fundamentalmente en el mantenimiento de la estructura celular y en la organización y la función de sus membranas para permitir el paso de iones a través de las mismas y, en definitiva, contribuir a su función primordial. Sin embargo, hasta el momento de plantear la hipótesis de esta Tesis Doctoral no existía ningún trabajo acerca de la expresión y/o de la función de la proteína AMPK en la célula germinal masculina.

Los resultados de esta Tesis muestran por primera vez que la proteína AMPK se expresa en espermatozoides, concretamente en una especie de mamíferos, el cerdo. El anticuerpo utilizado para ello, y que es específico para la subunidad catalítica α de la AMPK, reconoce en espermatozoides de cerdo dos bandas de peso molecular muy similar, lo que concuerda con lo que reconoce este mismo anticuerpo en células de músculo liso vascular de la misma especie (Rubin y cols., 2005). Como el anticuerpo anti-AMPKα utilizado reacciona con dos isoformas α de origen humano (α1 y α2), estas dos bandas inmunorreactivas en espermatozoides de cerdo probablemente se corresponden con las isoformas α1 y α2 de la AMPK en esta especie. Es interesante mencionar que el nivel de expresión de la proteína AMPK detectado en espermatozoides de cerdo es mayor que en otras células

somáticas porcinas, las cuales han sido también analizadas como controles experimentales de forma paralela, como son el corazón, cerebro y pulmón.

Una vez demostrado por western blott que la proteína AMPK se expresa en espermatozoides de cerdo, estudiamos su localización subcelular. Los resultados obtenidos con la técnica de inmunocitoquímica, utilizando el anticuerpo anti-AMPKα, muestran que su distribución en espermatozoides está restringida a determinadas estructuras. Así, en la cabeza del espermatozoide la AMPK se localiza en la totalidad del acrosoma, mientras que en el flagelo, la AMPK está localizada exclusivamente en la pieza intermedia.

Como se ha mencionado anteriormente en la Introducción, la activación enzimática de la AMPK se consigue, además de por activación alostérica, mediante la fosforilación del residuo Thr^{172}, localizado estructuralmente en el bucle de activación de la subunidad catalítica α. Utilizando un anticuerpo que reconoce específicamente este residuo fosforilado (p-Thr^{172}-AMPK), los resultados de esta Tesis Doctoral demuestran que la AMPK es fosforilada (enzimáticamente activa) en la Thr^{172} en espermatozoides de cerdo que se encuentran tanto en un medio sin estímulos (TBM), como en un medio que contiene estímulos fisiológicos para estas células germinales como son Ca^{2+} y HCO_3^- (TCM). En todo caso, un resultado interesante es que la AMPK se encuentra enzimáticamente activa (p-Thr^{172}-AMPK) a la temperatura fisiológica de los espermatozoides de esta especie, que es 38,5°C. El nivel de fosforilación de AMPK a esta temperatura varía en función del tiempo experimental analizado, aumentando inicialmente durante los primeros 60 minutos y manteniéndose detectable al tiempo máximo analizado, 24h. Por otro lado, los resultados de inmunocitoquímica muestran que la fosforilación de la AMPK, además de inducir su actividad enzimática, conlleva una redistribución de su expresión en la cabeza del espermatozoide. Así, la localización de la forma activa de AMPK (p-Thr^{172}-AMPK) se restringe al segmento subecuatorial y, sobre todo, a la parte más apical del acrosoma, donde se visualiza un elevado nivel de expresión. Sin embargo, la fosforilación de la AMPK no conlleva cambios en la localización en la pieza

intermedia del flagelo, donde el nivel de expresión de p-Thr^{172}-AMPK es similar al detectado para la AMPK.

RUTAS DE SEÑALIZACIÓN Y MECANISMOS INTRACELULARES QUE REGULAN LA FOSFORILACIÓN DE LA AMPK EN EL ESPERMATOZOIDE DE CERDO

En el momento de plantear la hipótesis de esta Tesis Doctoral solo se conocían las rutas de señalización que conducen a la activación de la AMPK en células somáticas. En este sentido se han identificado varias quinasas que fosforilan y, por tanto, aumentan la actividad de la AMPK en la Thr^{172}: La quinasa LKB1 (Woods y cols., 2003), codificada por el gen supresor de tumores responsable del síndrome hereditario *Peutz-Jeghers*; las proteínas CAMKKα y CAMKKβ quinasas de la quinasa activada por calcio y calmodulina (Hawley y cols., 2005); TAK1, quinasa-1 activada por el factor de crecimiento transformante TGFβ (Xie y cols., 2006) y la quinasa KSR2 (Costanzo-Garvey y cols., 2009).

Los resultados de esta Tesis Doctoral muestran por primera vez las rutas de señalización involucradas en la actividad de la AMPK en espermatozoides y que se resumen en un esquema mostrado en la figura 20.

Así, en primer lugar, hemos demostrado la implicación de la vía cAMP/PKA. La activación de la adenilato ciclasa soluble característica de espermatozoides (sAC), que se induce por los estímulos Ca^{2+} y HCO_3^- (incluidos en el medio TCM), provoca la síntesis intracelular de cAMP. Nuestros resultados muestran que tanto la incubación con un análogo no hidrolizable de cAMP (8Br-cAMP), como con un inhibidor de las fosfodiesterasas, IBMX, provocan un marcado incremento de la fosforilación de la AMPK en espermatozoides. Esto sugiere que un incremento en los niveles intracelulares de cAMP conduce a la activación de la AMPK en espermatozoides (Figura 20).

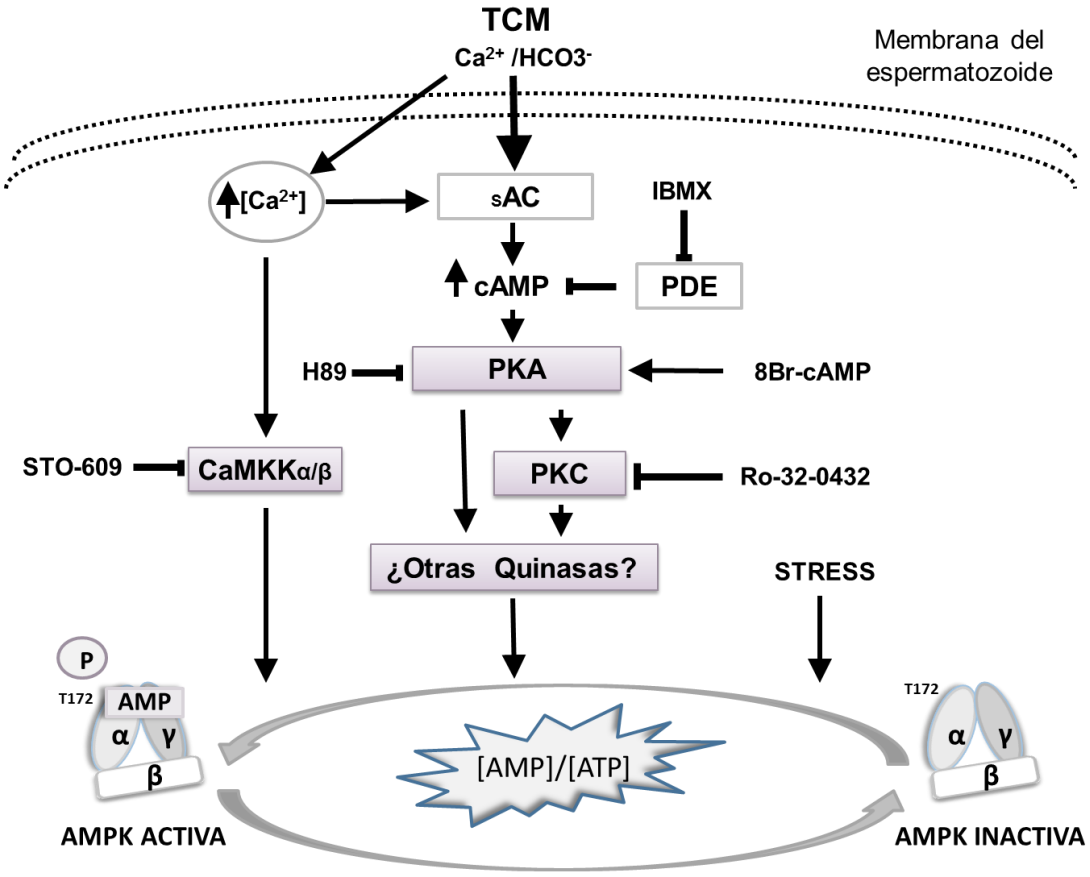


Figura 20: *Esquema propuesto de las rutas de señalización intracelular que regulan la vía de la AMPK en espermatozoides de mamíferos.*

El cAMP es un efector alostérico de la PKA que tiene un papel central en la regulación de los procesos funcionales del espermatozoide. Nuestros resultados sugieren que los efectos del cAMP en la actividad de la AMPK del espermatozoide están efectivamente mediados por la activación de la PKA, ya que su inhibidor H89 bloquea los efectos inducidos por cualquier estímulo que incremente el cAMP en estas células germinales: por 8Br-cAMP, por calcio y por bicarbonato (TCM) y por IBMX. Hasta la fecha, la implicación de la ruta de PKA en la cascada de fosforilación de la AMPK ha sido demostrada exclusivamente en células somáticas como en adipocitos (Sanders y cols., 2007b), hepatocitos (Kimball y cols., 2004; Wu y cols., 2011), células CV-1 (Collins y cols., 2000), células musculares (Williamson y cols., 2006) y en células 293T (Nin y cols., 2012). Recientemente se ha propuesto una comunicación bioquímica entre la PKA y la AMPK que actuarían de forma conjunta como una red de señalización capaz de controlar el metabolismo celular en algunas

células somáticas, como las de músculo liso vascular (Stone y cols., 2012) o adipocitos (Djouder y cols., 2010). La activación de AMPK dependiente de cAMP/PKA en espermatozoides podría ocurrir a través de la quinasa LKB1, como se ha demostrado que puede ocurrir en células somáticas, donde LKB1 es a su vez directamente fosforilada en la Ser^{431} por la PKA en respuesta a la activación de la adenilato ciclasa por forskolina (Collins y cols., 2000; Sapkota y cols., 2001) o IBMX (Collins y cols., 2000). En relación a la LKB1, aún no se ha identificado su presencia en espermatozoides de cerdo, aunque sí se ha demostrado que una isoforma corta (LKB1s) se expresa en altos niveles en espermátidas haploides de testículos de ratón, donde desempeña un papel relevante en la espermiogénesis y en la fertilidad (Towler y cols., 2008). Un posible mecanismo adicional que podría parcialmente explicar la activación de la AMPK por un aumento en los niveles de cAMP es a través de la regulación de la degradación del cAMP (Omar y cols., 2009). Tras una elevación del cAMP intracelular, la cantidad de este mensajero rápidamente vuelve a los niveles basales a través de su degradación a AMP por las fosfodiesterasas. En definitiva, como ha sido sugerido en células somáticas (Nin y cols., 2012; Omar y cols., 2009), nosotros también postulamos que cualquier estímulo que cause en espermatozoides un incremento en el cAMP intracelular provocaría un aumento en la actividad de la AMPK debido a i) activación directa de la PKA por alosterismo, ii) estimulación de las fosfodiesterasas que a su vez provocaría un incremento de los niveles de AMP y éste activaría alostéricamente la AMPK, iii) mediante ambos mecanismos conjuntamente (Figura 20). El hecho de que al inhibir las fosfodiesterasas en espermatozoides con IBMX se observe un incremento en los niveles de p-Thr^{172}-AMPK sugiere que, al menos en esas condiciones experimentales, la implicación de la actividad de las fosfodiesterasas en la actividad de la AMPK no parece probable en esta célula germinal.

Un regulador esencial de cualquier proceso funcional del espermatozoide es el ión Ca^{2+}, mensajero intracelular que, como se ha comentado con anterioridad, activa la adenilato ciclasa soluble expresada en estas células germinales. Los resultados de esta Tesis sugieren que el calcio intracelular está implicado en una

ruta de señalización que activa potentemente la AMPK en espermatozoides. Este resultado en la célula germinal está apoyado por trabajos previos en células somáticas en los que el calcio directamente regula la activación de la AMPK (Ghislat y cols., 2012; Hawley y cols., 2005; Woods y cols., 2005). Los resultados de esta Tesis demuestran que la ruta inducida por calcio y que conduce a la AMPK está mediada por la activación de PKA (Figura 20). La explicación más lógica es que el calcio estimula directamente la adenilato ciclasa soluble del espermatozoide, catalizando así la síntesis de cAMP que provocaría la activación alostérica de PKA. Además de esta ruta, el calcio también podría provocar la fosforilación de la AMPK en espermatozoides a través de la estimulación de las CAMKKα/β, las cuales activan AMPK en células somáticas (Hawley y cols., 2005; Woods y cols., 2005). En este estudio hemos utilizado STO-609, que actúa como un inhibidor específico de ambas CAMKKs (Tokumitsu y cols., 2002). Aunque la expresión de ambas isoformas CAMKKα/β se ha descrito en espermatozoides de diferentes especies de mamíferos, como la rata (Ackermann y cols., 2009) y el ratón (Schlingmann y cols., 2007), hasta la fecha los niveles de expresión en espermatozoides de cerdo no se conocen. Por ello, y puesto que no podemos atribuir de manera inequívoca el efecto a una o las dos isoformas de esta quinasa, en esta discusión nos referiremos a ambas isoformas, utilizando CAMKKα/β. El inhibidor STO-609 provoca una inhibición completa de la actividad de la AMPK inducida por Ca^{2+} en espermatozoides, en consonancia con estudios previos realizados en células somáticas (Hawley y cols., 2005; Woods y cols., 2005). Esto sugiere que CAMKKα/β están implicadas en la vía de señalización que regula la actividad de la AMPK en la célula germinal. De forma previsible, en ausencia de calcio extracelular (quelado por el EGTA presente en el medio TBM) los niveles de p-Thr^{172}-AMPK que existen a la temperatura fisiológica del espermatozoide no están regulados por CAMKKα/β.

Entre otras rutas intracelulares que tienen gran relevancia en el control de la función del espermatozoide de cerdo se encuentra la proteína quinasa C, PKC. Los resultados obtenidos en la presente Tesis Doctoral muestran que la fosforilación de la AMPK se estimula en espermatozoides de cerdo por la activación directa de la

PKC con el éster de forbol PMA, lo que sugiere que al menos una o más isoformas de la PKC están regulando la actividad de la AMPK en estas células germinales (Figura 20). Apoyando esta conclusión se encuentra el hecho de que un inhibidor de PKCs, RO-32-0432, bloquea tanto la actividad enzimática que presenta la AMPK en un medio con estímulos para el espermatozoide (TCM), como la actividad inducida por el activador A769662 en ese mismo medio (TCM). La evidencia de que PMA estimula la fosforilación de la AMPK ha sido descrita recientemente en células somáticas (Chang y cols., 2012) donde, además, se demuestra que la AMPK no es un sustrato de la PKC. En este sentido, Xie y cols., demostraron en el 2008 que la PKCζ atípica es una quinasa que fosforila LKB1 en la Ser^{428} (Xie y cols., 2008) y también en la Ser^{307} en diferentes tipos de células somáticas (Xie y cols., 2009). Muy recientemente, tras el descubrimiento de las diferentes isoformas de LKB1 ($LKB1_L$, $LKB1_s$) se ha especificado que la PKCζ fosforila la $Ser^{428-431}$ en la isoforma larga, $LKB1_L$ y la Ser^{399} en la corta, $LKB1_s$. Dichos residuos son esenciales para la exportación de LKB1 desde el núcleo al citoplasma, donde consecuentemente desempeña su función de activación de la AMPK (Zhu y cols., 2013). En células germinales, la actividad de PKC se ha descrito en espermatozoides humanos (Rotem y cols., 1990) y de otros mamíferos (Breitbart y cols., 1992), donde la PKC contribuye a la regulación de la reacción acrosómica (Breitbart y cols., 1992; Rotem y cols., 1990) y también de la movilidad (Bragado y cols., 2010) e hiperactivación (Harayama y Miyake, 2006). Entre las isoformas de PKC identificadas en espermatozoides de mamíferos se encuentran PKCα y PKCβ1 en toro (Naor y Breitbart, 1997) y PKCζ en hámster (NagDas y cols., 2002) y ratón (Jungnickel y cols., 2007). Es por ello plausible que la PKCζ pueda tener en espermatozoides un papel similar al que desempeña en las células somáticas: incrementar la actividad de la AMPK a través de la fosforilación de la quinasa inmediatamente por encima, LKB1. Una explicación alternativa para describir la contribución de la PKC(s) en la vía de la AMPK en la célula germinal masculina está basada en diferentes estudios realizados en espermatozoides de cerdo. Por un lado, nuestro grupo de investigación ha demostrado que la PKC está situada por debajo de la PKA en la ruta que controla la

movilidad de estas células germinales (Bragado y cols., 2010). Por otro lado, Harayama y Miyake (2006) habían demostrado que la señalización mediada por la ruta cAMP/PKA puede inducir la activación de PKCs que son sensibles al calcio y responsables de la hiperactivación del espermatozoide de cerdo (Harayama y Miyake, 2006). Teniendo en cuenta que la PKCζ anteriormente propuesta, no es sensible a calcio, nosotros planteamos en esta Tesis que otras isoformas de PKC sensibles a calcio podrían estar adicionalmente implicadas en la ruta de la AMPK en espermatozoides de cerdo, al menos en respuesta al aumento de cAMP.

Otra ruta de señalización que contribuye a la regulación de la función del espermatozoide es la PI3K (Bragado y cols., 2010; Breitbart y cols., 2010). Nuestro grupo ha descrito que la PI3K ejerce un efecto negativo en los niveles de cAMP en la vía de señalización que controla la movilidad de los espermatozoides de cerdo, ya que el tratamiento de los espermatozoides con el inhibidor LY294002 provoca un incremento en los niveles de cAMP tanto en un medio basal como con estímulos (Bragado y cols., 2010). De esta manera, el hecho de que el LY294002 potencie el efecto del activador de la AMPK, A769662, se explicaría a través de la activación adicional de la vía de la PKA, mediada por un aumento del cAMP provocado por el LY294002 en espermatozoides de cerdo (Bragado y cols., 2010). Apoyando esta idea de una conexión bioquímica entre las vías de PKA y PI3K se ha demostrado un cruce entre las vías de señalización de la PKA, PKC y PI3K en espermatozoides de otras especies (Breitbart y cols., 2010).

Además de proponer las rutas de señalización que regulan la actividad de la AMPK en condiciones fisiológicas, esta Tesis aporta evidencias claras de que la AMPK también se activa en espermatozoides bajo condiciones que causan algún tipo de estrés celular (Figura 20). Así, tanto la inhibición de la actividad mitocondrial mediante el bloqueo de la cadena transportadora de electrones con rotenona o cianuro, como el estrés hiperosmótico inducido por sorbitol, causan un claro incremento en la fosforilación de la AMPK en estas células germinales. En células somáticas la activación de la AMPK depende del tipo de estrés y puede estar

mediada por i) un incremento en los niveles de AMP, ii) por la generación de las especies reactivas de oxígeno (ROS) que actúan como moléculas que activan la AMPK (Emerling y cols., 2009) a través de las vías dependientes de LKB1 y de CAMKKs, iii) o por ambos mecanismos. De manera sorprendente, la ausencia de calcio intracelular en espermatozoides de cerdo (incubación con BAPTA-AM en un medio libre de Ca^{2+}) provoca un fuerte incremento en la actividad de la AMPK. Una posible explicación se basa en un estudio realizado en espermatozoides humanos (de Lamirande y cols., 2009), donde se ha demostrado que el BAPTA provoca un aumento de la producción de óxido nítrico ($NO^{\cdot}$), que, en el caso de que lo provocara también en espermatozoides de cerdo, a su vez el $NO^{\cdot}$ podría inducir la fosforilación de la AMPK. Apoyando esta última hipótesis, un estudio reciente realizado en células somáticas demuestra que la activación de la AMPK está también influida directamente por el estatus redox celular, así el H_2O_2 activa la AMPK a través de la oxidación de residuos de cisteína en su subunidad catalítica α (Zmijewski y cols., 2010). Una explicación alternativa es que el $NO^{\cdot}$ producido por el BAPTA en espermatozoides de cerdo podría interaccionar con la vía del cAMP como ocurre en humanos (Belen y cols., 2000), provocando de esta manera la activación de la AMPK, como hemos mencionado anteriormente.

IMPLICACIÓN DE LA AMPK EN LOS PRINCIPALES PARÁMETROS Y PROCESOS FUNCIONALES DEL ESPERMATOZOIDE

El estudio de la posible contribución de la AMPK en los procesos claves que determinan la función del espermatozoide se ha abordado en esta Tesis Doctoral mediante una aproximación farmacológica, utilizando un inhibidor (Compuesto C) y un activador (A769662) de la AMPK. Centrándonos inicialmente en la viabilidad de los espermatozoides, nuestros resultados sugieren que la actividad de la AMPK está implicada en el control de la misma en determinadas condiciones extracelulares. Así, en aquellas condiciones en las que los espermatozoides se incuban en un medio sin estímulos añadidos, una inhibición de la actividad de la AMPK es capaz de prevenir el descenso en la viabilidad espermática; sin embargo en estas mismas

condiciones una actividad de la AMPK por encima de los niveles "fisiológicos" no tiene efecto en la viabilidad. Estos resultados sugieren que en condiciones extracelulares que provocan un descenso en el número de espermatozoides viables, la actividad de la AMPK, al menos a tiempos cortos, puede ser crucial en el mantenimiento de la viabilidad del espermatozoide.

El potencial de membrana mitocondrial (ΨΔm) se usa generalmente como un indicador del estado de las mitocondrias, puesto que al ser una medida del transporte de iones refleja la actividad metabólica y la integridad de la membrana mitocondrial (Amaral y Ramalho-Santos, 2010; Gillan y cols., 2005; Guthrie y Welch, 2008). Los resultados de esta Tesis demuestran que, en condiciones no estimulantes para el espermatozoide, una inhibición de la actividad de la AMPK no tiene ningún efecto en el ΨΔm, mientras que un incremento a corto plazo en la actividad de la AMPK provoca un ligero, pero reproducible descenso en el ΨΔm. Por otro lado, cuando los espermatozoides se encuentran en condiciones estimulantes, un incremento en la actividad de la AMPK previene el descenso en el número de espermatozoides con alto ΨΔm a corto plazo, mientras que una inactivación de la AMPK provoca un descenso del ΨΔm a las 24 horas. Este último resultado está apoyado por el reciente estudio de Tartarin y cols., (2012) donde describen que la inactivación de la AMPK llevada a cabo en ratones *Knockout* que no expresan su subunidad catalítica α, provoca un descenso en el ΨΔm en espermatozoides, acompañado de un menor consumo basal de oxígeno. Apoyando también una función de la AMPK en la actividad mitocondrial de los espermatozoides está el hecho de que la forma activa de la AMPK (p-Thr^{172}-AMPK) se localiza en la pieza intermedia del flagelo, donde las mitocondrias están exclusivamente localizadas y dispuestas en forma helicoidal en esta célula germinal. Nuestros datos concuerdan con la idea que relaciona la actividad de la AMPK y el ΨΔm, y sugieren que la actividad de la AMPK es esencial en espermatozoides de cerdo para mantener un ΨΔm adecuado a las cambiantes condiciones extracelulares, al igual que ha sido postulado en espermatozoides de ratón (Tartarin y cols., 2012). De manera similar a lo que ocurre en la viabilidad de los espermatozoides, las consecuencias de las

variaciones en el nivel de actividad de la AMPK en el ΨΔm dependen completamente de las condiciones extracelulares en las que se encuentre el espermatozoide, apoyando la idea de que la AMPK actúa como un punto de encuentro metabólico al integrar la señalización intracelular, desencadenada por los diferentes estímulos extracelulares, con el metabolismo de la célula germinal.

Para lograr con éxito la fecundación el espermatozoide necesita experimentar una serie de modificaciones fisiológicas y bioquímicas que afectan mayoritariamente a la membrana plasmática. Entre los cambios bioquímicos se encuentran, por un lado, un aumento en la fluidez de la membrana provocada por una desorganización lipídica, un mayor intercambio de fosfolípidos entre las dos caras de la membrana plasmática mediado, entre otras enzimas, por las escramblasas, y una pérdida de colesterol. La merocianina 540 (M540) es una sonda lipofílica capaz de penetrar aquellas membranas plasmáticas que han sufrido o están en proceso de una desorganización de sus fosfolípidos. Esta desorganización de la membrana plasmática ocurre de forma fisiológica durante diferentes procesos celulares en células somáticas y germinales que están asociados con el reconocimiento celular y un aumento de la capacidad fusogénica (Guthrie y Welch, 2005b). Nuestros datos muestran que la inhibición de la actividad de la AMPK aumenta la desorganización lipídica de la membrana plasmática de espermatozoides, medida con M540, de una forma dependiente del tiempo y del medio de incubación. Así, la desorganización lipídica inducida por el compuesto C ocurre más rápidamente (a las 4h) en presencia de Ca^{2+} y HCO_3^- en el medio. Sin embargo, la inhibición de la actividad de la AMPK a tiempos posteriores también causa desorganización lipídica de la membrana plasmática en un medio sin estímulos. Por otro lado, esta Tesis muestra que un aumento de la actividad de la AMPK (inducido por A769662) no tiene ningún efecto en la organización lipídica a corto plazo, sin embargo, a largo plazo provoca una mayor desorganización lipídica de la membrana plasmática, independientemente del medio de incubación. Todos estos datos sugieren la idea de que la organización lipídica de la membrana plasmática del espermatozoide es totalmente dependiente, al menos a corto plazo,

de la actividad enzimática de la AMPK. Con los resultados actuales no disponemos de ningún argumento científico que pueda explicar el hecho de que tanto una inhibición como un incremento en la actividad de la AMPK a largo plazo provocan un aumento en la desorganización lipídica de la membrana plasmática del espermatozoide.

Los niveles fisiológicos de HCO_3^- producen un rápido colapso de la asimetría de la membrana plasmática de los espermatozoides atribuible a la activación de enzimas como las escramblasas que translocan fosfolípidos, como la fosfatidilserina (PS) y la fosfatidiletanolamina (Gadella y Harrison, 2000), en las dos direcciones entre las dos caras de la membrana plasmática. Nuestros resultados en espermatozoides de cerdo demuestran que modificaciones que reducen o inhiben el nivel de actividad de la AMPK afectan a la asimetría de la membrana plasmática de una forma dependiente del tiempo. Así, a tiempos cortos (0-4 horas) una inhibición de la actividad de la AMPK provoca una inhibición de la exposición de PS en la cara externa de la membrana plasmática, mientras que en un incremento en su actividad no presenta ningún efecto. Estos datos sugieren que es necesario un determinado nivel ("mínimo") de actividad de la AMPK, que podríamos considerar fisiológico a efectos de esta Discusión, para mantener la correcta organización asimétrica de fosfolípidos en la membrana plasmática de los espermatozoides. Si la actividad de AMPK aumenta por encima de los valores fisiológicos no parece existir un control adicional de la translocación de PS hacia la cara externa de la membrana. Sin embargo, unos niveles menores de actividad de AMPK por debajo de los fisiológicos, o una inhibición de la actividad a corto plazo, podrían estar provocando una inhibición de las actividades escramblasa/s o flopasa en la membrana plasmática de los espermatozoides. En este sentido, Vucicevic y cols., (Vucicevic y cols., 2009) han descrito que el compuesto C causa un incremento en la exposición de PS en células de glioma, acompañado de una parada del ciclo celular y apoptosis. Adicionalmente, en eritrocitos de ratón carentes de la actividad de la AMPK al ser deficientes en su subunidad catalítica α1 ($AMPK^{-/-}$), la exposición de PS en la cara externa se encuentra también estimulada (Zelenak y cols., 2011). Las diferencias

entre estos últimos trabajos con nuestros resultados pueden ser explicadas por varias razones: i) los estudios mencionados se han realizado en células somáticas, ii) en el estudio previo donde usan CC la exposición de PS inducida por este inhibidor va acompañada de un efecto apoptótico, mientras que en espermatozoides de cerdo el CC no induce ningún tipo de muerte celular. Por otro lado, cuando el incremento en la actividad de la AMPK es sostenido en el tiempo (24 h) se constata una mayor translocación de PS a la cara externa de la membrana plasmática del espermatozoide. Aunque este efecto se observa en los dos medios de incubación utilizados en esta tesis, es importante destacar que la mayor translocación de PS ocurre en un medio sin estímulos. Estos resultados sugieren que un incremento sostenido en el tiempo de la actividad de la AMPK por encima de los niveles fisiológicos contribuiría al control de la organización asimétrica de los lípidos de la membrana plasmática del espermatozoide, y que ese control estaría modulado por las condiciones extracelulares. La ruta de señalización de AMPK implicada en el control de la organización de los lípidos de la membrana plasmática (asimetría) podría posiblemente ocurrir a través de la regulación de la actividad enzimática de las escramblasas o de las flopasas. En nuestra opinión, esta interesante función de la AMPK que hemos descrito en el mantenimiento de la asimetría y organización de la membrana plasmática del espermatozoide puede ser determinante para aquellos procesos que realiza esta célula germinal en los que esta estructura celular está implicada. Por ello, consideramos esencial plantear futuras investigaciones que establezcan cómo ocurre este control por parte de la AMPK, estableciendo con detalle la ruta de señalización y también si las escramblasas, u otras enzimas como las flopasas, están involucradas.

El grado de la integridad de la membrana externa del acrosoma es otro parámetro indicador de la funcionalidad del espermatozoide que puede ser determinado por citometría de flujo con la sonda PNA-FITC, de forma que una pérdida de dicha integridad (evaluada como espermatozoides vivos que son PNA^{+}) puede ser atribuida a acrosomas que han reaccionado espontáneamente sin estímulos, o bien a acrosomas que presentan un daño en su membrana externa.

Nuestros resultados muestran que el efecto de la actividad de la AMPK en la integridad del acrosoma es totalmente dependiente de la presencia de estímulos en el medio. Por un lado, en un medio sin estímulos, cualquier modificación de los niveles de actividad de la AMPK (aumento o inhibición) tanto a corto como a largo plazo, no tiene ningún efecto en este parámetro, lo que indica que cuando el espermatozoide se encuentra en un medio sin estímulos la actividad de la AMPK no parece necesaria para el mantenimiento de la integridad de la membrana del acrosoma. Por otro lado, nuestros datos sugieren que un determinado nivel de actividad de la AMPK (fisiológico) es necesario para controlar la integridad de la membrana del acrosoma cuando los espermatozoides se encuentran en un medio con estímulos como Ca^{2+} y HCO_3^-, ya que tanto la inhibición de AMPK, de modo más rápido (4h), como un aumento de su actividad provocan una clara pérdida de la integridad del acrosoma. Es interesante mencionar que estos cambios en la membrana del acrosoma inducidos por fluctuaciones en los niveles de actividad de la AMPK, ocurren de forma paralela a los observados en la fluidez de la membrana plasmática y en la exposición de la fosfatidilserina en su cara externa. La función que ejerce la AMPK en la membrana del acrosoma y que postulamos en esta Tesis, está apoyada por la inmunolocalización de una gran mayoría de la AMPK activa (p-Thr^{172}-AMPK) en la región más apical del acrosoma del espermatozoide.

Como se ha mencionado en la Introducción, para que un espermatozoide pueda experimentar la reacción acrosómica en el tracto genital de la hembra, es necesario que previamente esté capacitado. De manera experimental, no existe una única técnica que evalúe la mayor predisposición fusogénica de las membranas del espermatozoide que se alcanza durante la capacitación. Por ello, la evaluación del estado de capacitación de un espermatozoide se realiza indirectamente a través del análisis de varios parámetros, como la desorganización lipídica de la membrana plasmática (merocianina M540) junto a la pérdida de integridad de la membrana del acrosoma (PNA). Es interesante destacar que aunque a las 4 horas de incubación con CC en un medio con estímulos capacitantes (Ca^{2+} y HCO_3^-) se produce tanto una pérdida de integridad de la membrana del acrosoma como un aumento de la

desorganización lipídica de la membrana plasmática, esto no indica que la inhibición de la AMPK provoque necesariamente un estado capacitado de los espermatozoides. Nuestra explicación se basa en el hecho de que en estas condiciones, la inhibición de la actividad de la AMPK no afecta en absoluto al porcentaje de espermatozoides que experimentan la reacción acrosómica cuando es inducida *in vitro* con un ionoforo de calcio. Basándonos en la idea de que únicamente en aquel espermatozoide que está capacitado se puede disparar la reacción acrosómica en respuesta a un estímulo inductor, postulamos que si la inhibición de la AMPK provocara realmente un estado capacitado en el espermatozoide, sería esperable que aumentara el número de espermatozoides en los que se dispara la reacción acrosómica inducida, y como se muestra en esta Tesis, eso no se produce. Por todo ello, nuestros resultados sugieren que los cambios inducidos por la actividad de la AMPK en las membranas plasmática y del acrosoma del espermatozoide no parecen estar relacionados con el proceso de capacitación del espermatozoide. Adicionalmente, los resultados de esta Tesis sugieren que la actividad de la AMPK no está implicada en la reacción acrosómica inducida experimentalmente en el espermatozoide.

Teniendo en cuenta la función clave de la AMPK en el control de la carga energética en células somáticas (Carling y cols., 2008; Hardie y cols., 2006; Hardie, 2011; Kahn y cols., 2005; Solaz-Fuster y cols., 2008), es lógico asumir que esta quinasa pueda estar involucrada de manera relevante en aquellas funciones celulares del espermatozoide, como la movilidad, que son altamente dependientes de los niveles de energía. En relación a la movilidad de esta célula germinal, a continuación se discuten los escenarios experimentales relacionados con los tres niveles diferentes de actividad de la AMPK que se han estudiado en esta Tesis:

a) Actividad de la AMPK próxima a niveles fisiológicos: a efectos de esta discusión, consideraremos como fisiológica (o próxima a la fisiológica) la actividad quinasa de la AMPK que presentan los espermatozoides incubados experimentalmente en los diferentes medios usados en esta Tesis (TBM,

TCM a 38,5°C) y siempre en ausencia del activador y del inhibidor de la AMPK. En estas condiciones, el nivel de actividad de AMPK es el óptimo para mantener una movilidad del espermatozoide que es la adecuada a las diferentes condiciones ambientales en las que se encuentre, entre las que se incluyen la presencia o ausencia de Ca^{2+}, HCO_3^- y/o BSA. Consideramos que la actividad de la AMPK en el rango fisiológico es idónea para poder responder a los diferentes niveles de demanda de energía que probablemente se requieren en las diversas condiciones extracelulares en las que el espermatozoide puede encontrarse a lo largo de su tránsito por el tracto reproductor femenino. Se han intentado mimetizar experimentalmente en esta Tesis las condiciones que conllevan a las diferentes demandas de energía que pueden probablemente existir *in vivo* al incubar los espermatozoides en un medio sin estímulos (TBM), que le conferirá una determinada carga energética, o en un medio con estímulos (Ca^{2+} y HCO_3^-), que lógicamente conlleva a esta célula germinal a requerir un mayor nivel de energía necesario para poder desempeñar los procesos funcionales que se encuentren inducidos por dichos estímulos.

b) Actividad de la AMPK por debajo de los niveles fisiológicos: esta aproximación se ha realizado experimentalmente en esta Tesis incubando la célula germinal masculina en presencia del inhibidor CC. En esta situación, la AMPK se encuentra total o mayoritariamente inactiva, lo que implica que, aunque pueda ser capaz de detectar la diferente carga energética (AMP/ATP) que tenga el espermatozoide en un medio TBM o en TCM, esta quinasa es incapaz de responder a esta situación, provocando los ajustes metabólicos necesarios para controlar el mantenimiento de los niveles celulares de ATP que requieran los espermatozoides en cada ambiente extracelular. Esa ausencia de control en el mantenimiento de la carga energética sostenido en el tiempo provocaría, entre otras consecuencias, que una de las principales funciones del espermatozoide dependiente mayoritariamente de ATP, como es la movilidad, se vea afectada

negativamente. Esto explicaría la inhibición inducida en presencia del compuesto C de los principales parámetros que caracterizan la movilidad del espermatozoide (porcentaje de espermatozoides mótiles, descenso de las velocidades curvilínea VCL, rectilínea VSL y promedio VAP y, consecuentemente, un descenso del número de espermatozoides rápidos cuya VAP>80 µm/s).

Los resultados de esta Tesis sugieren que la AMPK ejerce un relevante papel funcional en el control de la movilidad de los espermatozoides. En este sentido, recientemente se ha postulado que la AMPK, además de fosforilar sustratos que intervienen en el control del metabolismo manteniendo los niveles de energía celular, puede también modular funciones celulares a través de la fosforilación de otras dianas que incluyen proteínas asociadas a los microtúbulos. Esta emergente función de la AMPK puede adquirir una gran relevancia en células mótiles como son los espermatozoides. Así, se ha descrito que una quinasa que pertenece a la familia de la AMPK, denominada TSSK2 y que se expresa en espermatozoides (Xu y cols., 2007), fosforila *in vitro* una proteína del aparato central del axonema, denominada SPAG16L (Zhang y cols., 2008), que es esencial para la movilidad de espermatozoides de ratón. Teniendo esto en consideración es razonable asumir que también la AMPK podría fosforilar otros sustratos como proteínas del aparato central del axonema, similares a SPAG16L, o de otras estructuras celulares que son cruciales para la movilidad del flagelo del espermatozoide de mamíferos.

c) Actividad de la AMPK incrementada por encima de los niveles fisiológicos: nuevamente, a efectos de esta discusión, nos basamos en el planteamiento de que la actividad de la AMPK, incrementada experimentalmente por encima de los niveles fisiológicos por el activador A769662, regularía las vías metabólicas en espermatozoides al igual que lo hace en células somáticas. Como muestran los resultados de esta Tesis, la movilidad del espermatozoide se ve afectada negativamente cuando la actividad de AMPK

se incrementa por encima de los niveles fisiológicos adecuados para funcionar en un determinado ambiente extracelular. Una posible explicación se basa en el planteamiento de que un incremento sostenido en el tiempo de la actividad de la AMPK por encima del nivel fisiológico origina una situación de desregulación del metabolismo del espermatozoide, causada por la estimulación sostenida de rutas que generan ATP y por la inhibición sostenida de las rutas anabólicas que consumen ATP. Dicha situación no puede considerarse fisiológica, ya que su origen es una actividad incrementada y permanente de una proteína quinasa (AMPK), que de forma fisiológica, y al igual que ocurre en cualquier cascada de señalización intracelular, se regularía por inactivación al cabo de un cierto tiempo. Esa situación de desregulación o descontrol de las vías metabólicas no parece en absoluto adecuada para mantener una correcta movilidad del espermatozoide. De hecho, en estas condiciones de incremento sostenido de la actividad de AMPK, observamos, en general, una reducción del número de espermatozoides mótiles, un descenso de las velocidades curvilínea VCL, rectilínea VSL y promedio VAP y, consecuentemente, un menor número de espermatozoides rápidos (cuya VAP > 80 µm/s). Excepcionalmente, el número de espermatozoides mótiles en un medio sin estímulos añadidos no se ve afectado por un incremento de la actividad de la AMPK, lo que sugiere, por otro lado, que la presencia o ausencia de estímulos en el medio extracelular del espermatozoide condicionan el efecto de un incremento de la actividad de AMPK en la movilidad. En este sentido, es lógico pensar que la carga energética del espermatozoide en un medio sin estímulos es bastante diferente a la que tendría el espermatozoide en un medio con estímulos como Ca^{2+} ó HCO_3^-. Por ello, es plausible asumir que en determinadas condiciones extracelulares del espermatozoide, mimetizadas experimentalmente en esta Tesis por la presencia o ausencia de estímulos (Ca^{2+}, HCO_3^- y/o BSA), la actividad incrementada y permanente de la AMPK pueda causar un efecto diferente en algún parámetro de movilidad. De

hecho, esta idea se ve apoyada por los resultados en los que se comprueba el diferente efecto del activador A769662 en presencia y ausencia de otro estímulo importante para el espermatozoide, como la BSA.

De manera adicional a estas tres situaciones experimentales descritas y, como ya se ha mencionado anteriormente, esta Tesis demuestra que, al igual que ocurre en células somáticas, determinados tipos de estrés celular también son capaces de activar la AMPK en espermatozoides. Por otro lado, nuestro grupo de investigación ha demostrado recientemente que la actividad de la AMPK también está implicada en la regulación de la movilidad de los espermatozoides de cerdo conservados durante varios días a 17ºC (Martin-Hidalgo y cols., 2013), por ello extendemos nuestro planteamiento de que la actividad de la AMPK es esencial para mantener una óptima movilidad también a aquellas condiciones, como un descenso pronunciado de la temperatura, que pueden ser consideradas cómo estrés celular.

En resumen y en relación al papel de la AMPK en la movilidad de la célula germinal masculina de mamíferos, esta Tesis muestra que una actividad fisiológica de la AMPK en el espermatozoide es necesaria para poder desempeñar una movilidad óptima que les permitiría adecuarse a las cambiantes condiciones extracelulares a las que se ve sometido. Experimentalmente, estas diferentes condiciones se han abordado estudiando el papel de la AMPK en presencia o ausencia de calcio, bicarbonato o BSA, estímulos presentes, junto a otros, en el tracto genital femenino, lo que nos permitiría plantear que la función descrita de la AMPK en el mantenimiento de una correcta movilidad del espermatozoide podría desempeñarse también *in vivo.* Esta Tesis adicionalmente muestra que fluctuaciones importantes en el nivel de actividad de la AMPK en el espermatozoide que se alejan del nivel considerado fisiológico, tanto un exceso como una inhibición de dicha actividad, tienen un efecto negativo en la movilidad de dicha célula germinal.

Consideramos que este trabajo, que describe por primera vez la expresión y función de la AMPK en la célula germinal masculina, ha cumplido con el objetivo

último de una Tesis Doctoral que es, además de aportar nuevos resultados científicos y conclusiones inéditas, abrir nuevas líneas de investigación. En este sentido esperamos que esta Tesis suponga una base que permita en un futuro la investigación sobre la forma en que la AMPK regula los parámetros indicadores de la función y los procesos celulares que, en definitiva, permiten la función primordial del espermatozoide de mamífero.

Conclusiones/ Conclusions

- **1. La proteína quinasa activada por AMP, AMPK, se expresa en el espermatozoide de cerdo y se encuentra enzimáticamente activa en condiciones fisiológicas.**

- **2. La proteína AMPK se localiza principalmente en todo el acrosoma y en la pieza intermedia del flagelo del espermatozoide de cerdo. Cuando está activa, su localización en la pieza intermedia del flagelo se mantiene, sin embargo, en la cabeza del espermatozoide su localización está restringida a la parte más apical del acrosoma.**

- **3. La ruta de señalización que provoca la activación de AMPK por fosforilación en el espermatozoide de cerdo está regulada por mensajeros intracelulares como el calcio y el cAMP, así como por la proteína quinasa A (PKA), proteína quinasa C (PKC) y las proteínas quinasas α y/o β de la quinasa dependiente de calcio y calmodulina (CaMKKα/β).**

- **4. La proteína AMPK está involucrada en la regulación de la movilidad del espermatozoide de cerdo.**

- **5. La proteína AMPK desempeña una importante función en las membranas del espermatozoide de cerdo. Por un lado, está implicada en el mantenimiento de la integridad de la membrana externa del acrosoma y, por otro, contribuye tanto al mantenimiento de la organización lipídica de la membrana plasmática, como al control de la translocación de la fosfatidilserina hacia su cara externa.**

- **6. La proteína quinasa AMPK está implicada en la regulación del potencial de membrana mitocondrial del espermatozoide de cerdo.**

- **7. La proteína quinasa AMPK se activa por fosforilación en el espermatozoide de cerdo en repuesta a determinados tipos de estrés celular.**

- **1. AMP-activated protein kinase AMPK is expressed in boar spermatozoa and is active under physiological conditions.**

- **2. AMPK protein is mainly localized at the whole acrosome and in the middle piece of boar spermatozoa flagellum. When active, p-Thr172-AMPK remains localized at the middle piece of flagellum, while in spermatozoa head its localization is restricted to the most apical part of the acrosome.**

- **3. Intracellular mechanisms leading to AMPK activation by phosphorylation in boar spermatozoa involved intracellular messengers such as calcium and cAMP, as well as signalling pathways of the protein kinase A (PKA), protein kinase C (PKC), protein kinase α and/or β of calcium-calmodulin dependent kinase (CaMKKα/β).**

- **4. AMPK protein Kinase is involved in the regulation of motility in boar spermatozoa.**

- **5. AMPK protein plays a relevant function in boar spermatozoa membranes. In one hand, AMPK is involved in the maintenance of the outer acrosome membrane integrity, and on the other hand, it contributes to the maintenance of both the lipid organization of plasma membrane, and the phosphatidylserine translocation to its outer face.**

- **6. AMPK protein kinase is involved in the control of mitochondrial membrane potential in boar spermatozoa.**

- **7. AMPK protein kinase becomes activated by phosphorylation in boar spermatozoa in response to several germ cell stresses.**

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Anexos

Anexo I

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PLoS one

AMP-Activated Kinase AMPK Is Expressed in Boar Spermatozoa and Regulates Motility

Ana Hurtado de Llera, David Martin-Hidalgo, María C. Gil, Luis J. Garcia-Marin*, María J. Bragado*

Research Group of Intracellular Signalling and Technology of Reproduction (SINTREP), Veterinary School, University of Extremadura, Caceres, Spain

Abstract

The main functions of spermatozoa required for fertilization are dependent on the energy status and metabolism. AMP-activated kinase, AMPK, acts a sensor and regulator of cell metabolism. As AMPK studies have been focused on somatic cells, our aim was to investigate the expression of AMPK protein in spermatozoa and its possible role in regulating motility. Spermatozoa from boar ejaculates were isolated and incubated under different conditions (38,5°C or 17°C, basal medium TBM or medium with Ca^{2+} and bicarbonate TCM, time from 1–24 hours) in presence or absence of AMPK inhibitor, compound C (CC, 30 μM). Western blotting reveals that AMPK is expressed in boar spermatozoa at relatively higher levels than in somatic cells. AMPK phosphorylation (activation) in spermatozoa is temperature-dependent, as it is undetectable at semen preservation temperature (17°C) and increases at 38,5°C in a time-dependent manner. AMPK phosphorylation is independent of the presence of Ca^{2+} and/or bicarbonate in the medium. We confirm that CC effectively blocks AMPK phosphorylation in boar spermatozoa. Analysis of spermatozoa motility by CASA shows that CC treatment either in TBM or in TCM causes a significant reduction of any spermatozoa motility parameter in a time-dependent manner. Thus, AMPK inhibition significantly decreases the percentages of motile and rapid spermatozoa, significantly reduces spermatozoa velocities VAP, VCL and affects other motility parameters and coefficients. CC treatment does not cause additional side effects in spermatozoa that might lead to a lower viability even at 24 h incubation. Our results show that AMPK is expressed in spermatozoa at high levels and is phosphorylated under physiological conditions. Moreover, our study suggests that AMPK regulates a relevant function of spermatozoa, motility, which is essential for their ultimate role of fertilization.

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* E-mail: ljgarcia@unex.es (LJGM); jbragado@unex.es (MJB)

These authors contributed equally to this work.

Introduction

The spermatozoon is a germ cell that is highly specialized for cellular processes, motility, capacitation, hyperactivation and acrosome reaction that promote its essential function of oocyte fertilization. All these cellular processes are dependent on the energetic cellular state, determined by the ratio between cellular AMP and ATP, [1], [2] and regulated by biochemical mechanisms such as phosphorylation of proteins. Spermatozoa possess an elaborated intracellular compartmentalization and, in the last phase of development, are transcriptionally inactive and thus unable to synthesize proteins. Therefore, the intracellular pathways that regulate those cellular processes based in post translation modifications of pre-existing proteins, such as phosphorylation, catalyzed by kinases, are especially important in these germ cells.

The AMP activated protein kinase AMPK is an evolutionarily conserved serine/threonine kinase that acts as a sensor that detects the cell energy state and subsequently regulates metabolism [3]. AMPK is a heterotrimeric protein that has a catalytic α and two regulatory subunits, β and γ. One of the essential features of the AMPK kinase as a sensor and metabolic regulator is its extreme sensitivity to AMP, as any increase in the ratio AMP/ATP that means a decrease in cellular energy state, activates AMPK [3], [4]. Optimal allosteric activation of AMPK, which is induced by binding of AMP to the γ subunit, requires formation of the αβγ complex [3], [5], [6], [7]. In addition to allosteric activation by AMP, phosphorylation of the Thr^{172} residue, located at the critical activation loop of the α subunit, is absolutely required for full AMPK activation [8]. Phosphorylation of AMPK is carried out by an upstream kinase that functions as a tumor suppressor called LKB1 (Peutz-Jerhers protein). Additionally, AMP binding to AMPK inhibits dephosphorylation of Thr^{172}. When AMPK becomes activated it stimulates catabolic pathways that produce ATP, while simultaneously inhibits ATP-consuming anabolic pathways [9], [10], therefore the overall metabolic consequences of AMPK activation is the maintenance of cellular energy stores. The best known substrates of AMPK are acetyl CoA-carboxylase [11] and hydroxymethylglutaryl CoA-reductase, which are the most regulated enzymes in the synthesis pathways of fatty acids and cholesterol, respectively, and also the phosphofructokinase 2, key enzyme in the carbohydrate metabolism [3], [4], [12]. However, AMPK is a ser/thr kinase and may regulate processes outside metabolism [13]. Recently it has been demonstrated that AMPK activity is also induced by several types of stimuli involving metabolic stresses such as glucose deprivation, hypoxia, ischemia, oxidative or hyperosmotic stress [4], heat shock or alterations of

mitochondrial oxidative production [3], [14], [15]. Some AMPK stimuli as hyperosmotic stress do not alter AMP/ATP ratio suggesting that other mechanisms are involved in its activation. Recent studies identified the calcium calmodulin-dependent protein kinase kinase (CaMKK) as an enzyme that also activates AMPK [3], [4] by an increase in calcium concentration, with no appreciable change in the AMP/ATP ratio. As CaMKK is expressed at very high levels in the central nervous system and to a lesser extent in other tissues, it is postulated that the intracellular pathway of AMPK may be regulated by multiple mechanisms that are possibly cell type specific.

All AMPK studies have been conducted exclusively in somatic cells, and there is no work performed in spermatozoa to date. However, there are some studies in germ cells with AMPK-related kinases, as recently it has been reported that a shorter isoform of LKB1, called LKB1s, is expressed predominantly in haploid sperm cells from testes of mammals [16]. LKB1s knockout mice have a dramatic reduction in the number of mature spermatozoa in the epididymis, and the few spermatozoa produced are not mobile, have an abnormal head morphology and resulted sterile [16]. These data suggest that this variant of the LKB1 has a crucial role in spermiogenesis and fertility in mice. Moreover, members of "ser/thr kinase testis specific" TSSK family, which belongs to the AMPK branch in the human kinome tree, have been identified in human spermatozoa: TSSK2, TSKS and SSTK [17]. Deletion of TSSK1 and 2 causes male infertility in chimera mice due to haploinsufficiency [18].

Besides the mentioned upstream kinases LKB1 and CaMKK in somatic cells, it has been demonstrated that protein kinase A (PKA) regulates AMPKα activity by phosphorylation at Ser-173 in mouse adipocytes [19]. The fact that PKA pathway, which is stimulated by HCO_3^- [20] or Ca^{2+} in spermatozoa, highly regulates spermatozoa function [21], further supports the idea that AMPK might play a role in male germ cells.

The study of mechanisms by which spermatozoa regulates their energy status through AMPK is very important for the understanding of the ability of these germ cells to survive and adapt to external extreme conditions such as the transit through the female genital tract. In this regard, our hypothesis is that AMPK would act a sensor molecule of the cell energetic state of spermatozoa and subsequently would regulate their most relevant cellular processes required for successful fertilization. Therefore, the aim of this work is to study the protein expression and activity status of AMPK in spermatozoa, and in addition to investigate its role in the regulation of one of the most important functional procesess of these germinal cells: motility.

Results

Expression of AMP Activated Kinase, AMPK, in Boar Spermatozoa

A western blotting analysis was performed to determine whether AMPK is expressed in boar spermatozoa. Two cross reactive bands are detected in boar spermatozoa lysates using a specific antibody against the α catalytic subunit of AMPK (Figure 1, lane 1). Positive controls for this antibody were tested including proteins extracted from other porcine tissues such as brain, lung or heart, where a single reactive band is detected and corresponds to the smaller molecular weight band identified in spermatozoa. Negative control for this antibody was performed omitting the primary antibody and blot was probed with secondary antibody (anti-rabbit-HRP) only. Results show that no band is detected with the secondary antibody and confirm that bands visualized are due to the AMPKα antibody used (data not shown). It is interesting to mention that the expression level of AMPK protein detected in male germ cells (7 μg proteins were loaded into the SDS-PAGE) is likely higher than in somatic cells derived from other porcine tissues (20 μg proteins).

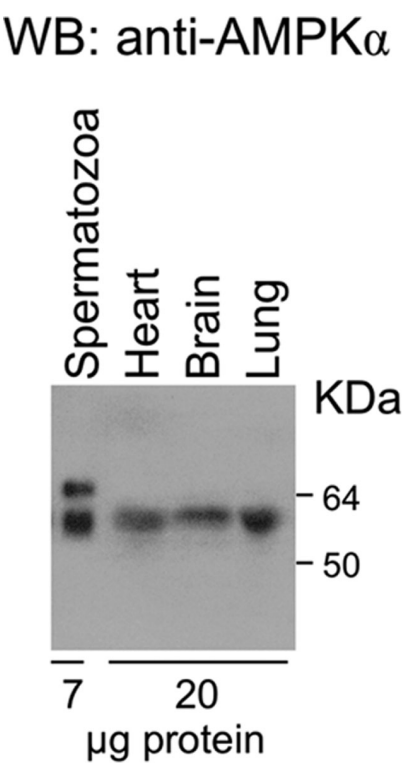

Figure 1. Expression of AMP-activated kinase protein, AMPK, in boar spermatozoa. Protein from boar spermatozoa lysates (lane 1) were analyzed by western blotting using anti-AMPKα as primary antibody. Other porcine tissues were homogenized and used as positive controls (hearth, lane 2; brain, lane 3 and lung, lane 4). Molecular weight markers are indicated in kDa and the amount of protein (μg) loaded into each lane is shown at the bottom. This experiment was performed 7 times and a representative film is shown.
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AMPK is Phosphorylated at Thr^{172} (Activated) in Basal Physiological Conditions in Boar Spermatozoa

The level of phosphorylation of AMPK in Thr^{172} was analyzed at physiological temperature of boar spermatozoa (38.5°C) as an assessment of its enzymatic activity. As seen in Figure 2A, two cross-reactive bands are detected with anti-phospho-Thr^{172}-AMPK antibody, being the upper band the AMPK phosphorylated at Thr^{172}, as i) the molecular weight is the proper to the α subunit of AMPK and ii) this upper band is also recognized with the anti AMPKα antibody used in Figure 1. Negative control was also performed omitting the primary antibody and blot was probed with secondary antibody (anti-rabbit-HRP) only. No bands are detected with the secondary antibody and confirm that bands visualized are due to the anti-phospho-Thr^{172}-AMPK antibody used (data not shown). As shown in Figure 2A, a clear band of phospho-Thr^{172}-AMPK is detected when spermatozoa are incubated at 38.5°C in TBM or in a medium with calcium and bicarbonate (TCM). The intensity of the AMPK phosphorylated band results dependent on the incubation time at 38.5°C, reaching highest levels of phosphorylation between 30–60 minutes. When the same pools

of spermatozoa are incubated either in a TBM or TCM at lower temperature, 17°C (considered as minute 0 in the Figure), which is the routine value for porcine semen preservation with low energy consumption, AMPK phosphorylation is very low or even not detectable. This effect is independent of the incubation time of spermatozoa at 17°C (data not shown). A loading control of protein is showed in lower panel of Figure 2A using an anti-GSK3β antibody, as we have previously shown that level of this protein does not change under these experimental conditions [30].

The AMPK Inhibitor, Compound C, Effectively Blocks AMPK Phosphorylation in 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, selective, reversible, and ATP-competitive inhibitor of AMPK. Thus, we initially decided to confirm that CC (30 μM) effectively blocks the phosphorylation (activity state) of AMPK in male germ cells. Based on the range of CC concentrations used in the literature in somatic cells, we initially tried several concentrations of CC (1, 10 and 30 μmol/l) in boar spermatozoa. Our results are showed in Figure 2B and demonstrate not only that AMPK remains phosphorylated at physiological temperature after 24 h of incubation but also that 30 μmol/l CC blocks phosphorylation of this kinase in

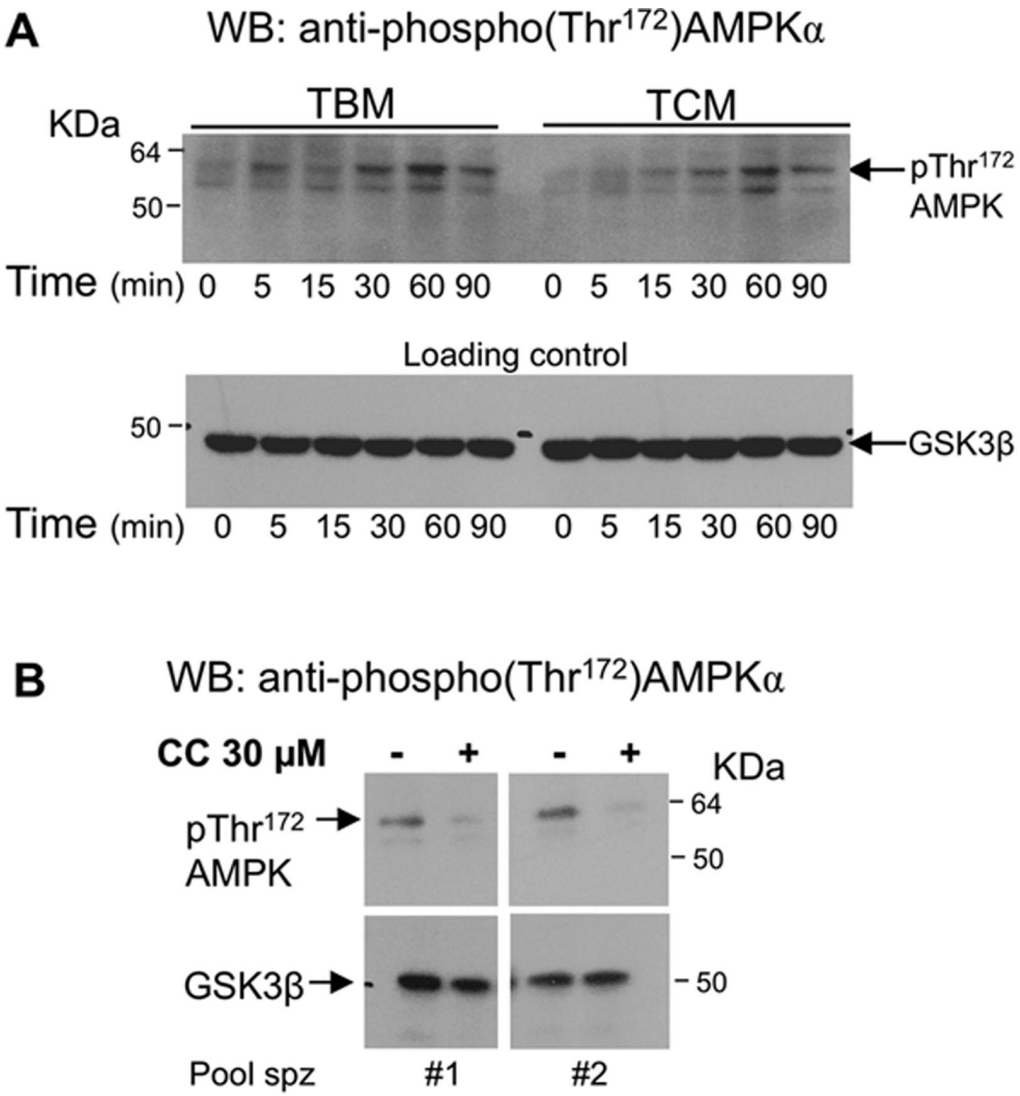

Figure 2. Phosphorylation of AMPK at Thr^{172} is regulated at physiological temperature in boar spermatozoa and is effectively inhibited by the compound C. 2A: Spermatozoa were incubated in TBM or TCM medium at 38.5°C for indicated times and then lysed. Samples at 17°C were considered as time 0. Proteins (20 μg) from lysates were analyzed by western blotting using anti-phospho-Thr^{172}-AMPKα as primary antibody. Arrow indicates the cross-reactive band of phospho-Thr^{172}AMPK, recognized by the anti-AMPKα. This experiment was performed 6 times and a representative film is shown. 2B: AMPK phosphorylation was evaluated in spermatozoa incubated in TBM in the presence (+) or absence (−) of AMPK inhibitor, compound C (CC 30 μM) at 38.5°C during 24 h. This experiment was performed 3 times and a representative film is shown. Loading controls were performed for each experiment in the same membranes (with different time of exposure) using anti-GSK3β antibody and are showed at lower panels in 2A and 2B.
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spermatozoa. Moreover, under these conditions CC does not affect the levels of AMPKα in spermatozoa (data not shown).

AMPK Inhibition by Compound C Significantly Reduces the Percentage of Motile Spermatozoa

To evaluate the effect of the AMPK inhibition by CC (30 μM) in motility, boar spermatozoa were incubated in TBM or TCM, in the presence or absence of CC for different times at physiological temperature 38,5°C. In addition, we have analyzed motility parameters at semen preservation temperature 17°C and included as time 0. It is well described in the literature that the increase in the temperature up to the physiological 38,5°C in boar is a potent stimulator of spermatozoa motility [21]. As observed in Figure 3, short treatment (0–4 h) with CC at 38,5°C leads to a significant reduction in the percentage of motile spermatozoa in either TBM (3A) or TCM (3B). The reduction of the percentage of motile spermatozoa is time-dependent and results statistically significant as rapid as at 1 hour in TCM (3B) or 2 h in TBM (3A). The fall in this percentage caused by AMPK inhibition is clearly greater in TCM (where there is a 66% of reduction in the percentage of motile spermatozoa after AMPK inhibition at 4 h; Fig. 3B) than in TBM (where there is a 26% of reduction in the percentage of motile spermatozoa after AMPK inhibition at 4 h; Fig. 3A).

AMPK Inhibition Significantly Decreases the Percentage of Rapid Spermatozoa

Rapid spermatozoa are defined as the percentage of those motile spermatozoa with velocity VAP higher than 80 μm/s. As seen in Figure 4 and according to previous literature [21], the time-course of the percentage of rapid spermatozoa incubated in TBM (Fig. 4A) clearly differs from the time-course in TCM (Fig. 4B). The inhibition of AMPK by CC in either TBM or TCM leads to a significant reduction in the percentage of those motile spermatozoa that move in a rapid manner in a time-dependent manner. However, whereas in TBM the reduction is detected at 2 h and maximum at 4 h of AMPK inhibition (reduction of number of rapid spermatozoa by 44%), in TCM the CC inhibitory effect is detected as rapid as 1 h and remains constant at any time studied (about 30% of reduction of the percentage of rapid spermatozoa).

AMPK Inhibition Significantly Reduces Spermatozoa Velocities and Affects other Motility Parameters

As seen in other motility parameters, the time-course of the different velocities analyzed in spermatozoa incubated in TBM clearly differs from the time-course in TCM (Figs. 5 and 6; Tables 1 and 2). Inhibition of AMPK by CC in spermatozoa incubated either TBM or TCM significantly reduces any spermatozoa velocity studied, including average path velocity VAP (Figure 5), curvilinear velocity VCL (Figure 6) and straight linear velocity VSL (Tables 1 and 2) in a time dependent manner. This CC inhibitory effect is detected in TBM (Figs. 5A and 6A; Table 1) at 2 h and the highest reduction in the velocity is achieved at 4 h of AMPK inhibition (VAP by 23%, VCL by 26% and VSL by 13%). However, in TCM (Figs. 5B and 6B; Table 2) the CC inhibitory effect is detected as rapid as 1 h and remains constant at any time studied (22% of reduction of VAP, 24% reduction in the case of VCL and 15% reduction in VSL).

Moreover, AMPK inhibition by treatment of spermatozoa with CC incubated in either TBM or TCM for short term (up to 4 h) significantly affects in different ways other spermatozoa motility parameter analyzed: LIN, STR, WOB, ALH and BCF (Tables 1 and 2).

Effect of the Long-term AMPK Inhibition by CC in Spermatozoa Motility Parameters

As literature describes that effects of AMPK inhibition in somatic cells can be obtained not only a short-term but at long-term [22], we next evaluated the action of AMPK inhibition in spermatozoa motility at longer times than 4 h by treatment with CC for 24 hours (Table 3). Inhibition of AMPK for longer time in TBM induces a higher and significant effect than short term in all spermatozoa motility parameters mentioned. Thus, potent reduction of 82% in the percentage of motile spermatozoa, as well as

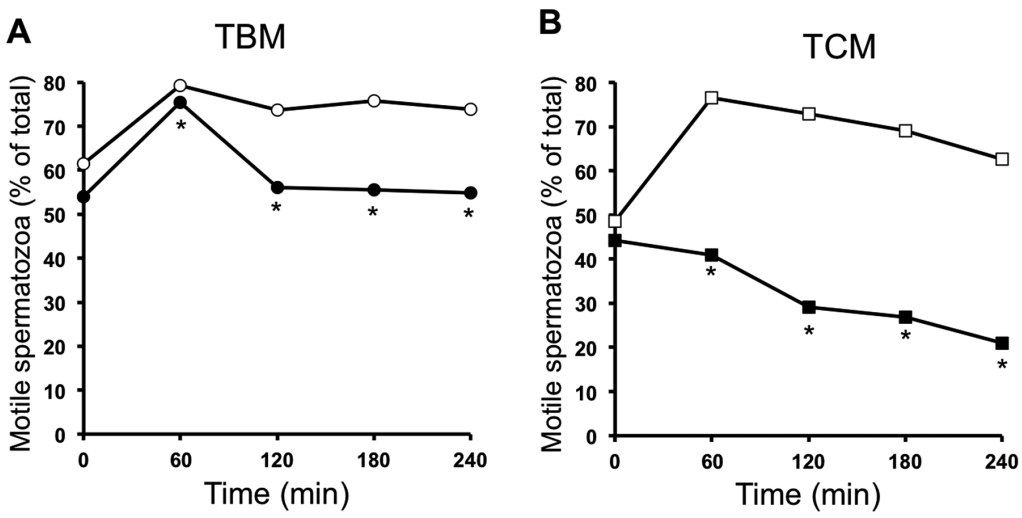

Figure 3. Short time effect (0–4 h) of the AMPK inhibition by CC in the percentage of motile spermatozoa. Spermatozoa were incubated in TBM (A, circles) or TCM medium (B, squares) at 38.5°C in the absence (white) or presence (black) of the AMPK inhibitor, compound C, (CC 30 μM) during 4 h. Samples at 17°C were considered as time 0. The percentage of motile spermatozoa was measured by the ISAS system as described. This experiment was performed at least 6 times and the results express the percentage of motile spermatozoa from the total analyzed (4.000–5.000). Statistical differences were considered when $p<0.05$ and showed with an asterisk.
doi:10.1371/journal.pone.0038840.g003

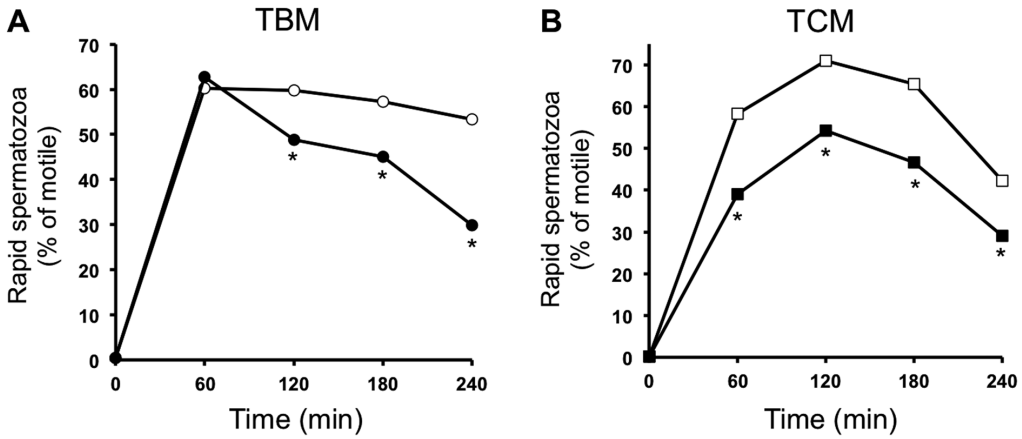

Figure 4. Short time effect (0–4 h) of the AMPK inhibition in the percentage of rapid spermatozoa. Spermatozoa were incubated in TBM (A, circles) or TCM medium (B, squares) at 38.5°C in the absence (white) or presence (black) of the AMPK inhibitor, compound C, (CC 30 μM) during 4 h. Samples at 17°C were considered as time 0. The percentage of those motile spermatozoa with VAP>80 μm/s, defined as rapid spermatozoa, was measured. This experiment was performed at least 6 times and the results express the percentage of rapid spermatozoa from the total spermatozoa motile analyzed (4.000–5.000). Statistical differences were considered when $p<0.05$ and showed with an asterisk.
doi:10.1371/journal.pone.0038840.g004

a full reduction of the percentage of rapid spermatozoa were obtained by long term CC treatment (Table 3). Concomitantly, AMPK inhibition by 24 h causes a clear and significant diminution in any spermatozoa velocity analyzed: VCL as an estimate of instantaneous sperm swimming speed, by 45%, VSL by 61% and VAP by 53%. The rest of spermatozoa motility parameters were also significantly reduced by 24 h of AMPK inhibition with CC.

Effect of the AMPK Inhibition by CC in the Viability of Boar Spermatozoa

We have studied the effect of AMPK inhibition by CC in spermatozoa viability in order to correlate it with motility studies and in addition to know whether CC treatment might cause spermatozoa side effects that lead to germ cell death. According to our previous results [21], viability of boar spermatozoa is sensitive to both temperature and the presence of Ca^{2+} and/or bicarbonate in the medium in a time dependent manner, as confirmed in Table 4. Thus, spermatozoa viability measured as the number of SYBR-14^{+}/IP^{-} spermatozoa, decreases after 4 hours at 38,5°C by 30% in TBM and by 42% in TCM. Short-term exposure to compound C does not affect in a significant manner the percentage of viable spermatozoa in any experimental condition (Table 4). However, a slight but reproducible effect of CC preventing the loss of cell viability is observed in TBM, were 66% of sperm cells remain viable after 4 h with CC versus 59% in the absence of CC. This protective effect of compound C in the loss of spermatozoa viability in TBM becomes significant at 24 h of treatment (Table 4), where 63% of spermatozoa remain viable in the presence of compound C compared with 49% in its absence.

Discussion

The control of cell metabolism in spermatozoa is achieved by dynamic mechanisms able to adapt to environmental changes and

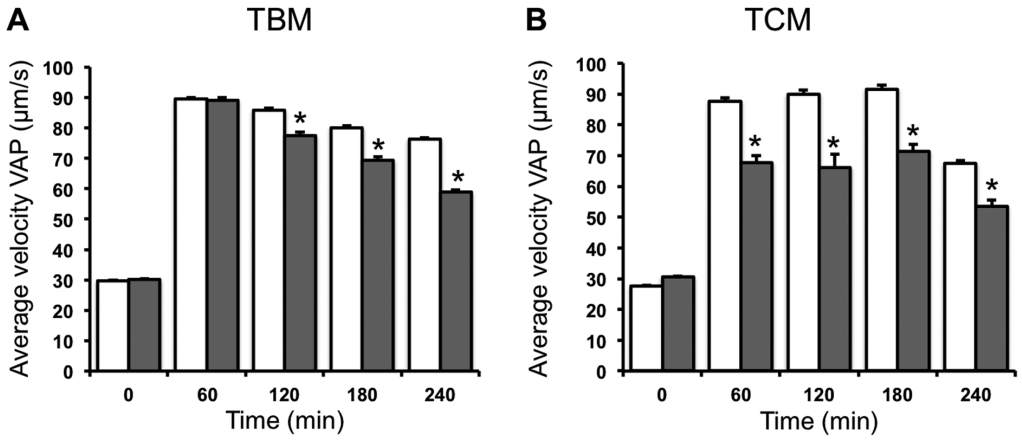

Figure 5. Short time effect (0–4 h) of the AMPK inhibition in the average path velocity, VAP, of boar spermatozoa. Spermatozoa were incubated in TBM (A) or TCM medium (B) at 38.5°C in the absence (unfilled bars) or presence (filled bars) of the AMPK inhibitor compound C, (CC 30 μM) during 4 h. Samples at 17°C were considered as time 0. The average path velocity (VAP) was measured and expressed as μm/s. This experiment was performed at least 6 times and the results express the mean ± standard error of the mean. Statistical differences were considered when $p<0.05$ and showed with an asterisk.
doi:10.1371/journal.pone.0038840.g005

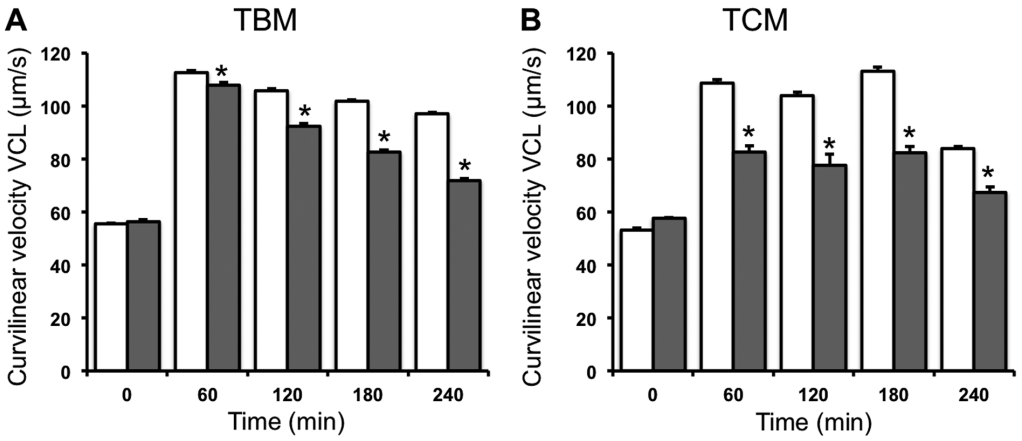

Figure 6. Short time effect (0–4 h) of the AMPK inhibition by CC in the curvilinear velocity, VCL, of boar spermatozoa. Spermatozoa were incubated in TBM (A) or TCM medium (B) at 38.5°C in the absence (unfilled bars) or presence (filled bars) of the AMPK inhibitor compound C, (CC 30 μM) during 4 h. Samples at 17°C were considered as time 0. The curvilinear velocity VCL was evaluated and expressed as μm/s. This experiment was performed at least 6 times and results express the mean ± standard error of the mean. Statistical differences were considered when $p<0.05$ and showed with an asterisk.
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related with cellular structures such as mitochondria or plasmalemma [23]. Therefore, regulation of the energy levels during changing extracellular conditions, such as those leading to oocyte fertilization, is of essential importance in the understanding of spermatozoa function. In mammalian tissues the protein AMPK controls metabolism [8], [24] by activating metabolic pathways that produce ATP and simultaneously by inhibiting those pathways that consume ATP [9], [10], however, to date there are not data about the presence or function of AMPK in male germ cells.

The present study shows for the first time that the metabolic sensor AMPK is expressed in spermatozoa. The AMPK antibody against α subunit recognizes two bands with close molecular weight in boar spermatozoa, in agreement with the data obtained in vascular smooth muscle from the same specie [25]. As the AMPKα antibody reacts with two α isoforms of human origin (α1 and α2), these two reactive bands in porcine spermatozoa likely include α1 and α2 isoforms of AMPK. It is interesting to mention that the expression level of AMPKα protein detected in male germ cells is likely higher than in somatic cells derived from porcine heart, brain and lung, where a unique reactive band is detected.

Our results demonstrate that AMPK is phosphorylated at Thr^{172}, and therefore subsequently activated, at physiological temperature of boar spermatozoa (38,5°C). Although the phosphorylation level of the kinase varies with the time of incubation at 38,5°C increasing mainly in the first 60 minutes, AMPK phosphorylation (activation) remains clearly detectable after 24 h of treatment. Our results suggest that phosphorylation of AMPK in spermatozoa does not require Ca^{2+} and/or bicarbonate in the extracellular medium to occur, as it is detected either in presence or in absence of these ions. The fact that AMPK is phosphorylated at physiological temperature and that phosphorylation occurs both in TBM and TCM, which contains Ca^{2+} and bicarbonate, suggests that activation of this kinase likely occurs *in vivo* under those physiological conditions of spermatozoa when transiting through the female reproductive tract that are experimentally mimicked by these two media.

Potential side effects of the treatment with a pharmacological inhibitor as CC can be rule out in this study as spermatozoa viability was analyzed in parallel experiments. In this sense, even the longest time of CC incubation (24 h) does not cause any side effect that might lead to a loss in spermatozoa viability. By contrary, our results suggest that AMPK activity might be involved

Table 1. Short term effect (0–4 h) of the AMPK inhibition by compound C (CC) in boar spermatozoa motility parameters in TBM.

	VSL (μm/s)		LIN (%)		STR (%)		WOB (%)		ALH (μm)		BCF (Hz)	
Min.	**TBM**	**TBM + CC**	**TBM**	**TBM + CC**	**TBM**	**TBM + CC**	**TBM**	**TBM + CC**	**TBM**	**TBM + CC**	**TBM**	**TBM + CC**
0	16.9±0.2	17.7±0.3	31.5±0.2	32.6±0.4	56.9±0.3	58.2±0.6	54.4±0.2	54.6±0.3	2.8±0.0	2.8±0.0	6.4±0.1	6.9±0,1
60	66.2±0.6	68.3±0.9	54.8±0.4	58.9±0.6*	68.8±0.4	71.6±0.5	75.9±0.2	79.2±0.3*	3.6±0.0	3.4±0.0*	7.2±0.1	7.3±0.1
120	67.2±0.7	62.4±1.2*	58.7±0.4	61.6±0.7*	72.4±0.4	73.8±0.7	77.3±0.3	79.3±0.5*	3.3±0.0	2.7±0.0*	7.8±0.1	7.3±0.1*
180	62.5±0.5	58.0±1.0*	57.5±0.4	63.6±0.6*	72.4±0.4	76.3±0.6*	75.8±0.2	79.7±0.4*	3.4±0.0	2.6±0.0*	8.1±0.1	7.7±0.1*
240	60.6±0.5	49.5±0.8*	58.7±0.4	62.7±0.6*	73.9±0.4	76.7±0.6*	75.9±0.2	78.5±0.4*	3.2±0.0	2.5±0.0*	8.3±0.1	7.9±0.1*

Spermatozoa were incubated in TBM at 38.5°C in the presence or absence of the AMPK inhibitor, CC, (30 μM) during 4 hours. Samples measured at 17°C were considered as time 0. Spermatozoa motility parameters including straight-line velocity (VSL, expressed in μm/s) and coefficients LIN (Linearity coefficient in %), STR (Straightness coefficient in %), WOB (Wobble coefficient in %), ALH (Amplitude of lateral head displacement in μm) and BCF (Beat cross frequency in Hz) were measured by the ISAS system. This experiment was performed at least 6 times and results express the mean ± standard error of the mean. Statistical differences were considered when $p<0.05$.
doi:10.1371/journal.pone.0038840.t001

Table 2. Short term effect (0–4 h) of the AMPK inhibition by compound C (CC) in boar spermatozoa motility parameters in TCM.

	VSL (μm/s)		LIN (%)		STR (%)		WOB (%)		ALH (μm)		BCF (Hz)	
Min.	**TCM**	**TCM + CC**	**TCM**	**TCM + CC**	**TCM**	**TCM + CC**	**TCM**	**TCM + CC**	**TCM**	**TCM + CC**	**TCM**	**TCM + CC**
0	14.4±0.3	16.5±0.2	27.8±0.4	29.1±0.3	52.5±0.6	53.2±0.4	52.6±0.3	53.9±0.2	2.8±0.0	2.9±0.0	5.9±0.1	6.2±0.1
60	64.0±1.1	54.6±2.1*	53.9±0.6	58.8±1.2*	67.2±0.6	71.4±1.1*	76.9±0.4	78.4±0.8	3.6±0.0	2.7±0.1*	6.8±0.1	5.9±0.2*
120	63.9±1.1	73.3±3.1*	57.5±0.6	68.2±1.6*	71.4±0.6	79.3±1.4*	76.5±0.4	83.1±1.0*	3.3±0.0	2.7±0.1*	6.9±0.1	7.3±0.2
180	71.8±1.3	62.7±2.2*	61.8±0.7	68.5±1.3*	75.5±0.7	80.4±1.2*	79.4±0.4	82.2±0.8	3.6±0.0	2.5±0.1*	8.2±0.1	7.1±0.2*
240	55.1±0.9	45.5±2.1*	60.6±0.3	53.5±1.7	75.1±0.6	74.4±1.6	77.3±0.4	75.9±1.2	2.9±0.0	2.4±0.1*	7.5±0.1	6.7±0.2*

Spermatozoa were incubated in TCM at 38.5°C in the presence or absence of the AMPK inhibitor, CC, (30 μM) during 4 hours. Samples measured at 17°C were considered as time 0. Spermatozoa motility parameters including straight-line velocity (VSL, expressed in μm/s) and coefficients LIN (Linearity coefficient in %), STR (Straightness coefficient in %), WOB (Wobble coefficient in %), ALH (Amplitude of lateral head displacement in μm) and BCF (Beat cross frequency in Hz) were measured by the ISAS system. This experiment was performed at least 6 times and results express the mean ± standard error of the mean. Statistical differences were considered when $p<0.05$.

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in the modulation of the control of spermatozoa viability under some conditions, as compound C significantly prevents the loss in viability induced by time and temperature in TBM. Interestingly, compound C has not effect in the viability of spermatozoa incubated in TCM, which suggest that the possible regulatory role of AMPK in germ cell viability is dependent on the presence of Ca^{2+} and/or bicarbonate in the medium. This is not surprising, as Ca^{2+} is a major regulator of AMPK activity in somatic cells.

The fact that CC treatment causes a clear and significant reduction of the percentage of motile spermatozoa implies that AMPK inhibitor induces a potent increase in the number of motionless spermatozoa. Under these conditions, those remnant spermatozoa that still are motile move with significantly lower speed, as AMPK inhibition leads to a significant decrease in the number of rapid spermatozoa (VAP>80 μm/s). Concomitantly, AMPK inhibitor reduces spermatozoa curvilinear velocity VCL (an estimate of instantaneous sperm swimming speed) and average path velocity VAP. Although we rule out potential side effects of CC in spermatozoa, we cannot exclude the possibility that the effects observed on spermatozoa motility following CC treatment might result from disruption of other pathways, independently of AMPK. Therefore, our results, showing that the AMPK inhibitor exerts a potent anti-motility effect in boar spermatozoa, evaluated by different parameters, allow us to suggest that AMPK activity is likely necessary for optimal spermatozoa motility. Moreover, they also suggest that AMPK is not sufficient to achieve proper sperm motility. This idea is expected as our previous studies and others have established that the control of spermatozoa motility is achieved by the contribution, in a convergent or parallel manner, of several signalling kinase pathways [26], [27], [28], [29], [30].

The cellular action most related to spermatozoa motility induced by the AMPK inhibitor CC found in the literature is the inhibition of cell migration in different type of cancer cells such as glioma [31] or ovarian cancer cells [32]. As mentioned, there are not previous reports about the role of AMPK in spermatozoa motility.

Having in mind the energy-regulating role of AMPK in somatic cells, it logical to assume that it may play a role in those spermatozoa functions that are particularly dependent on the energy levels, such as motility. Specifically, AMPK plays a central role in the maintenance of cell energy levels by regulating among others pathways, the glycolysis [33]. Thus, as spermatozoa motility is totally dependent on ATP supply, generated mainly via glycolylis [34] or by mitochondrial activity, it is reasonable to

Table 3. Long-term effect (24 h) of the AMPK inhibition by CC in boar spermatozoa motility.

SPERMATOZOA MOTILITY PARAMETERS	TBM	TBM + CC (30 μM)
Motile spermatozoa (%)	58.1[a]	10.1[b]
Rapid spermatozoa (VAP>80 μm/s) (%)	7.7[a]	0.5[b]
VCL (μm/s)	56.2±0.4[a]	31.2±0.9[b]
VSL (μm/s)	38.1±0.4[a]	14.9±0.6[b]
VAP (μm/s)	45.6±0.4[a]	21.4±0.6[b]
LIN (%)	64.2±0.5[a]	49.5±1.3[b]
STR (%)	78.5±0.4[a]	65.8±1.4[b]
WOB (%)	78.9±0.3[a]	70.9±0.9[b]
ALH (μm)	2.0±0.01[a]	1.7±0.03[a]
BCF (Hz)	8.1±0.07[a]	4.1±0.1[b]

Spermatozoa were incubated in TBM at 38.5°C in the presence or absence of the AMPK inhibitor, CC, (30 μM) during 24 h. Spermatozoa motility parameters including the percentages of motile and rapid spermatozoa, curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL) and coefficients LIN, STR, WOB, ALH and BCF were measured by the ISAS system. This experiment was performed at least 5 times and results express the mean ± standard error of the mean. Statistical differences are shown as a, b when $p<0.0001$ between treatments (columns).

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Table 4. Effect of the AMPK inhibition by compound C in spermatozoa viability.

Cell Viability	TBM	TBM + CC (30 μM)	TCM	TCM + CC (30 μM)
17°C	83.22±2.0[a]	83.22±2,.[a]	82.57±1.9[a]	82.57±1.9[a]
1 h (38.5°C)	77.22±1.4[a]	77.80±2.6[a]	70.72±2.1[a]	67.30±3.1[a]
4 h (38.5°C)	58.97±6.5[a]	66.32±3.2[a]	48.20±5.7[a]	51.25±5.7[a]
24 h (38.5°C)	48.77±2.1[a]	62.49±1.9[b]	54.83±3.7[a]	56.63±3.4[a]

Spermatozoa were incubated in TBM or TCM medium at 17°C or 38.5°C in absence or presence of the AMPK inhibitor CC (30 μM) for different times (1–24 h). Spermatozoa viability was measured by flow cytometry using SYBR-14 and IP as probes. This experiment was performed at least 4 times and the results expressed as percentage of viable cells are shown as mean ± standard error of the mean. Statistical differences are shown as a,b when $p<0.001$ between treatments (columns).

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assume that AMPK activity is likely required for a optimal spermatozoa motility. In this regard, it is interesting to mention that intracellular mediator Ca^{2+}, which plays an essential role in spermatozoa motility [35], exerts a modulator function in the AMPK- regulated spermatozoa motility. However, we cannot exclude a possible non-metabolic effect of the AMPK pathway that could control spermatozoa motility in a parallel or synergistic way, as AMPK is a serine/threonine kinase with several known downstream substrates and therefore may regulate processes outside of cell metabolism. Recently, it has been demonstrated that the kinase TSSK2, which belongs to the AMPK branch in the human kinome tree and is expressed in spermatozoa [17], phosphorylates *in vitro* the axoneme central apparatus protein called SPAG16L [36], that is essential for flagellar motility in mouse spermatozoa [37]. Thus, it can be conceivable that its close related protein, AMPK might phosphorylate downstream substrates involved in the axoneme central apparatus similar to SPAG16L, or in other related structures that are essential for spermatozoa flagellar motility.

Our study points to a regulatory role of AMPK for proper spermatozoa motility, and therefore it is important to understand what factor(s) trigger the activation of AMPK and how this activation is modulated in male germ cells. We believe that this study opens future investigations about AMPK in spermatozoa and we consider that further work is necessary to elucidate mentioned key questions. In conclusion, the present study demonstrates that AMPK protein is expressed in boar spermatozoa and is phosphorylated at Thr^{172} (active) under physiological conditions of these male germ cells. As inhibition of AMPK clearly causes a potent inhibition of spermatozoa motility, our findings suggest that AMPK activity likely plays an important role in the regulation of optimal spermatozoa motility. Motility is essential for the ultimate function of spermatozoa, oocyte fertilization, therefore we propose for the first time that AMPK protein might play an important and necessary regulatory role in the mammalian spermatozoa function.

Materials and Methods

Chemicals and Sources

Complete, EDTA-free, protease inhibitor cocktail was purchased from Roche Diagnostics (Penzberg, Germany). Tris/Glycine/SDS buffer (10X) and Tris/Glycine buffer (10X) 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 Schleicher & Schuell, BioScience (Keene, NH). Compound C (6-[4-(2-Piperidin-1-ylethoxy) phenyl]-3-pyridin-4-ylpyrazolo[1,5-a]pyrimidine) were from Sigma-Aldrich® (St Louis, MI, USA). 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). Live/dead spermatozoa viability kit from Molecular Probes (Leiden, The Netherlands); Coulter Isoton II Diluent from Beckman coulter Inc. (Brea, CA, USA).

Spermatozoa Incubation Media

Tyrode's basal medium (TBM) was prepared as following: 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 3 mg/ml BSA. A variant of TBM medium, which includes direct activators of spermatozoa adenylyl cyclase sAC, was made by adding 1 mmol/l $CaCl_2$ and 10 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}.

Collection and Washing of Semen

Semen from Duroc boars (2–4 years old) was used. Animals were housed at a commercial insemination station (Tecnogenext, S.L, Mérida, Spain) and maintained according to institutional and European regulations. All boars were housed in individual pens in an environmentally controlled building (15–25°C) 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 minutes, 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 of motility. When required, a preincubation of spermatozoa with compound C was performed for 1 hour 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.

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 minutes at 4°C samples were centrifuged at 10.000 *g* (15 minutes, 4°C) and the supernatant (lysate) was used for analysis of protein concentration. Proteins from porcine spermatozoa lysates were resolved by SDS-PAGE and electro-transferred to nitrocellulose membranes. Western blotting was performed as previously described [38] using anti AMPKα (1:1.000), anti phospho-Thr^{172}-AMPKα (1:500), anti GSK3β (1:2.000) polyclonal antibodies as primary antibodies.

Evaluation of Spermatozoa Motility by Computer Assisted Sperm Analysis (CASA) System

After incubation of spermatozoa in TBM or TCM with 5% CO_2 at 38,5°C during different times, a total of 2 µl of sample was placed in a pre-warmed counting chamber (Leja®, Luzernestraat, The Netherlands). Sperm motility analysis is based on the examination of 25 consecutive digitalized images obtained from a single field using a X10 negative-phase contrast objective, and at least 400 spermatozoa per sample were analyzed. Images were taken with a time lapse of 1 s and objects incorrectly identified as spermatozoa were eliminated from the analysis. Motility parameters evaluated with the ISAS® program (Projectes i Serveis R+D, SL; Valencia, Spain) were as following: VCL (curvilinear velocity, in µm/s), VSL (straight-line velocity in µm/s), VAP (average path velocity, in µm/s), LIN

(linearity coefficient in %), STR (straightness coefficient in %), ALH (amplitude of lateral head displacement in μm), WOB (wobble coefficient in %), BCF (Beat cross frequency in Hz). Those spermatozoa with VAP<10 μm/s were considered immobile, while spermatozoa with a velocity >10 μm/s were considered mobile; spermatozoa with a VAP velocity >80 μm/s were considered as rapid spermatozoa. Spermatozoa motility was considered progressive (MP) when STR >80%.

Analysis of Spermatozoa Viability by Flow Cytometry

As described previously [21], fluorescent staining using the Live/Dead Sperm Viability kit was performed to assess porcine spermatozoa viability. Briefly, 5 μl of SYBR-14 (2 μM) and 10 μl of PI (5 μM) were added to 500 μl of spermatozoa sample diluted to 35×10^6 cells/ml in isotonic buffered diluent and incubated 20 min at room temperature in the darkness. After incubation, sperm cells were analyzed in the flow cytometer and results were expressed as the average of the percentage of SYBR14-positive and propidium iodide-negative spermatozoa ± SEM. 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 SYBR-14 were collected in the FL1 sensor using a 525 nm bad pass (BP) filter. Propidium Iodide (PI) fluorescence was collected in the FL3 sensor using a 620 nm BP filter. Flow cytometry data were analysed using a FacStation computer and EXPO^{TM} 32 ADC software (Beckman Coulter, Inc.).

Statistical Analysis

The mean and standard error of the mean were calculated for descriptive statistics, whenever was possible. The effect of treatment and incubation time on the motility variables was assessed using a General Linear Model. To analyze the percentage of motile, progressive and rapid spermatozoa from the total of spermatozoa analyzed we used the Pearson Chi-square test. All analyses were performed using SPSS v11.0 for MacOs X software (SPSS Inc. Chicago, IL). The level of significance was set at $p<0.05$.

Author Contributions

Conceived and designed the experiments: LJG-M MJB. Performed the experiments: AHdL DM-H. Analyzed the data: MCG LJG-M MJB. Wrote the paper: LJG-M MJB.

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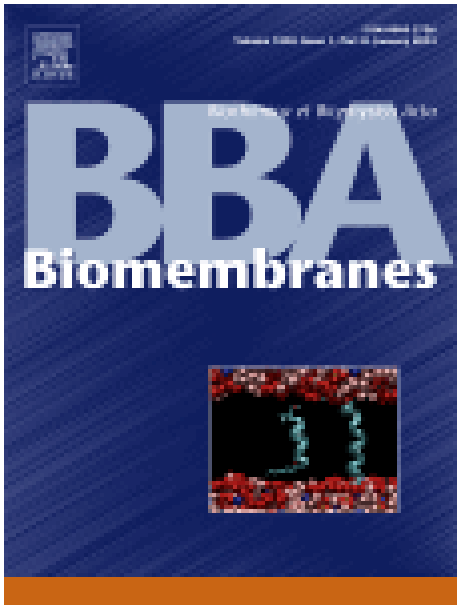
Anexo II



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

Ana Hurtado de Llera [a], David Martin-Hidalgo [a], Joan E. Rodriguez-Gil [b], M. Cruz Gil [a], Luis J. Garcia-Marin [a,*,1], M. Julia Bragado [a,**,1]

[a] Research Group of Intracellular Signaling and Technology of Reproduction (SINTREP), School of Veterinary Medicine, University of Extremadura, Caceres, Spain
[b] Department of Animal Medicine and Surgery, School of Veterinary Medicine, Autonomous University of Barcelona, Bellaterra, Spain

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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 zone 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

* Correspondence to: L.J. Garcia-Marin, Research Group of Intracellular Signaling and Technology of Reproduction, Department of Physiology, School of Veterinary Medicine, University of Extremadura, 10003 Caceres, Spain. Tel.: +34 927257100; fax: +34 927257110.
** Correspondence to: M.J. Bragado, Research Group of Intracellular Signaling and Technology of Reproduction, Department of Biochemistry and Molecular Biology and Genetics, School of Veterinary Medicine, University of Extremadura, 10003 Caceres, Spain.
E-mail addresses: anahl@unex.es (A. Hurtado de Llera), davidmh@unex.es (D. Martin-Hidalgo), juanenrique.rodriguez@uab.cat (J.E. Rodriguez-Gil), crgil@unex.es (M.C. Gil), ljgarcia@unex.es (L.J. Garcia-Marin), jbragado@unex.es (M.J. Bragado).
[1] Both authors contributed equally as senior investigators of this study.

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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.

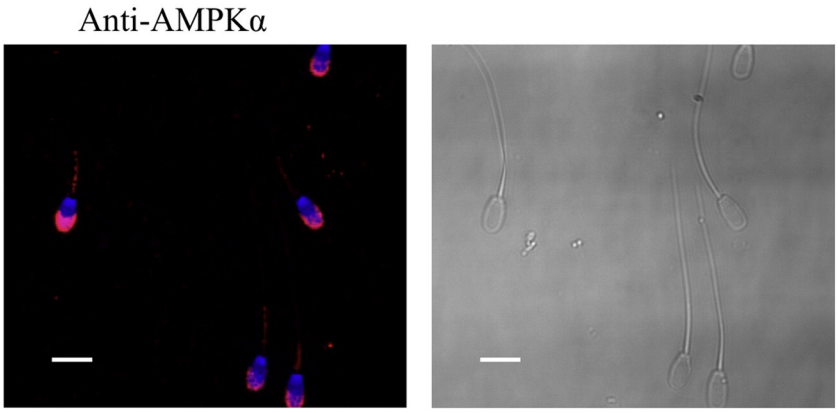

Fig. 1. Immunolocalization of AMP-activated kinase protein, AMPK, in boar spermatozoa. Fresh boar spermatozoa maintained at physiological temperature 38.5 °C were pooled and fixed in 4% paraformaldehyde and immunostaining was performed using antibody against the catalytic subunit of AMPKα. The immunofluorescence 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

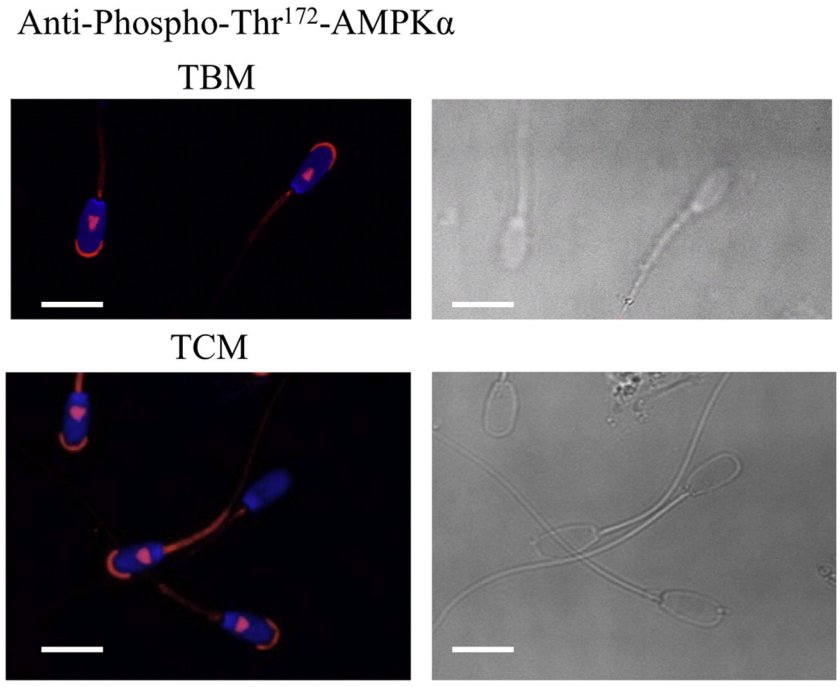

Fig. 2. Immunolocalization of the Thr^{172} phosphorylated (active) form of AMP-activated kinase, AMPK, in boar spermatozoa. After 2 h incubation in TBM (upper image) or TCM (lower image) at 38.5 °C, boar spermatozoa were fixed in 4% paraformaldehyde and immunostaining was performed using anti phospho-Thr^{172}-AMPKα 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).

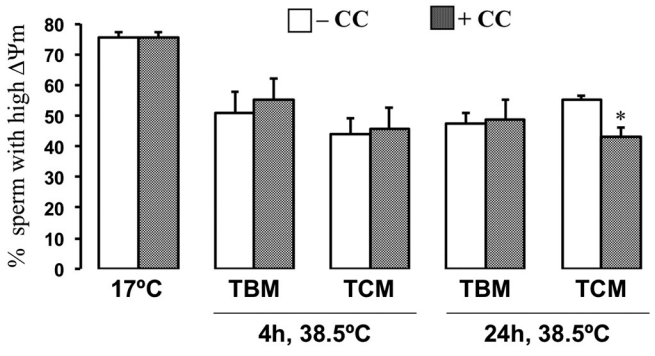

Fig. 3. Effect of the AMPK inhibition in the mitochondrial membrane potential of boar spermatozoa. Spermatozoa were incubated in TBM or TCM at 17 °C or in a CO_2 incubator at 38.5 °C in the presence or absence of the AMPK inhibitor CC (30 μM, filled histograms) for indicated times. Mitochondrial membrane 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 $p < 0.05$.

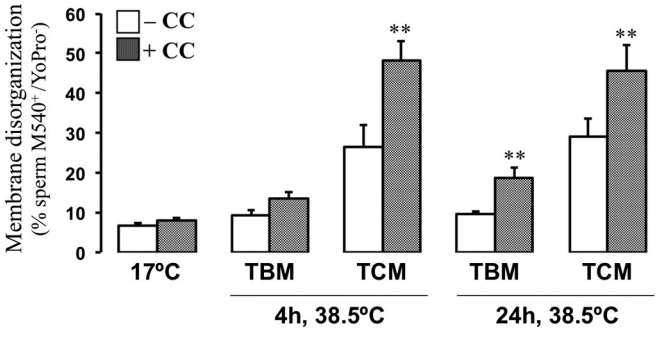

Fig. 4. Effect of the AMPK inhibition in the degree of lipid organization of plasma membrane in boar spermatozoa. Spermatozoa were incubated in TBM or TCM at 17 °C or in a CO_2 incubator at 38.5 °C in the presence or absence of the AMPK inhibitor CC (30 μM, filled histograms) for indicated times. The 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 $p < 0.01$.

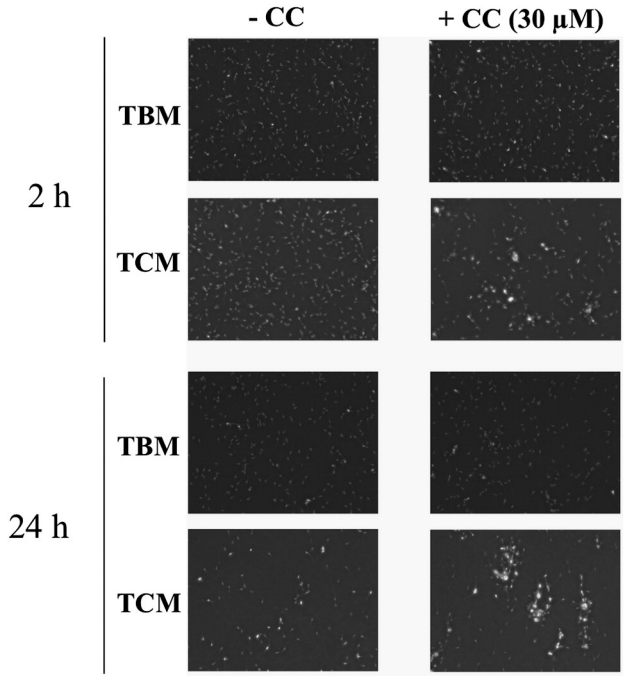

Fig. 5. The AMPK inhibitor, compound C rapidly confers spermatozoa-aggregation ability. Spermatozoa from several boars were pooled, incubated in TBM (upper images) or TCM (lower images) in the presence (right images) or absence (left images) of AMPK inhibitor, compound C (CC 30 μM) in a CO_2 incubator 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

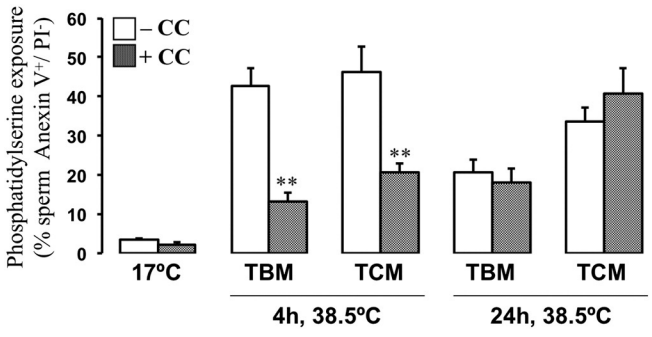

Fig. 6. Effect of the AMPK inhibition in the phosphatidylserine externalization at the plasma membrane of boar spermatozoa. Spermatozoa were incubated in TBM or TCM at 17 °C or in a CO_2 incubator at 38.5 °C in the presence or the AMPK inhibitor CC (30 μM, filled histograms) for indicated times. The 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 $p < 0.01$.

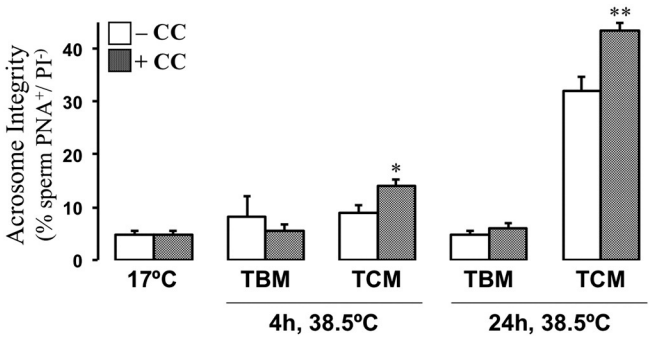

Fig. 7. Effect of the AMPK inhibition in the acrosomal integrity of boar spermatozoa. Spermatozoa were incubated in TBM or TCM at 17 °C or in a CO_2 incubator at 38.5 °C in the presence or absence of the AMPK inhibitor CC (30 μM, filled histograms) for indicated times. Acrosomal integrity was measured 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

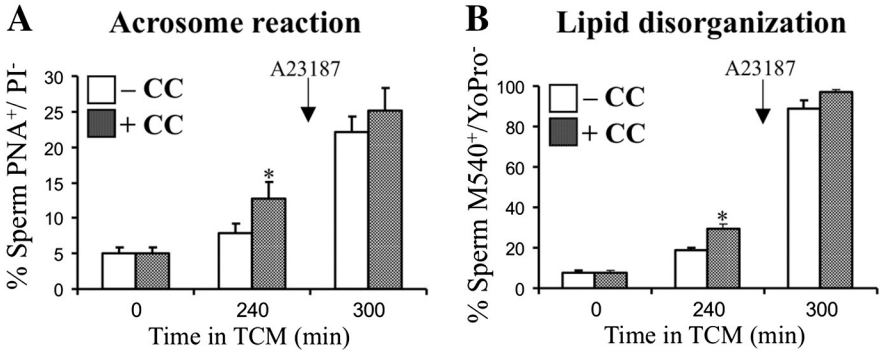

Fig. 8. Effect of the AMPK inhibition in the acrosome reaction and the plasma membrane lipid disorganization induced by calcium ionophore A23187. Spermatozoa were induced to capacitate by 4 h incubation in TCM in a CO_2 incubator at 38.5 °C in the presence or absence of the AMPK inhibitor CC (30 μM, 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

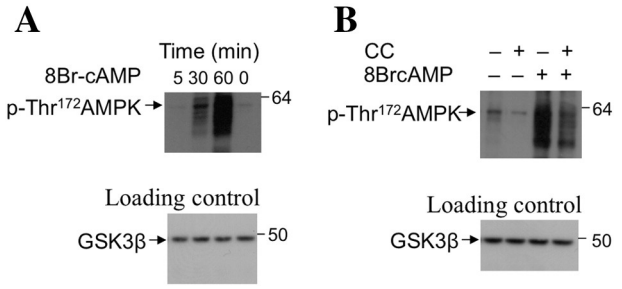

Fig. 9. PKA activation by 8Br-cAMP stimulates the phosphorylation of AMPK, which is inhibited by compound C in boar spermatozoa. Spermatozoa were incubated in TBM at 17 °C (0 min) or in a CO_2 incubator at 38.5 °C in the presence or absence of PKA activator, the cAMP analog, 8Br-cAMP for indicated times (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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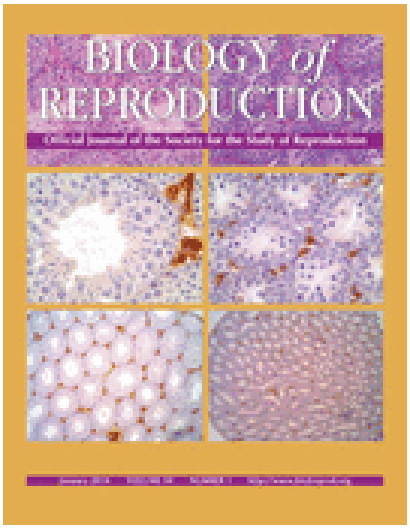
Anexo III



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The calcium/CaMKKα/β and the cAMP/PKA pathways are essential upstream regulators of AMPK activity in boar spermatozoa[1]

Ana Hurtado de Llera, David Martin-Hidalgo, Maria Cruz Gil, Luis J. Garcia-Marin,[2,4] and Maria Julia Bragado[3,4]
Research Group of Intracellular Signalling and Technology of Reproduction (SINTREP), School of Veterinary Medicine, University of Extremadura, Caceres, Spain

Running title: Activation pathways of AMPK in spermatozoa.
Summary sentence: The AMPK activity in spermatozoa is centrally/essentially controlled by intracellular messengers Ca^{2+} and cAMP, which downstream act through the PKA, CaMKKα/β, PKC and PI3K pathways.
Keywords: spermatozoa, AMPK phosphorylation and activity, cAMP, Ca^{2+}, PKA, PKC, PI3K, CaMKKα/β.

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[2]Correspondence: Luis J. Garcia-Marin, Research group of Intracellular Signaling and Technology of Reproduction, Department of Physiology, School of Veterinary Medicine, University of Extremadura, 10003 Caceres, Spain. E-mail: ljgarcia@unex.es
[3]Correspondence: Maria Julia Bragado, Research group of Intracellular Signaling and Technology of Reproduction, Department of Biochemistry and Molecular Biology and Genetics, School of Veterinary Medicine, University of Extremadura, 10003 Caceres, Spain. E-mail: jbragado@unex.es
[4]Both authors contributed equally as senior investigators of this study.

ABSTRACT

Spermatozoa successfully fertilize oocyte depending on cell energy sensitive processes. We recently showed that the cell energy sensor the AMP-activated protein kinase, AMPK, plays a relevant role in spermatozoa by regulating motility as well as plasma membrane organization and acrosomal integrity, and contributes to the maintenance of mitochondrial membrane potential. As the signaling pathways that control AMPK activity has been studied exclusively in somatic cells, our aim is to investigate the intracellular pathways that regulate AMPK phosphorylation at Thr172 (activity) in male germ cells. Boar spermatozoa were incubated under different conditions in presence or absence of Ca^{2+}, 8Br-cAMP, IBMX, PMA, the AMPK activator A769662 or inhibitors of PKA, PKC or CaMKKalpha/beta. AMPK phosphorylation was evaluated by western blot using anti-phospho-Thr^{172}-AMPK antibody. Data show that AMPK phosphorylation in spermatozoa is potently stimulated by an elevation of cAMP levels through the activation of PKA as the PKA inhibitor H89 blocks phospho-Thr^{172}-AMPK. Another mechanism to potently activate AMPK is Ca^{2+} that acts through two pathways, PKA (blocked by H89) and CaMKKalpha/beta (blocked by STO-609). Moreover, phospho-Thr^{172}-AMPK levels greatly increased upon PKC activation induced by PMA and the PKC inhibitor Ro-32-0432 inhibits TCM-induced AMPK activation. Different stimuli considered as cell stresses (rotenone, cyanide, sorbitol and complete absence of intracellular Ca^{2+} by BAPTA-AM) also cause AMPK phosphorylation in spermatozoa. In summary, AMPK activity in boar spermatozoa is regulated

upstream by different kinases such as PKA, CaMKKalpha/beta and PKC, as well as by the essential intracellular messengers for spermatozoa function, Ca^{2+} and cAMP levels.

INTRODUCTION

In order to successfully achieve a fertilization competent status, spermatozoa requires to undergo several biochemical changes within the female genital tract that are called capacitation. This process allows the spermatozoa to acquire the ability to reach the oocyte, penetrate the cumulus oophorus and to bind to the zona pellucida of the oocyte, triggering the acrosome reaction and subsequently leading to egg fertilization. Biochemical changes during spermatozoa capacitation include cholesterol loss from the plasma membrane, increased plasma membrane fluidity due to phospholipid scrambling, changes in intracellular ions concentration, hyperpolarization of plasma membrane and increased tyrosine phosphorylation, among others [1]. The acquisition of spermatozoa functionality during capacitation is mainly dependent on i) post-translational modifications of pre-existing proteins, such as phosphorylation in either tyrosine [2-4] or serine/threonine [5] and/or ii) the male germ cell energetic state, determined by the ratio between cellular AMP and ATP [6].

The AMP-activated protein kinase AMPK is an evolutionary conserved serine/threonine kinase that acts as a regulator of energy balance at both, the cellular and the whole body levels [7-9]. AMPK responds to a rise in AMP levels by increasing ATP-generating pathways and reducing ATP-consuming metabolic pathways [8, 10], thus the overall metabolic consequence of AMPK activation is the maintenance of cellular energy state under ATP-limiting conditions. AMPK is a heterotrimeric protein composed of a catalytic α subunit and two regulatory subunits, β and γ. The binding of AMP to the two "Bateman" domains in the γ subunit [11] triggers increased phosphorylation at Thr-172 located in the activation loop of the α subunit, causing its enzymatic activation (more than 100 fold) [12]. To date, the following kinases have been identified that phosphorylate AMPK at Thr172 in somatic cells: i) the tumour suppressor responsible for the inherited cancer disorder Peutz-Jeghers syndrome, LKB1 [13], ii) the Ca^{2+}/calmodulin-dependent protein kinase kinases CaMKKα and β [14] and iii) the transforming growth factor TGF-β-activated kinase-1, TAK1 [15]. Although AMP binding was previously thought both to induce phosphorylation [16] and to inhibit dephosphorylation [17] later studies suggest that the AMP effect is exclusively mediated by an inhibition of Thr172 dephosphorylation [18] by unknown mechanism. AMPK is highly sensitive to its allosteric effector AMP, as any increase in the ratio AMP/ATP due to a decrease in cellular energy state, increases AMPK activity by up to 10-fold [12].

AMPK might regulate processes outside metabolism [8, 10], as its enzymatic activity is also switched on by different types of cellular and metabolic stresses [8, 19, 20]. Some of these stimuli, as hyperosmotic stress or an increase in calcium concentration [8, 20], do not modify the ratio AMP/ATP, suggesting that other mechanisms, which are likely cell type specific, are involved in AMPK activation.

Any AMPK studies had been conducted exclusively in somatic cells until 2008, where Towler [21] demonstrated in male germ cells that a short splice variant of LKB1, the upstream kinase of AMPK, called LKB1s, is expressed predominantly in haploid sperm cells from testes of mammals. LKB1s knockout mice have a dramatic reduction in the number of mature spermatozoa in the epididymis, and the few spermatozoa produced are non-motile, have an abnormal head morphology and resulted sterile [21]. These data suggest that this variant, LKB1s, has a crucial role in spermiogenesis and fertility in mice. Moreover, members of "ser/thr kinase

testis specific" TSSK family, which belongs to the AMPK branch in the human kinome tree, have been identified in human spermatozoa: TSSK2, TSKS and SSTK [22]. Deletion of TSSK1 and 2 causes male infertility in chimera mice due to haploinsufficiency [23]. Recently we have demonstrated for the first time that AMPK protein is relatively highly expressed in mammalian ejaculated spermatozoa and regulates one of their most important functions: motility [24], as well as contributes effectively to the maintenance of mitochondrial membrane potential, spermatozoa plasma membrane fluidity and organization and acrosomal integrity at physiological temperature [25] or during semen preservation at 17ºC [26]. Supporting a relevant role of AMPK in the function of the male germ cells, Tartarin [27] demonstrated that mice lacking the AMPK catalytic subunit α1 gene [α1AMPKα1knockout (KO)] show a decreased fertility in parallel with an alteration in spermatozoa morphology as well as decreased spermatozoa motility.
As mentioned, spermatozoa need to adapt to external changing conditions such as the transit through the female reproductive tract. The study of the signalling pathways that regulate AMPK phosphorylation (activity) in spermatozoa is crucial for the understanding of those functions of these germ cells in which this cell energy sensor kinase is involved [24-26]. Therefore, the aim of this work is to investigate the involvement of intracellular messengers such as Ca^{2+} and cAMP, as well as different intracellular pathways CaMKKα/β, PKA, PKC and PI3K in the activation of AMPK in male germ cells.

MATERIALS AND METHODS

Chemicals and Sources

8 Bromoadenosine 3´5´-cyclic monophosphate sodium salt (8BrcAMP), 3-Isobutyl-1-methylxanthine (IBMX), dihydrochloride hydrate (H-89), STO-609-acetic acid, phorbol 12-myristate 13-acetate (PMA), D-sorbitol, rotenone and cyanide were from Sigma-Aldrich (St Louis, MI, USA); LY 294002 from Cayman Chemical Company; Ro-32-0432 and BAPTA-AM from Calbiochem EMD; A769662 was from Tocris Bioscience (Bristol, UK); anti-GSK3β antibody were from Cell Signaling (Beverly, CA); anti-P-Thr^{172}-AMPK antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA); complete, EDTA-free, protease inhibitor cocktail was purchased from Roche Diagnostics (Penzberg, Germany). Tris/Glycine/SDS buffer (10X) and Tris/Glycine buffer (10X) 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).

Spermatozoa incubation media.

Tyrode's basal medium (TBM) was prepared as following: 96 mmol/l NaCl, 4.7 mmol/l KCl, 0.4mmol/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 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). For experiments designed to study the effect of addition of Ca^{2+} 5mM, a particular EGTA-free TBM medium was prepared. 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}.

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 for 12h at 17ºC before use and, in order to minimize individual boar variations, semen from up to 3 animals was pooled using ejaculates from a minimum of 19 boars in different combinations. Only ejaculates containing at least 80% of morphologically normal spermatozoa, 70% of motile spermatozoa and a total number of spermatozoa higher than 10×10^9 were used. Semen pools were centrifuged at 2000*g* for 4 minutes, 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. When required, a pre-incubation of spermatozoa with different inhibitors (H89, Ro-32-0432, LY294002, STO-609 or BAPTA-AM) was performed for 1 hour 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 (ethanol for rotenone or DMSO ≤ 0.1% for the rest of compounds) was included. We have previously demonstrated that ethanol [28] or DMSO [24, 25] at concentrations used in this study or higher do not modify the spermatozoa parameters analyzed.

Western blotting.
Spermatozoa under different treatments were centrifuged 20s at 7000*g*, washed with phosphate buffered saline (PBS) supplemented with 0.2mM Na_3VO_4 and then lysated in a lysis buffer consisting in 50mmol/L Tris/HCl, pH 7.5, 150mmol/L NaCl, 1% Triton X-100, 1% deoxycholate, 1mmol/L EGTA, 0.4mmol/L EDTA, protease inhibitors cocktail (Complete, EDTA-free), 0.2mmol/L Na_3VO_4, and 1mmol/L PMSF by sonication for 5s at 4ºC. After 20 minutes at 4ºC samples were centrifuged at 10.000*g* (15 minutes, 4°C) and the supernatant (lysate) was used for analysis of protein concentration. Proteins from porcine spermatozoa lysates were resolved by SDS-PAGE and electro-transferred to nitrocellulose membranes. Western blotting was performed as previously described [24, 29] using anti phospho-Thr^{172}-AMPKα (1:500) and anti GSK3β (1:2.000) polyclonal antibodies as primary antibodies. The intensity of bands in the film was measured using a scanning densitometer and quantified using the software ImageJ for Macintosh (Research Service Branch, NIMH, NIH, Bethesda).

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 $p<0.05$.

RESULTS

AMPK activity is physiologically regulated by an increase in cAMP levels through the activation of protein kinase A in spermatozoa.

The physiology of mammalian spermatozoa is essentially regulated by the protein kinase A, PKA. Our recent work suggests that AMPK lies downstream of PKA in boar spermatozoa as the non hydrolysable cAMP analogue 8Br-cAMP, which elevates intracellular cAMP levels, greatly increases phospho-Thr^{172}-AMPK [25]. We further investigated the role of PKA pathway in the AMPK activity by using a pharmacological inhibitor of PKA, H89 (IC_{50}=48nM), at concentration of 100 μM that possesses demonstrated effect in boar spermatozoa [30]. As seen in Figure 1, phospho-Thr^{172}-AMPK (activity) in boar spermatozoa is minimal at 17ºC in TBM medium. Interestingly, under these non stimulated conditions of spermatozoa, AMPK activity is not dependent on PKA, as pre-treatment with inhibitor H89 does not modify AMPK phosphorylation levels. However, phospho-Thr^{172}-AMPK rapidly increases (2,7-3 times) when boar spermatozoa are incubated at physiological temperature (38,5ºC), confirming previous data [24]. AMPK activity induced by physiological temperature is regulated by PKA activity as H89 inhibits phospho-Thr^{172}-AMPK levels and seems independent of the spermatozoa incubation medium, although slight differences between media can be pointed (Figure 1). Whereas in TCM, a stimulating (capacitating) medium for spermatozoa, a clear inhibition of AMPK activity is observed by H89, in TBM medium the PKA inhibitor totally abolishes the temperature induced-AMPK phosphorylation, reaching minimal values comparable to those at 17ºC or even lower.
In order to further demonstrate the involvement of PKA in AMPK activity, we incubated spermatozoa in TBM with 3-isobutyl-1-methylxanthine (IBMX, 100 μM), a phosphodiesterases inhibitor that leads to an increase in cAMP, as well as with 8Br-cAMP (1 mM) as a positive control and evaluated phospho-Thr^{172}-AMPK levels (Figure 1). Although in both cases a potent increase in phospho-Thr^{172}-AMPK levels is achieved, the induction of the AMPK activity due to the cAMP analogue is almost twice than IBMX (densitometry analysis in Figure 1). The fact that an intracellular elevation of cAMP levels in spermatozoa leads to PKA stimulation and subsequent AMPK activity is further demonstrated in Figure 1, where it is showed that pre-incubation of spermatozoa with the PKA inhibitor H89 totally blocks the induction of AMPK activity due to elevated cAMP levels induced by either IBMX or 8Br-cAMP.

An increase in intracellular calcium levels leads to the phosphorylation and activation of AMPK through CaMKKα/β and PKA pathways in spermatozoa.

The involvement of the intracellular Ca^{2+} in AMPK activity in spermatozoa was studied by incubating these germ cells in a non stimulating medium TBM (slightly modified as EGTA was omitted) with the addition of different concentrations (mM) of extracellular Ca^{2+}, although only the effect of 5mM Ca^{2+} is showed (Figure 2). The phosphorylation of AMPK potently increases by 2 fold in spermatozoa incubated at 38,5ºC in a medium with 5mM Ca^{2+} as unique stimulus. This enhanced AMPK activity due to Ca^{2+} is mediated at least partially by the activation of PKA as H89 blocks the increase in phosphorylation induced by Ca^{2+} although the remaining levels of AMPK phosphorylation are higher than after H89 treatment alone in absence of Ca^{2+}.
Two known kinases member of the Ca^{2+}-activated pathways are the Ca^{2+}/calmodulin-dependent kinase kinase-α and β, CaMKKα/β, which lie upstream of AMPK in somatic cells. Therefore, we next studied the contribution of CaMKKα/β to the Ca^{2+}-stimulated AMPK activity in male germ cells by using a specific inhibitor of these kinases, STO-609 (K_i= 80 ng/ml for CaMKKα and K_i=15ng/ml for CaMKKβ). Results in Figure 2 show that the Ca^{2+}-induced AMPK activity under

physiological conditions (38,5ºC) is mediated by the activity of CaMKKα or β as STO-609 (40 μM) abolishes the increase in phosphorylation induced by 5mM Ca^{2+}. As expected, the increase in phospho-Thr^{172}-AMPK level due to the physiological temperature in absence of any Ca^{2+} (neutralized by EGTA present in TBM) is not affected by the CaMKKα/β inhibitor.

AMPK activity is physiologically regulated by protein kinase C in spermatozoa.
The involvement of the protein kinase C (PKC) in AMPK activity in spermatozoa was studied by incubating these germ cells in TCM medium, which contains Ca^{2+} and HCO_3^-, with the addition of a well known activator of PKC activity, the phorphol ester PMA (1 and 10 μM). As seen in Figure 3, the incubation of spermatozoa under physiological conditions of temperature with PMA potently increases phospho-Thr^{172}-AMPK levels in a concentration dependent manner, showing a maximal effect at 10 μM PMA (2,5 fold). In order to further demonstrate the involvement of PKC in AMPK activity in male germ cells, we incubated spermatozoa in TCM in the presence of a selective cell-permeable PKC inhibitor Ro-32-0432 (IC_{50}=9nM for PKC_α primary target; IC_{50}=28nM for $PKC_{\beta1}$; IC_{50}=108nM for PKC_ϵ secondary targets) at concentration previously used in these germ cells, 50 μM [30] and evaluated phospho-Thr^{172}-AMPK levels. As seen in Figure 3, the PKC inhibitor blocks the increase in AMPK activity induced by the presence of spermatozoa stimuli such as Ca^{2+} and HCO_3^- present in TCM.

Effect of the inhibitor LY294002 in AMPK activity in spermatozoa.
The possible involvement of the phosphatidylinositol 3-kinase (PI3K) in the AMPK activation pathway was studied by incubating male germ cells in TCM medium in the presence of LY294002 (IC_{50} for PI3K= 1.4μM) at 100 μM (Figure 4), concentration with demonstrated effect in the motility of boar spermatozoa [30]. The AMPK activity due to physiological stimuli of spermatozoa (temperature 38,5ºC and activators of sAC, Ca^{2+} and HCO_3^-) seems not mediated by the activation of PI3-K as LY294002 does not significantly affect phospho-Thr^{172}-AMPK levels induced by TCM.

The activity of AMPK induced pharmacologically by A769662 is regulated by PKA, PKC in spermatozoa.
We further investigated the signalling pathways leading to AMPK activity in boar spermatozoa by using a novel direct and potent AMPK activator, A769662 [31] in a TCM medium (Figure 5). It has been showed that A769662 activates AMPK by inhibiting its dephosphorylation at Thr-172, as well as allosterically activating AMPK [32, 33]. Incubation of boar spermatozoa for 60 min in TCM with A769662 (200 μM) potently increases the phosphorylation at Thr^{172} of AMPK, reaching values higher than those in TCM alone. The A769662-induced AMPK activity is inhibited by the PKA inhibitor, H89 as well as the PKC inhibitor Ro-32-0432 (Figure 5).

Different cellular stresses induce the activation of AMPK in boar spermatozoa.
Additionally we investigated whether known cellular stresses induce AMPK phosphorylation (activity) in male germ cells. As seen in Figure 6, different blockers of mitochondrial electron transport such as rotenone (Rot, 10 μM for 5 min) that inhibits NADH:ubiquinone oxidoreductase, and cyanide (CN, 2mM for 5 min), an inhibitor of cytochrome oxidase complex, clearly increase AMPK phosphorylation in boar spermatozoa. AMPK activity is also induced when spermatozoa are incubated 60 min under hyperosmotic stress caused by the addition of 500mM sorbitol. Interestingly, a specific cellular stress for male germ cells, which is the total

absence of intra and extracellular calcium due to pre-incubation with the permeable Ca^{2+}-quelator BAPTA-AM (50 μM) in a Ca^{2+}-free media (TBM contains EGTA), potently activates AMPK in a time-dependent manner.

DISCUSSION

Maintaining energy balance is an essential process at cellular level as well as the whole body level. A necessary and key molecule in the regulation of energy homeostasis is AMPK, which has been investigated exclusively in somatic cells until recently. In 2012 our group has demonstrated for the first time that AMPK is highly expressed in male germ cells [24] at the entire acrosome in spermatozoa head as well as in the midpiece of flagellum, whereas its active form, phospho-Thr^{172}-AMPK, is specifically localized at the apical part of acrosome, remaining also in the midpiece of flagellum [25].The physiological role(s) of AMPK in mammalian male germ cells has been demonstrated under different spermatozoa conditions (basal, capacitating and/or preservation at 17ºC) and includes an important function in the regulation of spermatozoa motility in boar [24, 26] and in mice [27], as well as an essential role in the maintenance of both the mitochondrial membrane potential [25-27] and the basal oxygen consumption of spermatozoa [27]. Moreover, we have demonstrated additional and relevant functions of AMPK in boar spermatozoa [25, 26] which include a relevant role in the maintenance of the physiological organization of plasma membrane by regulating i) its lipid organization and fluidity and ii) the suitable outward translocation of phosphatidylserine. The role of AMPK in spermatozoa membrane reaches the acrosomal region where AMPK activity is localized and controls the integrity of acrosomal membrane [25, 26]. As these AMPK-regulated spermatozoa processes are required under different environmental conditions of male germ cells when transiting through the female reproductive tract to achieve oocyte fertilization, as well as during 17ºC boar semen preservation [26], these previous studies clearly point to AMPK as an essential regulator of spermatozoa function. However, to date signalling pathways involved in the control of AMPK activity in male germ cells are unknown.

As mentioned, activation of AMPK requires phosphorylation on Thr-172 within the α catalytic subunit [34, 35]. Our recent work [25] suggests that cAMP-mediated pathway, likely through PKA, is upstream regulator of AMPK in boar spermatozoa. In the present study we have further demonstrated that an elevation of intracellular cAMP levels not only caused by a non hydrolysable analogue of cAMP, but due to an inhibition of phosphodiesterases rapidly leads to a clear increase in phospho-Thr^{172}-AMPK in boar spermatozoa. The best know downstream target of cAMP is PKA, which plays a central and essential role in spermatozoa physiology by regulating any functional process occurring in these germ cells: motility, capacitation, biochemical changes at the acrosomal and plasma membrane, acrosome reaction and fertilization [1, 6]. Furthermore, this study, using PKA inhibitor H89, demonstrates for the first time that an elevation of intracellular cAMP levels in boar spermatozoa effectively increases AMPK phosphorylation through the activation of PKA. To date, the involvement of PKA pathway in the upstream regulation of AMPK phosphorylation has been demonstrated exclusively in somatic cells such as adipocytes [36], hepatocytes [37, 38], CV-1 cells [39], muscle cells [40] and recently 293T cells [41]. Moreover, it has been recently proposed that PKA and AMPK communicate biochemically and act in concert as a signalling network capable of controlling cellular metabolism in somatic cells, such as vascular smooth muscle cells [42] or adipocytes [43]. The cAMP/PKA-dependent AMPK activation in spermatozoa might occur through its upstream kinase LKB1, which in somatic cells can be directly phosphorylated at Ser 431 by PKA

in response to activation of adenylate cyclase by forskolin [39, 44] or IBMX [39]. Regarding this upstream kinase LKB1, it has been showed that its short splice variant $LKB1_S$ is highly expressed in haploid spermatids in mice testis [21] where it has a relevant role in spermiogenesis and male fertility.

Another possible mechanism that might explain AMPK activation by an increase in cAMP levels is through regulation of cAMP degradation [45]. After any elevation of intracellular cAMP levels, the amount of this intracellular messenger rapidly returns to basal levels because of its degradation to 5´-AMP by phosphodiesterases. Thus, as it has been suggested in somatic cells [41, 45], we postulate that any stimulus leading to an increase in intracellular cAMP in spermatozoa could result in AMPK activation either by direct activation of PKA, by an phosphodiesterases-induced increase in AMP levels which activate allosterically AMPK, or both mechanisms.

An essential regulator of any spermatozoa functional process is the intracellular messenger Ca^{2+} which activates the specific soluble adenylate cyclase sAC, expressed in these germ cells. Our results now provide strong evidence that intracellular Ca^{2+} acts as an additional signalling pathway to potently activate AMPK in spermatozoa. This finding in germ cells is supported by previous works in somatic cells where Ca^{2+} directly regulates the activation of AMPK [14, 46, 47]. This work demonstrate that the Ca^{2+}-induced signalling pathway leading to AMPK activation in spermatozoa is mediated by the activation of PKA. A plausible explanation is the direct stimulation by Ca^{2+} of the spermatozoa sAC that catalyzes the synthesis of cAMP, which in turn allosterically activates PKA. Besides through the PKA pathway, Ca^{2+} also might lead to the phosphorylation of AMPK through the activation of Ca^{2+}-calmodulin dependent kinase kinases II, CaMKKα/β, which lie upstream of AMPK in somatic cells [14, 47]. In this work we have used STO-609 that acts a specific inhibitor of both CaMKKs, CaMKKα and β [48]. Although the expression of both CaMKKs isoforms has been showed in spermatozoa from different mammalian species, such as rat [49] and mouse [50], to date it is unknown the expression level of each CaMKK isoform in boar spermatozoa; therefore we will cautiously refer in this work to both isoforms, using CaMKKα/β. The CaMKKs inhibitor STO-609 caused a complete inhibition of Ca^{2+}-induced AMPK activity in spermatozoa, in agreement with previous studies in somatic cells [14, 47]. As expected, in absence of any extracellular Ca^{2+} (neutralized by EGTA present in TBM), phospho-Thr^{172}-AMPK levels due to spermatozoa physiological temperature are not regulated by CaMKKα/β, although they are effectively blocked by PKA inhibitor, further confirming a physiological role of PKA in AMPK activation under spermatozoa basal conditions.

Additionally, this work describes that AMPK phosphorylation is dose-dependently stimulated by direct activation of PKC with phorbol 12-myristate 13-acetate (PMA) in boar spermatozoa, which indicates that at least one or more isoforms of PKC are upstream of AMPK in these male germ cells. This finding is further confirmed by the fact that PKC inhibitor Ro-32-0432 abolishes AMPK activation in response to a stimulating medium TCM or to a higher AMPK activation induced by A769662 in TCM. The observation that PMA stimulates AMPK phosphorylation had been previously reported in somatic cells [51], where it has been also demonstrated that AMPK is not a substrate for PKC. Instead, Xie [52] first demonstrated that the atypical PKCζ is a kinase that phosphorylates LKB1 at Ser428 and later that also phosphorylates LKB1 at Ser307 in several somatic cell types [53]. Very recently, when different LKB1 isoforms (long, $LKB1_L$ and short, $LKB1_S$), have been described, it has been specified that PKCζ phosphorylates Ser428/431 in the long form of $LKB1_L$ and Ser399 in the short form $LKB1_S$, residues that are essential for

nucleocytoplasmic export of LKB1 and the consequent AMPK activation [54]. Regarding male germ cells, the activity of PKC has been reported in human [55] and other mammalian spermatozoa [56] where PKC regulates acrosome reaction [55, 56], and also motility [30] and hyperactivation [57]. Among PKC isoforms that have been identified in mammalian male germ cells are PKCα and PKCβI in bovine [58], PKCζ in hamster [59] and mouse sperm [60]. It is therefore plausible that PKCζ might play in spermatozoa a similar role than in somatic cells leading to AMPK activation through the phosphorylation of LKB1. An alternative explanation describing the pathway by which PKC is upstream of AMPK activity in male germ cells is based on different studies in boar spermatozoa. More recently, Bragado [30] demonstrate that PKC activity lies downstream of PKA in the control of motility of these germ cells. Previously, Harayama and Miyake [57] demonstrated that the cAMP/PKA signalling can induce the activation of calcium-sensitive PKCs, which are responsible for spermatozoa hyperactivation. Thus, we propose that another PKC isoform(s) besides PKCζ, which is not calcium sensitive, could be likely mediating AMPK activation, at least in response to an elevation of cAMP levels, on boar spermatozoa.

A different signalling pathway that regulates several processes in spermatozoa function is the PI3K [30, 61]. We have previously described in boar spermatozoa that PI3K exerts a negative effect at the cAMP level in the signalling pathway that controls spermatozoa motility, as treatment of spermatozoa with the inhibitor LY294002 leads to a significant increase in cAMP levels under stimulating or basal conditions [30]. Thus, the finding that the inhibitor LY294002 leads to a higher increase in A769662-induced phospho-Thr172 AMPK levels than the increase due to A769662 alone is explained through the activation of PKA pathway due to the LY294002-induced elevated cAMP levels, as we demonstrated that occurs in boar spermatozoa [30]. Despite this, and supporting the idea that PI3K may regulate cAMP levels, it has been demonstrated a crosstalk between PKA and PKC to regulate PI3K activity in spermatozoa from other species [61]. Given that LY294002 is a weak inhibitor with only micromolar potency and loses specificity at high concentrations, we cannot make any strong conclusion about our data.

Besides the demonstration of which signaling pathways lead to AMPK activation under physiological conditions of boar spermatozoa, as well as by A766962, this work additionally provides clear evidence that AMPK becomes activated in male germ cells under conditions that cause some type of cellular stress. Thus, inhibition of spermatozoa mitochondrial activity by blocking electron transport chain, as well as hypersomotic stress induced by sorbitol cause a marked increase in AMPK phosphorylation in spermatozoa. In somatic cells, the activation of AMPK depends on the type of cell stress and can be mediated by either i) an increase in AMP levels, ii) by the generation of reactive oxygen species ROS that act as signaling molecules to activate AMPK [62] through LKB1 and CaMKKs dependent pathways, or iii) both. Surprisingly, the absence of intracellular Ca^{2+} in boar spermatozoa by incubation with BAPTA-AM in a Ca^{2+}-free media, leads to a strong increase in AMPK activity in these germ cells. A plausible explanation is that BAPTA-AM might lead to AMPK phosphorylation in boar spermatozoa through an increase in nitric oxide NO· production, as demonstrated in human sperm [63] where BAPTA-AM incubation promotes the production of a reactive oxygen specie, the nitric oxide NO·. In this regard, recently it has been demonstrated in somatic cells that AMPK activation is also directly influenced by cellular redox status, thus H_2O_2 activates AMPK through oxidative modification of cysteine residues in the AMPKα subunit [64]. An alternative or simultaneous explanation is that NO· produced by BAPTA-AM in boar spermatozoa might interacts with the cAMP pathway as it occurs in human [65] leading to the AMPK activation as mentioned before.

In summary, the results presented here provide mechanistic insight into the signalling pathways leading to AMPK activation in boar spermatozoa under physiological and pharmacological conditions (Fig. 7). Thus, AMPK phoshorylation is regulated upstream by intracellular messengers Ca^{2+} and cAMP, as well as by PKA, PKC and CaMKKα/β signalling pathways in spermatozoa. Moreover, we show that different cellular stresses, including the complete absence of intracellular Ca^{2+} in spermatozoa lead to the activation of AMPK in these germ cells.

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FIGURE LEGENDS

Figure 1. Phosphorylation of AMPK at Thr-172 is greatly increased by an elevation of cAMP levels due to IBMX or 8BrcAMP and is blocked by PKA inhibitor H89 in boar spermatozoa. Male germ cells from several boars were pooled and incubated in TBM (**B** upper films) or TCM medium (**B** lower films) at 17°C or in a CO_2 incubator at 38.5°C for 60 min in the presence or absence of 8BrcAMP (1mM) or IBMX (100 μM) and then lysed. Sperm samples treated with PKA inhibitor were previously preincubated for 1 h at RT with H89 (100 μM). Proteins (20μg) from spermatozoa lysates were analyzed by western blotting using anti-phospho-Thr^{172}-AMPKα as primary antibody. **B**) Arrow indicates the cross-reactive band of phospho-Thr^{172} AMPK in each experiment that was performed at least 6 times. A representative film of each experiment is shown at the right hand. Protein loading controls for each experiment using anti-GSK3β antibody are showed in lower films. **A**) Analysis of phospho-Thr^{172} AMPK bands in the films by densitometry is shown at the left. Results express the mean ± standard error of the mean. Statistical differences between treatments are showed with different letters when $p<0.05$.

Figure 2. Phospho-Thr172-AMPK levels are potently stimulated by an increase in intracellular calcium, via CaMKKα/β and PKA pathways in boar spermatozoa. Male germ cells from several boars were pooled and incubated in TBM medium at 17°C or in a CO_2 incubator at 38.5°C for 60 min in the absence or presence of extracellular 5 mM Cl_2Ca that was added to a modified TBM medium, without EGTA. Sperm samples treated with PKA inhibitor H89 (100 μM) or with CaMKKα/β inhibitor STO-609 (40 μM) were first preincubated for 1 h at RT. Proteins (20μg) from spermatozoa lysates were analyzed by western blotting using anti-phospho-Thr^{172}-AMPKα as primary antibody. **A**) Arrow indicates the cross-reactive band of phospho-Thr^{172} AMPK. This experiment was performed at least 6 times and a representative film is shown at the upper part. Protein loading control using anti-GSK3β antibody is showed in lower film. **B**) Analysis of phospho-Thr^{172} AMPK bands in the films by densitometry is shown at the bottom. Results express the mean ± standard error of the mean. Statistical differences between treatments are showed with different letters when $p<0.05$.

Figure 3. Phosphorylation of Thr-172-AMPK is markedly increased by direct stimulation of PKC with PMA and blocked by PKC inhibitor Ro-32-0432 in boar spermatozoa. Male germ cells were incubated in TCM medium at 17°C or in a CO_2 incubator at 38.5°C for 60 min in the absence or presence of indicated concentrations of PMA (**B**, upper films) or PKC inhibitor Ro-32-0432 (**B**, lower films). Sperm samples treated with PKC inhibitor Ro-32-0432 (50 μM) were previously preincubated for 1 h at RT. Proteins (20μg) from spermatozoa lysates were analyzed by western blotting using anti-phospho-Thr^{172}-AMPKα as primary antibody. **B**) Arrow indicates the cross-reactive band of phospho-Thr^{172} AMPK in each experiment that was performed at least 5 times. A representative film of each one is shown and protein loading controls for each experiment using anti-GSK3β antibody are showed in lower films. **A**) Analysis of phospho-Thr^{172} AMPK bands in the films was performed by densitometry and data are expressed as arbitrary units. Results express the mean ± standard error of the mean. Statistical differences between treatments are showed with different letters when $p<0.05$.

Figure 4. Effect of PI3K inhibitor LY294002 in the TCM-induced AMPK phosphorylation at Thr-172 in spermatozoa. Male germ cells were incubated in TCM medium at 17°C or in a CO_2 incubator at 38.5°C for 60 min in the absence or presence of PI3K inhibitor LY294002 100 μM. Sperm samples treated with PI3K inhibitor LY294002 were previously preincubated for 1 h at RT. Proteins (20μg) from spermatozoa lysates were analyzed by western blotting using anti-phospho-Thr^{172}-AMPKα as primary antibody. **A**) Arrow indicates the cross-reactive band of phospho-Thr^{172} AMPK. This experiment was performed at least 5 times and a representative film is shown. Protein loading control for each experiment using anti-GSK3β antibody is showed in the lower film. **B**) Analysis of phospho-Thr^{172} AMPK bands in the films by densitometry is shown. Results express the mean ± standard error of the mean. Statistical differences between treatments are showed with different letters when $p<0.05$.

Figure 5. AMPK phosphorylation at Thr-172 is potently enhanced by its pharmacological activator A769662 via PKA and PKC pathways in spermatozoa. Male germ cells were incubated in TCM medium at 17°C or in a CO_2 incubator at 38.5°C for 60 min in the presence or absence of AMPK activator A769662 (200 μM) and then lysed. Sperm samples treated with kinases inhibitors H89 (100 μM), Ro-32-0432 (50 μM) or LY294002 (100 μM) were previously preincubated for 1 h at RT. Proteins (20μg) from spermatozoa lysates were analyzed by western blotting using anti-phospho-Thr^{172}-AMPKα as primary antibody. **A**) Arrow indicates the cross-reactive band of phospho-Thr^{172} AMPK. This experiment was performed at least 6 times and a representative film is shown. Protein loading control using anti-GSK3β antibody is showed in the lower film. **B**) Analysis of phospho-Thr^{172} AMPK bands in the films by densitometry is shown. Results express the mean ± standard error of the mean. Statistical differences between treatments are showed with different letters when $p<0.05$.

Figure 6. AMPK activation is increased by different cellular stresses in spermatozoa. Male germ cells were incubated in TBM medium at 17°C or in a CO_2 incubator at 38.5°C for different times in the presence or absence of inhibitors of mitochondrial activity, rotenone (10 μM, 5 min) and cyanide (2mM, 5 min), inducer of hypersomotic stress, sorbitol (500 mM, 60 min) or a Ca^{2+} absence-induced sperm stress, BAPTA-AM (50 μM, time-course) and then lysed. Proteins (20μg) from spermatozoa lysates were analyzed by western blotting using anti-phospho-Thr^{172}-AMPKα as primary antibody. Arrow indicates the cross-reactive band of phospho-Thr^{172} AMPK

in each experiment that was performed 4-6 times. A representative film of each experiment is shown.

Figure 7. Proposed scheme of intracellular signalling pathways leading to regulation of AMPK activity in mammalian spermatozoa. Intracellular mechanisms involved in AMPK activity in boar spermatozoa that have been investigated in this work are shown. Activators such as 8Br-cAMP, Ca^{2+}, HCO_3^-, PMA, A769662 and different types of cellular stresses (hyperosmotic stress, absence of Ca^{2+}, and inhibition of mitochondrial activity), as well as inhibitors of different kinases (H89, IBMX, STO-609 and Ro-032-0432) are indicated.

Figure 1

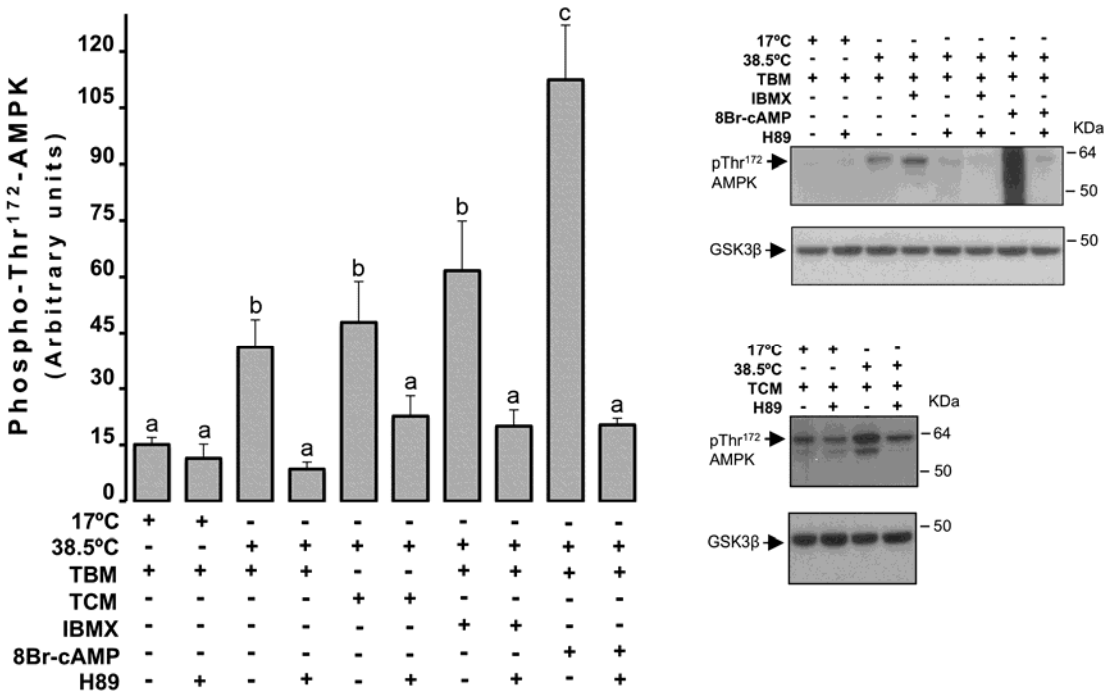

Phospho-Thr172-AMPK
(Arbitrary units)
120
105
90
75
60
45
30
15
0
a
a
b
a
b
a
b
a
c
a
17ºC + + - - - - - - - -
38.5ºC - - + + + + + + + +
TBM + + + + - - + + + +
TCM - - - - + + - - - -
IBMX - - - - - - + + - -
8Br-cAMP - - - - - - - - + +
H89 - + - + - + - + - +
17ºC + + - - - - - - - -
38.5ºC - - + + + + + + + +
TBM + + + + + + + + + +
IBMX - - - + - + - - - -
8Br-cAMP - - - - - - - - + +
H89 - + - - + + - + + +
KDa
pThr172
AMPK
64
50
GSK3β
50
17ºC + + - -
38.5ºC - - + +
TCM + + + +
H89 - + - +
KDa
pThr172
AMPK
64
50
GSK3β
50

Figure 2

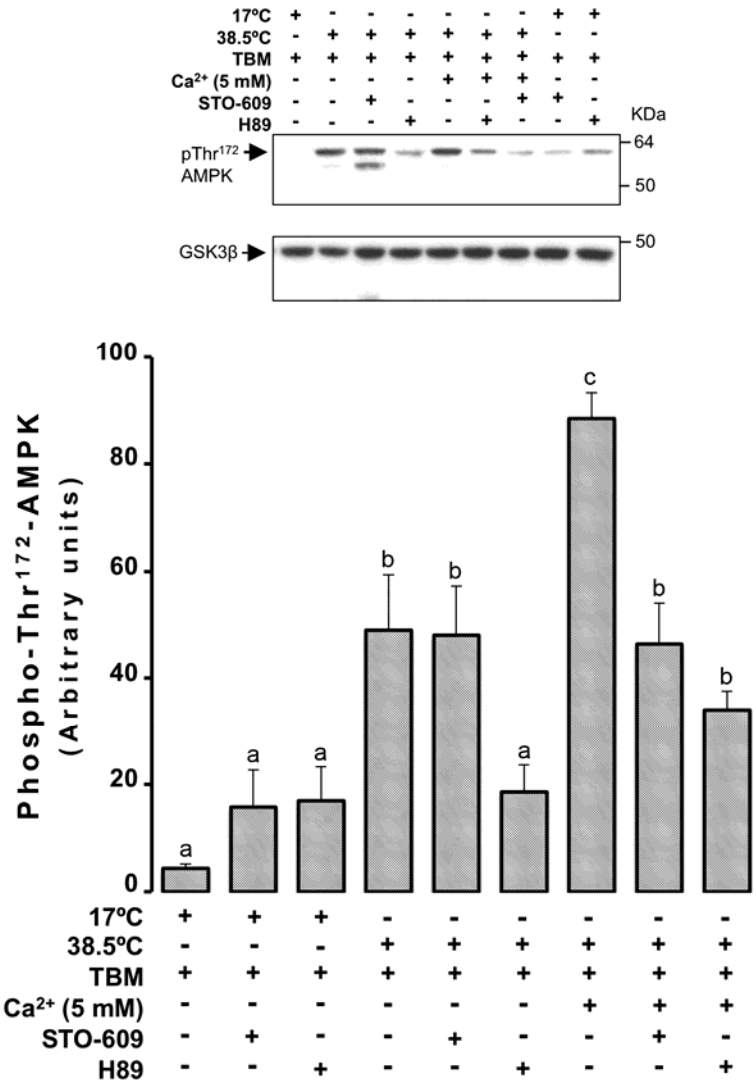
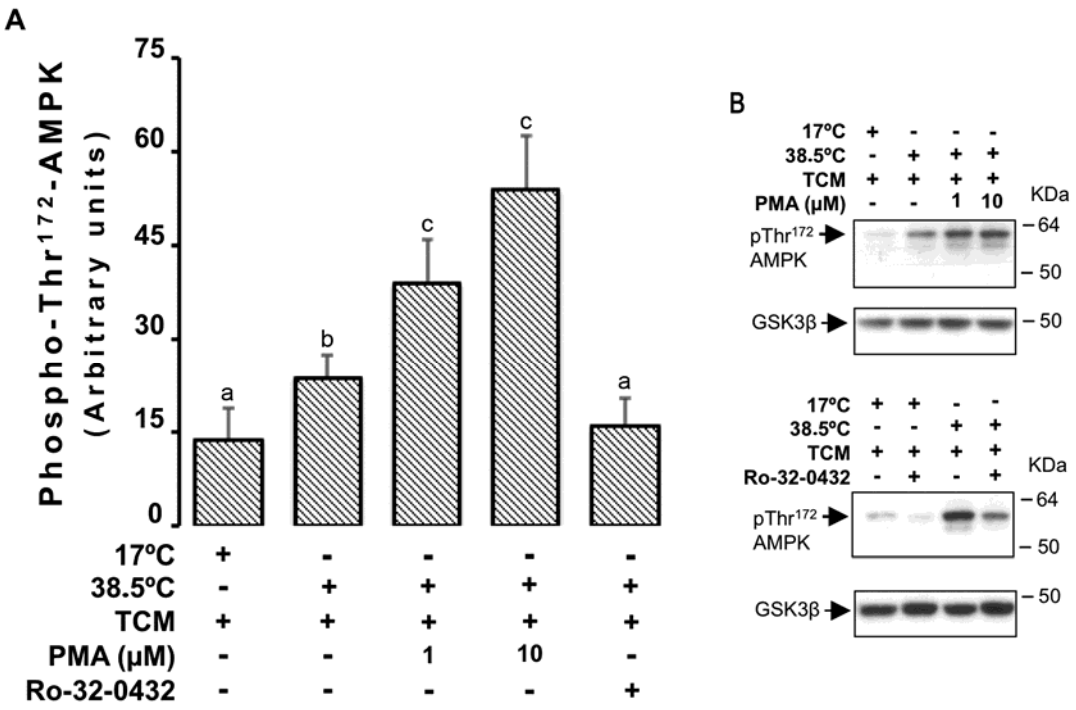

17ºC
38.5ºC
TBM
Ca2+ (5 mM)
STO-609
H89
KDa
pThr172
AMPK
64
50
GSK3β
50
Phospho-Thr172-AMPK
(Arbitrary units)
100
80
60
40
20
0
a
a
a
b
b
a
c
b
b
17ºC
38.5ºC
TBM
Ca2+ (5 mM)
STO-609
H89

Figure 3


A
Phospho-Thr172-AMPK
(Arbitrary units)
75
60
45
30
15
0
a
b
c
c
a
17ºC + - - - -
38.5ºC - + + + +
TCM + + + + +
PMA (μM) - - 1 10 -
Ro-32-0432 - - - - +
B
17ºC + - - -
38.5ºC - + + +
TCM + + + +
PMA (μM) - - 1 10
KDa
pThr172 AMPK
64
50
GSK3β
50
17ºC + + - -
38.5ºC - - + +
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KDa
pThr172 AMPK
64
50
GSK3β
50

Figure 4

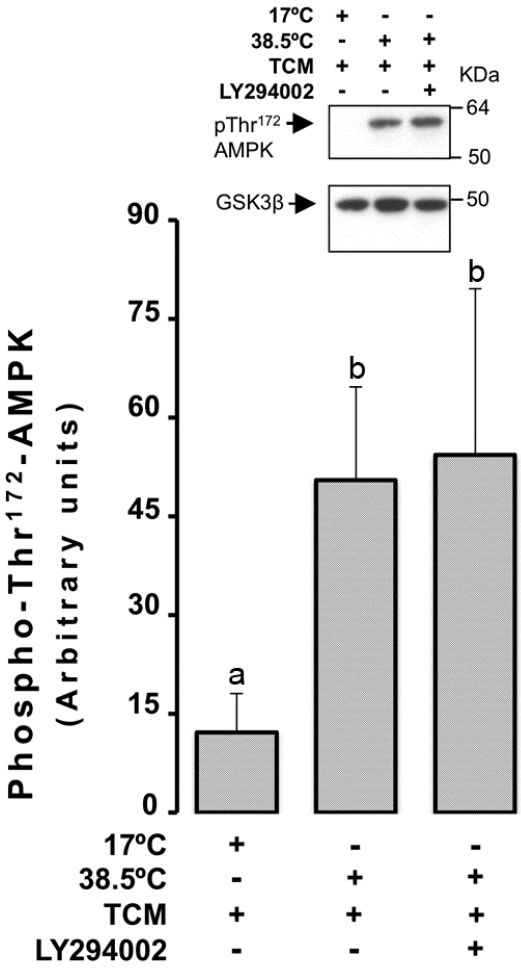

17ºC + - -
38.5ºC - + +
TCM + + +
LY294002 - - +
KDa
pThr172
AMPK
64
50
GSK3β
50
Phospho-Thr172-AMPK
(Arbitrary units)
90
75
60
45
30
15
0
a
b
b
17ºC + - -
38.5ºC - + +
TCM + + +
LY294002 - - +

Figure 5

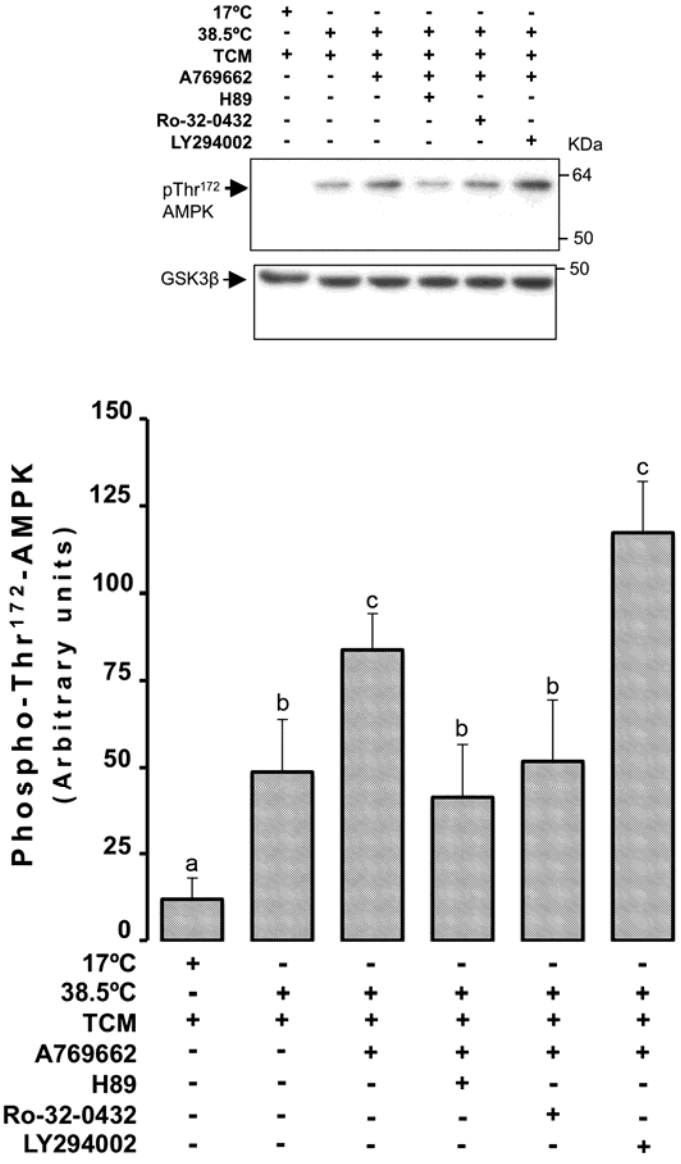

17ºC + - - - - -
38.5ºC - + + + + +
TCM + + + + + +
A769662 - - + + + +
H89 - - - + - -
Ro-32-0432 - - - - + -
LY294002 - - - - - +
KDa
pThr172 AMPK
64
50
GSK3β
50
Phospho-Thr172-AMPK (Arbitrary units)
150
125
100
75
50
25
0
a
b
c
b
b
c
17ºC + - - - - -
38.5ºC - + + + + +
TCM + + + + + +
A769662 - - + + + +
H89 - - - + - -
Ro-32-0432 - - - - + -
LY294002 - - - - - +

Figure 6

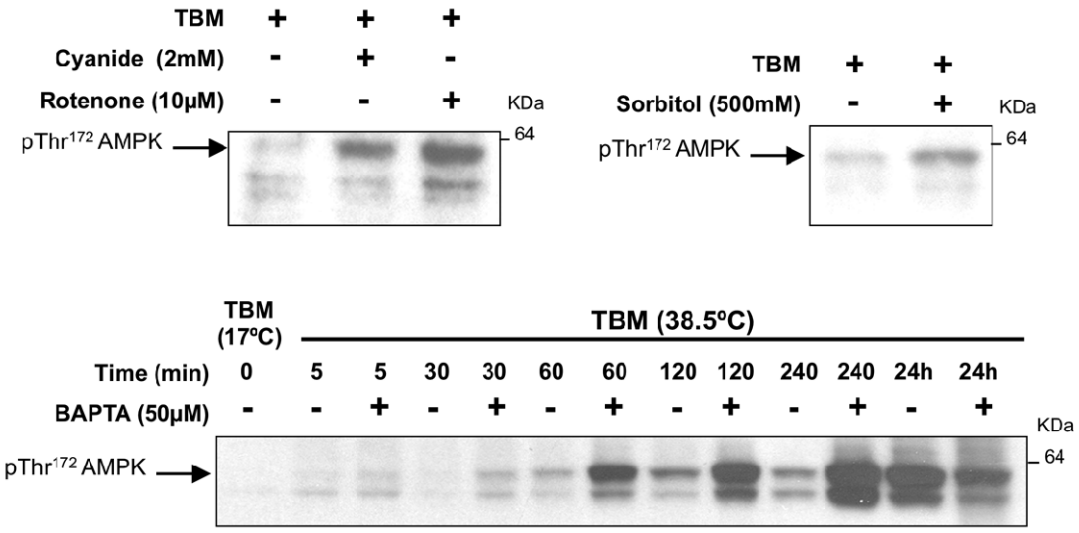
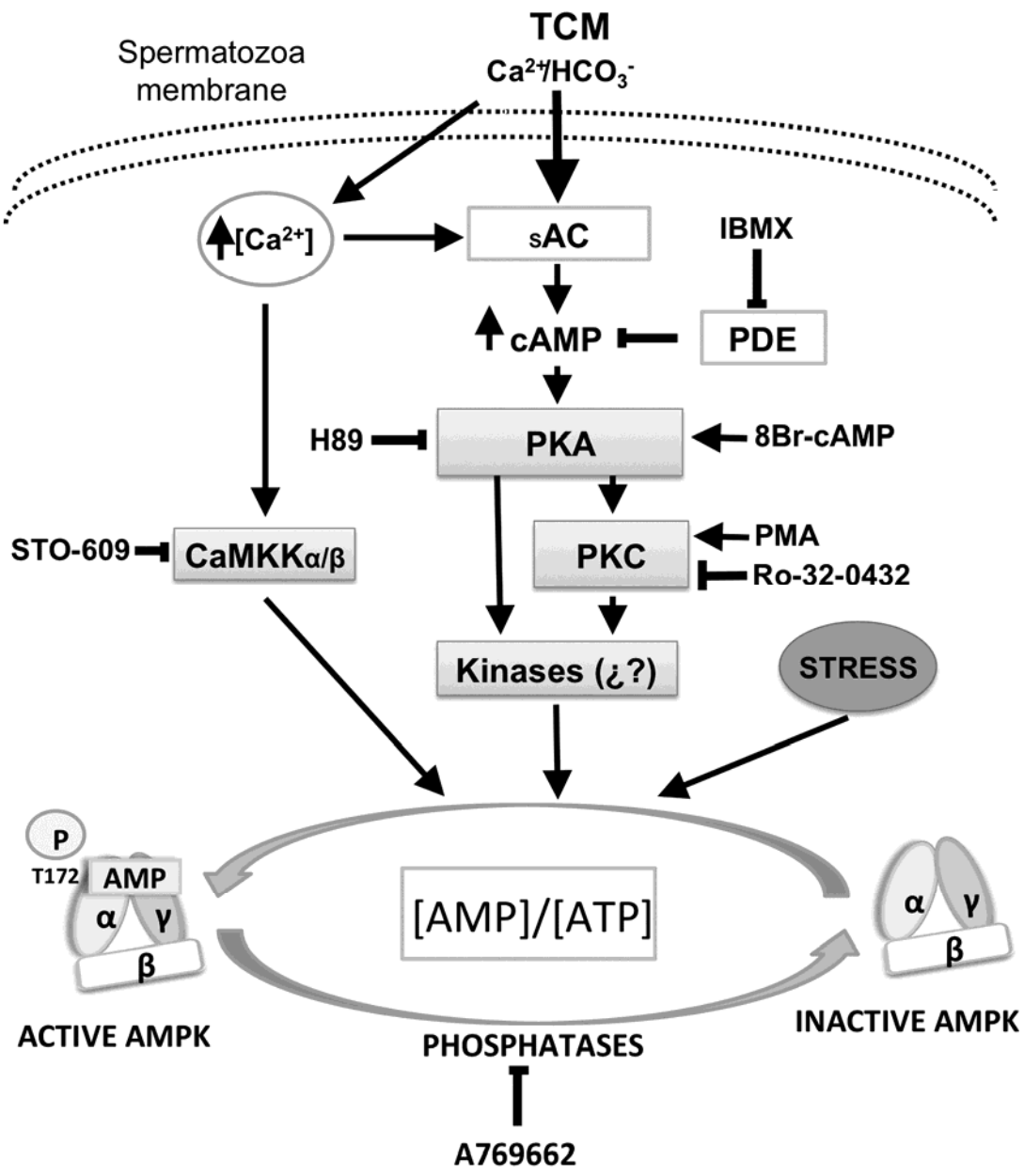

TBM + + +
Cyanide (2mM) - + -
Rotenone (10µM) - - +
KDa
pThr172 AMPK
64
TBM + +
Sorbitol (500mM) - +
KDa
pThr172 AMPK
64
TBM (17ºC)
TBM (38.5ºC)
Time (min) 0 5 5 30 30 60 60 120 120 240 240 24h 24h
BAPTA (50µM) - - + - + - + - + - + - +
KDa
pThr172 AMPK
64

Figure 7


TCM
Ca2+/HCO3-
Spermatozoa membrane
[Ca2+]
sAC
IBMX
cAMP
PDE
H89
PKA
8Br-cAMP
STO-609
CaMKKα/β
PKC
PMA
Ro-32-0432
Kinases (¿?)
STRESS
P
T172
AMP
α
γ
β
[AMP]/[ATP]
α
γ
β
ACTIVE AMPK
PHOSPHATASES
INACTIVE AMPK
A769662

Anexo IV

Up-activation of AMPK down regulates motility and controls essential cellular processes required for boar spermatozoa function.

Ana Hurtado de Llera[a], David Martin-Hidalgo[a], M. Cruz Gil[a], Luis J. Garcia-Marin[a]*, M. Julia Bragado[a]*.

[a] Research Group of Intracellular Signalling and Technology of Reproduction (SINTREP), School of Veterinary Medicine, University of Extremadura, Caceres, Spain.

E-mail address:

Ana Hurtado de Llera	anahl@unex.es
David Martin-Hidalgo	davidmh@unex.es
M. Cruz Gil	crgil@unex.es
Luis J. Garcia-Marin	ljgarcia@unex.es
M. Julia Bragado	jbragado@unex.es

*Both authors contributed equally as senior investigators of this study.

Addresses of correspondence:

Luis J. Garcia-Marin Ph.D.
Research group of Intracellular Signaling and Technology of Reproduction
Department of Physiology
School of Veterinary Medicine, University of Extremadura
10003 Caceres, Spain
Email: ljgarcia@unex.es

M. Julia Bragado Ph.D.
Research group of Intracellular Signaling and Technology of Reproduction
Department of Biochemistry and Molecular Biology and Genetics.
School of Veterinary Medicine, University of Extremadura
10003 Caceres, Spain
Email: jbragado@unex.es

ABSTRACT

We recently demonstrated that inhibition of the kinase acting as cell energy sensor AMPK in boar spermatozoa causes regulatory modifications in motility, plasma membrane organization, acrosome integrity, as well as in the mitochondrial membrane potential. Our aim is to investigate the effect of an increase in AMPK activity by A769662 in essential functional processes of boar spermatozoa. Spermatozoa were incubated under different media (non-stimulating TBM or Ca^{2+} and HCO_3^-- stimulating TCM) in presence or absence of the AMPK activator A769662 for different times (0-24h). AMPK activity, evaluated as Thr172 phosphorylation by western blot, is effectively induced by A769662 in spermatozoa in a time and concentration dependent manner. AMPK activation significant reduces the percentage of motile spermatozoa under Ca^{2+} and/or HCO_3^--stimulated conditions. Moreover, an increase in AMPK activity under both non-stimulating and stimulating conditions for spermatozoa causes a significant reduction in any velocity measured, curvilinear VCL, straight-line VSL and average VAP, which subsequently leads to a significant decrease in the percentage of rapid spermatozoa (VAP>80 μm/s). The effect of increased AMPK activity in motility is intensified by the absence of BSA in the medium. An increase in AMPK activity for short time (hours) prevents the fall in cell viability, as well as in the high mitochondrial membrane potential induced under spermatozoa stimulating conditions (Ca^{2+} and/or HCO_3^-), whereas sustained (24h) increase in AMPK activity causes a significant increase in lipid disorganization as well as phosphatidylserine externalization in spermatozoa plasma membrane, and a decrease in acrosome membrane integrity under both non stimulating and Ca^{2+} and/or HCO_3^--stimulating conditions. In summary, an increase in AMPK activity regulates essential spermatozoa processes such as motility, viability, mitochondrial membrane potential, acrosome membrane integrity, organization and fluidity of plasma membrane. As all these spermatozoa functions are required under different environmental conditions when transiting through the female reproductive tract to achieve oocyte fertilization, we conclude that balanced levels of AMPK activity are essential for regulating spermatozoa physiological function.

Key words: spermatozoa, A769662, AMPK activity, motility, viability, plasma membrane, mitochondrial membrane potential, acrosome integrity.

INTRODUCTION

Mammalian spermatozoa need to acquire a fertilizing competent status and therefore undergo several cellular processes within the female genital tract to successfully fertilize the oocyte. These spermatozoa processes include motility, capacitation, hyperactivation and acrosome reaction and allow spermatozoa to reach the oocyte, trigger the acrosome reaction and subsequently fertilize the oocyte. The acquisition of these spermatozoa functional competences is mainly accomplished by post-translational modifications of pre-existing proteins such as phosphorylation and is critically dependent on the cellular energy state, defined as the ratio between cellular AMP and ATP [7,19].

The energy sensor molecule acting as a regulator of energy balance at both, the cellular and the whole body levels is the AMP-activated protein kinase AMPK [9], an evolutionary conserved serine/threonine kinase that responds to a rise in AMP levels (which indicates low cell energy state) by stimulating ATP-generating pathways and inhibiting ATP-consuming anabolic pathways [2,9]. The overall metabolic consequence of AMPK activity is the maintenance of energy levels under ATP-limiting conditions. AMPK is a heterotrimeric protein with a catalytic α subunit and two regulatory subunits, β and γ. Two "Bateman" domains in the γ subunit bind its allosteric effector AMP [21]. The binding of AMP leads to an increase in phosphorylation at Thr-172 located in the activation loop of α subunit, causing its enzymatic activation [22]. In this regard, AMPK is highly sensitive to AMP, as any increase in the ratio AMP/ATP due to a decrease in cellular energy state, stimulates AMPK activity [22]. To date, the following kinases have been described to phosphorylate AMPK at Thr172 in somatic cells: i) the tumour suppressor responsible for the inherited cancer disorder Peutz-Jeghers syndrome, LKB1 [26], ii) the two Ca^{2+}/calmodulin-dependent protein kinase kinases CaMKKα and CaMKKβ [10] and iii) the transforming growth factor TGF-β-activated kinase-1, TAK1 [27]. AMPK might regulate cell processes outside metabolism [2,9], as its enzymatic activity is also switched on by different types of cellular and metabolic stresses [4,9,15].

The first work studying signalling pathway leading to AMPK activity in male germ cells was published in 2008, where Towler et al., (2008) proposed that a short splice variant of LKB1, the upstream kinase of AMPK, called LKB1s, plays a crucial role in spermiogenesis, motility and fertility in mice [24]. Moreover, deletion of two Ser/Thr kinases testis specific and related to AMPK, TSSK1 and 2 causes male infertility in chimera mice due to haploinsufficiency [28].

Recently, we have demonstrated for the first time that AMPK protein is highly expressed in mammalian ejaculated spermatozoa [11] and that it localizes in the acrosome region of boar spermatozoa head as well as in the midpiece of the flagellum [13]. Our work demonstrates that inhibition of AMPK leads to modifications in one of their most important functions, motility [11], as well as causes regulatory adjustments in the mitochondrial membrane potential, spermatozoa plasma membrane fluidity and organization and acrosome integrity at both boar physiological temperature [13] and also during boar semen preservation at 17ºC [18]. Very recently, supporting a relevant role of AMPK in the function of male germ cells, Tartarin et al., (2012) demonstrated that mice lacking the AMPK catalytic subunit α1 gene [α1AMPKα1knockout (KO)] show a decreased fertility in parallel with an alteration in spermatozoa morphology, reduced mitochondrial membrane potential, lower basal oxygen consumption as well as a decreased spermatozoa motility [23]. Regarding the intracellular regulation of AMPK activity in spermatozoa, we have recently demonstrated that AMPK activity is regulated by external Ca^{2+} and HCO_3^- that through the activation of soluble adenilylcyclase produce an increase in intracellular cAMP. Spermatozoa kinases involved in AMPK activity signalling pathway are that in turn activates PKACaMKKα/β, PKA, and PKC [12].

As mentioned, spermatozoa need to adapt to external changing conditions as well as metabolic or environmental stresses that might physiologically occur during their transit through the female reproductive tract. Therefore, the study of the functional consequences of AMPK activation in these germ cells is essential for the understanding of those spermatozoa processes in which this energy sensor kinase is likely involved. As few published studies about AMPK in mammalian spermatozoa have been performed under conditions where AMPK activity is inhibited [11,13,18] or by α1AMPKα1 knockout [23], the aim of this work is to investigate the role of a (short and long) increase in AMPK activity in those functional processes of male germ cells that are required to successfully accomplish the oocyte fertilization.

MATERIALS AND METHODS

Chemicals and Sources

A769662 was from Tocris Bioscience (Bristol, UK); anti-GSK3β antibodies was from Cell Signaling (Beverly, CA); anti-P-Thr^{172}-AMPK antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA); live/dead spermatozoa viability kit (including both propidium iodine (PI) and SYBR-14 probes), as well as M540 and YoPro-1 probes were purchased from Molecular Probes (Leiden, The Netherlands); PNA-FITC were from Sigma-Aldrich® (St Louis, MI, USA); annexin-V–FITC from Immunostep (Salamanca, Spain); JC-1 probe from Life Technologies Ltd (Grand Island, NY, USA); complete EDTA-free protease inhibitor cocktail was purchased from Roche Diagnostics (Penzberg, Germany); Tris/Glycine/SDS buffer (10X) and tris/Glycine buffer (10X) from Bio-Rad (Richmond, CA). Hyperfilm ECL were 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); coulter isoton II diluent from Beckman Coulter Inc. (Brea, CA, USA).

Spermatozoa incubation media.

Tyrode's basal medium (TBM) was prepared as following: 96 mmol/l NaCl, 4.7 mmol/l KCl, 0.4mmol/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 variant of this basal 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). A variant of both mediums was prepared omitting BSA. 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}.

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 17ºC before use and, in

order to minimize individual boar variations, samples from up to 3 animals were pooled using semen from no less than 19 boars in different combinations. Only semen pools with at least 80% morphologically normal spermatozoa were used. Semen was centrifuged at 2000*g* for 4 minutes, washed with PBS and placed in TBM or TCM medium. Samples of 1.5 ml containing 120 $x10^6$ spermatozoa/ml were incubated at 38.5°C in a stove without CO_2 (TBM) or in a CO_2 cell incubator (TCM) for different times until 24h for AMPK analysis by western blotting and lower volume (0.5ml) was used in spermatozoa samples prepared for flow cytometry analysis. In order to minimize possible experimental variations, every condition/treatment studied was performed in the same semen pool. A control sample with the final concentration of the solvent DMSO (≤ 0.1%) was included. We have previously demonstrated that DMSO [11,18] at concentration used in this study, or even higher, does not modify at all any spermatozoa functional parameters analyzed.

Western blotting.

Spermatozoa under different treatments were centrifuged 20s at 7000*g*, washed with phosphate buffered saline (PBS) supplemented with 0.2mM Na_3VO_4 and then lysated in a lysis buffer consisting in 50mmol/L Tris/HCl, pH 7.5, 150mmol/L NaCl, 1% Triton X-100, 1% deoxycholate, 1mmol/L EGTA, 0.4mmol/L EDTA, protease inhibitors cocktail (Complete, EDTA-free), 0.2mmol/L Na_3VO_4, and 1mmol/L PMSF by sonication for 5s at 4ºC. After 20 minutes at 4ºC samples were centrifuged at 10.000*g* (15 minutes, 4°C) and the supernatant (lysate) was used for analysis of protein concentration. Proteins from porcine spermatozoa lysates were resolved by SDS-PAGE and electro-transferred to nitrocellulose membranes. Western blotting was performed as previously described [11,13,18] using anti phospho-Thr^{172}-AMPKα (1:500) and anti GSK3β (1:2.000) polyclonal antibodies as primary antibodies.

Evaluation of spermatozoa motility by Computer Assisted Sperm Analysis (CASA) system.

After incubation of spermatozoa at 38,5°C in the absence (TBM) or presence of CO_2 (TCM) during different times, a total of 2µl of sample was placed in a pre-warmed counting chamber (Leja®, Luzernestraat, The Netherlands). Sperm motility analysis is based on the examination of 25 consecutive digitalized images obtained from a single field using a X10 negative-phase contrast objective, and at least 400 spermatozoa per sample were analyzed. Images were taken with a time lapse of 1s and objects incorrectly identified as spermatozoa were eliminated from the analysis. Motility parameters evaluated with the ISAS® program (Projectes i Serveis R+D, SL; Valencia, Spain) were as following: VCL (curvilinear velocity, in µm/s), VSL (straight-line velocity in µm/s), VAP (average path velocity, in µm/s), LIN (linearity coefficient in %),

STR (straightness coefficient in %), ALH (amplitude of lateral head displacement in μm), WOB (wobble coefficient in %), BCF (beat cross frequency in Hz). Those spermatozoa with VAP <10μm/s were considered immobile, while spermatozoa with a velocity >10μm/s were considered mobile; spermatozoa with a VAP velocity >80μm/s were considered as rapid spermatozoa.

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 200mV argon ion laser operating at 488nm. 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. Flow cytometry data were analysed using a FacStation computer and EXPO^{TM} 32 ADC software (Beckman Coulter, Inc.).

Analysis of spermatozoa viability by flow cytometry

As described previously [11,18] fluorescent staining using the Live/Dead Sperm Viability kit was performed to measure spermatozoa viability. Briefly, 5μl of SYBR-14 (2μM) and 10μl of propidium iodide PI (5μM) were added to 500 μl of spermatozoa sample diluted to 35 x 10^6 cells/ml in isotonic buffered diluent and incubated 20 min at room temperature in the darkness. After incubation, sperm cells were analyzed in the flow cytometer and results of viable spermatozoa were expressed as the average of the percentage of $SYBR14^+$ and PI^- spermatozoa ± SEM. The fluorescence values of SYBR-14 were collected in the FL1 sensor using a 525 nm bad pass filter, whereas PI fluorescence was collected in the FL3 sensor using a 620 nm bad pass filter.

Evaluation of the acrosome integrity by flow cytometry

The population of spermatozoa with reacted or -damaged acrosomes was assessed after staining these germ cells with phycoerythrin PNA-FITC as specific marker for acrosome membrane status and PI as a marker for cell death [17,25]. Aliquots of 100μl of each semen sample (35 x 10^6 cells/ml) were incubated at RT in the darkness for 5min with 1μg/mL of PNA-FITC and 6μmol/L of PI. Then, 400μl of isotonic buffered diluent were added to each sample and mixed before flow cytometry analysis. The fluorescence value of probe PNA-FITC was collected in the FL1 sensor using a 525nm bad pass filter. Results are expressed as the average of the percentage of PNA^+ and PI^- spermatozoa ± SEM.

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 iodine) [13,18]. This lipophilic cationic fluorochrome JC-1 is present as protomeric aggregates in mitochondria with high membrane potential that emitt in orange (590nm), whereas in mitochondria with low membrane potential, JC-1 is present as monomers that emitt in green (525nm) when excitted at 488nm. Briefly, an aliquot of 100µl from each spermatozoa sample (35 x 10^6 cells/ml) was diluted in 400µl of isotonic buffer containing 0.15mmol/L of JC-1 and then mixed and incubated at 38.5ºC for 30min. The samples were mixed again before flow cytometry analysis. The fluorescence value of probe JC-1 was collected in the FL1 sensor using a 525nm bad pass filter. 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 (high ΔΨm) spermatozoa ± SEM.

Evaluation of the degree of plasma membrane lipid organization of spermatozoa by flow cytometry

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 in the lipid architecture of spermatozoa plasma membrane, as we previously described [13,17,18]. Briefly, aliquots of 100µl of each semen sample (35 x 10^6cells/ml) were diluted in 400µl of isotonic buffer containing 75nmol/L of YoPro-1, mixed and incubated at 38.5ºC for 15min. Then, M540 was added to each sample to a final concentration of 2µmol/L, incubated for 2min and remixed before flow cytometry analysis. The fluorescence values of probes YoPro-1 and SYBR-14 were collected in the FL1 sensor using a 525nm bad pass filter. The spermatozoa were categorized by labelling 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 are expressed as the average of the percentage of YoPro-1^-/M540^+ spermatozoa ± SEM.

Evaluation of the phosphatidylserine externalization at the outer leaflet plasma membrane of spermatozoa by flow cytometry

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 boar sperm plasma membrane, as we have previously described [13]. Briefly, 60×10^6 sperm cells were incubated at 38.5°C with or without A766962 either in TBM

or TCM for different times, and then diluted to at a final concentration of 12×10^6 cells/ml in the following buffer: 96mmol/L NaCl, 4.7mmol/L KCl, 0.4mmol/L $MgSO_4$, 0.3mmol/L NaH_2PO_4, 5.5mmol/L glucose, 1mmol/L sodium pyruvate, 21.6mmol/L sodium lactate, 20mmol/L HEPES (pH 7.45), and 2.5mM $CaCl_2$. Then, a 100µl aliquot (2.4×10^6 sperm cells), was transferred to 5ml tubes and stained with 5µl of annexin V-FITC and 4µl of PI by incubation for 15min in the dark at room temperature. Finally, 400µl of above mentioned buffer were added to each sample and mixed before flow cytometry analysis. The fluorescence values of probes annexin V-FITC and PI were collected in the FL1 and FL3 sensors using a 520 and 620nm bad pass filter, respectively. The results are expressed as the average of the percentage of annexin V^+/PI^- spermatozoa ± SEM.

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. To analyze the percentage of motile and rapid spermatozoa we used the Pearson Chi-square test. All analyses were performed using SPSS v11.0 for MacOs X software (SPSS Inc. Chicago, IL). The level of significance was set at $p<0.05$.

RESULTS

A769662 increases physiological Thr172-AMPK phosphorylation (activation) in boar spermatozoa in a time and concentration dependent manner.

We have used a relatively novel compound that acts as a potent and selective AMPK activator in somatic cells: A769662 [3,8,20]. Initially we have studied the effect of different concentrations of A769662 (25-300 µM) in TBM medium during 24h at 38.5°C in boar spermatozoa AMPK activity, evaluated as phospho-Thr172-AMPK levels, as we have previously described in these germ cells [11,13,18]. As seen in Figure 1A, spermatozoa treatment with A769662 increases phospho-Thr172-AMPK levels in a concentration dependent manner, being 100 µM the minimum concentration with clear effect. Independent experiments show that the maximum AMPK activating effect is achieved at the range of 200-300 µM (data not shown), and therefore we decided to use 200µM in this study. Figure 1B shows that the activating effect of A769662 (200µM) on Thr172-AMPK phosphorylation is detectable as rapid as 60 minutes of spermatozoa incubation at 38.5ºC, reaching a maximum action about 4 hours. Thus, these data demonstrate that A769662 effectively activates AMPK in mammalian male germ cells by increasing its phosphorylation at Thr172 located in the α catalytic subunit.

The increase in AMPK activity induced by A769662 in boar spermatozoa causes a reduction of spermatozoa motility.

To evaluate the effect of an increase in the physiological levels of AMPK activity in spermatozoa motility, these germ cells were incubated in TBM or TCM in the presence or absence of A769662 (200µM) for different times at boar physiological temperature, 38.5°C. In addition, we have analyzed motility parameters at boar semen preservation temperature, 17°C, in the absence of stimulus (TBM) and included as time 0. As observed in Figure 2A, an increase in AMPK activity under stimulating conditions for spermatozoa (TCM medium, including Ca^{2+} and HCO_3^-, right graph) leads to a time-dependent reduction in the percentage of motile spermatozoa, reaching a maximum decreasing effect between 2-4hs. However, an increase in AMPK activity under non-stimulating conditions (TBM, left graph) does not significantly affect this parameter. Regarding the percentage of rapid spermatozoa, defined as those motile spermatozoa with average velocity VAP higher than 80µm/s (Figure 2B), a short or sustained increase in AMPK activity causes a significant reduction in this motility parameter, expressed as the percentage of motile spermatozoa, either in TBM (left) or TCM

(right). This effect of A769662 is clear and rapid as at 60 min of incubation reduces by 50% in TBM or by 40% in TCM the percentage of rapid spermatozoa, and at 4 hours the inhibitory effect is greater, about 60% and 80% reduction in TBM and TCM, respectively. Longer incubation times such as 24h at physiological temperature are not the optimal experimental conditions to detect any rapid spermatozoa, even at control conditions in the absence of A769662, (Figure 2B).

The analysis of spermatozoa motility parameters under stimulating or non-stimulating conditions reveals that any type of spermatozoa velocity measured is always reduced when an increase in AMPK activity is induced by A769662 (Figure 3). Thus, the curvilinear velocity VCL (Figure 3A), the straight-line velocity VSL (Figure 3B) and the average velocity VAP (Figure 3C) are always significantly reduced in the presence of the AMPK activator in a time-dependent manner. Thus, the A769662 effect is not detectable when motility parameters are measured at 17ºC (considered as time 0 in TBM) or during first 5 min incubation in TCM. However, as rapid as 60 min incubation at 38.5ºC there is a detectable and consistent inhibitory effect of A769662 in any spermatozoa velocity analyzed, which remains almost constant for longer incubation times. Moreover, AMPK activity induced by A769662 under either non-stimulating (Table 1) or stimulating (Table 2) spermatozoa conditions does not significantly affect other spermatozoa motility parameters analyzed: LIN, STR, WOB, ALH and BCF.

Effect of A769662-induced increase in AMPK activity in the viability of boar spermatozoa.

The effect of AMPK activity induced by A769662 in boar spermatozoa viability has been analysed to correlate it with motility and other functional studies, and in addition to know whether an increase over the physiological AMPK activity might cause spermatozoa side effects that compromise these germ cells viability. In the same experimental conditions that we have used in this study, the viability of boar spermatozoa is sensitive to the presence of Ca^{2+} and/or bicarbonate in the medium in a time dependent manner [1,11], therefore, we evaluated the A769662 effect under non-stimulating (Figure 4A) and HCO_3^- and Ca^{2+}-stimulated conditions for spermatozoa (Figure 4B). Results show that spermatozoa viability under non-stimulating conditions remains constant at any time analyzed (by 80% of total spermatozoa are viable) and is not affected by an increased AMPK activity (Figure 4A). Results confirmed that under HCO_3^- and Ca^{2+}-stimulated conditions, spermatozoa viability significantly decreases in a time dependent manner (Figure 4B) until 50% at 24h. However, under these stimulating conditions for spermatozoa, an increase in AMPK activity causes a

slight but reproducible effect preventing the loss of these germ cells viability observed at the short time of 2-4 hours, where about 80% of spermatozoa remain viable in the presence of A769662 compared with 65% in its absence (4 h). Interestingly, this protective effect against spermatozoa death due to a A769662-induced increase in AMPK activity is not observed at later time of 24 h (Figure 4B).

Effect of A769662-induced increase in AMPK activity in the mitochondrial membrane potential of boar spermatozoa.

The effect an increase in AMPK activity 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 A769662 (200µM) for different times at boar semen preservation temperature 17°C (time 0) or physiological temperature 38.5°C (Figure 5). Increased activity of AMPK under non-stimulating spermatozoa conditions leads to a slight but reproducible effect decreasing (by about 10%) the population of spermatozoa presenting high ΔΨm at any time studied (Figure 5A). However, an increase in AMPK activity in spermatozoa causes a significant protective effect at the short time, preventing the fall in the percentage of spermatozoa having high ΔΨm induced by HCO_3^- and Ca^{2+}-stimulated conditions (Figure 5B). Thus, about 75% of spermatozoa remain with high ΔΨm after 4h in the presence of A769662 in TCM compared with 45% in its absence. Similarly to the A769662 action showed in cell viability, this protective effect against the fall in the sperm population with high ΔΨm due to an increased AMPK activity does not occur at longer time of 24h (Figure 5B).

Effect of A769662-induced increase in AMPK activity in the integrity of acrosome membrane and in the lipid organization of spermatozoa plasma membrane.

An optimal function of spermatozoa is crucially dependent of a suitable lipid organization of their plasma membrane. We therefore evaluated the effect of an increased AMPK activity in plasma membrane lipid organization after incubation of spermatozoa in TBM or TCM in the presence or absence of 200µM A769662 by flow cytometry using merocyanine M540. As expected, under non-stimulating conditions the population of $M540^+$ viable spermatozoa is very low (<5%) and remains at minimum at any time studied (Figure 6A, white histograms), however it is clearly dependent of the presence of HCO_3^- and Ca^{2+} in the medium (Figure 6B, white histograms). Thus, data from $M540^+$ viable spermatozoa confirm that TCM incubation markedly increases the percentage of boar spermatozoa with plasma membrane lipid disorganization at in a time dependent manner (Figure 6B, white histograms), confirming previous results in this specie [5,13,18]. An increase in AMPK activity for short time (1-4h)

does not significantly affect the percentage of $M540^+$ viable spermatozoa independently of whether germ cells are incubated in non-stimulating (Figure 6A, black histograms) or stimulating conditions (Figure 6B, black histograms). However, a sustained increase in AMPK activity (24h) of boar germ cells induces a marked and significant increase in the population of live spermatozoa with plasma membrane lipid disorganization either in TBM (4 fold) or in TCM (2 fold), as seen in Figure 6.

We next analyzed the effect of an increase in AMPK activity in the integrity of acrosome membrane of boar spermatozoa incubated in TBM or TCM. Spermatozoa labelling with PNA is indicative of the integrity of acrosome membrane and might reflect both damaged as reacted acrosome. Acrosome membrane integrity is generally maintained under non-stimulating conditions over the time, as observed in Figure 7A (where about 90% of live spermatozoa are PNA^-, white histograms). However, when spermatozoa are incubated in the presence of HCO_3^- and Ca^{2+} the percentages of live spermatozoa PNA^+ are always higher than in TBM, between 2-4 fold depending of the time analyzed (Figure 7B, white histograms). Increased AMPK activity during short time does not affect the integrity of spermatozoa acrosome in either TBM or TCM (Figure 7, black histograms). However, a sustained increase in AMPK activity for 24h in any medium causes a significant loss in acrosome integrity, analyzed as an increase in the percentage of PNA^+/PI^- spermatozoa, being 3.3 fold higher under non-stimulating conditions (Figure 7A) and 1.7 fold increase under stimulating conditions (Figure 7B)

Effect of A769662-induced increase in AMPK activity in the phosphatidylserine externalization in spermatozoa plasma membrane.

We further investigated the effect of an increased AMPK activity in the phosphatidylserine (PS) externalization at spermatozoa plasma membrane, process that indicates plasma membrane scrambling, which physiologically occurs in relevant spermatozoa functions. As observed in Figure 8, the level of phosphatidylserine externalized in spermatozoa plasma membrane is very low (by 10% of total) at semen preservation temperature 17ºC (time 0), independently of the incubation media. The incubation of spermatozoa at 38.5ºC under non-stimulating conditions does not induce PS externalization at any time studied (Figure 8A, white histograms) and is unaffected by A769662 at short time. However, a sustained increase in AMPK activity for 24h causes a significant increase in the PS translocation to the outer leaf of plasma membrane (black histograms). As expected, the presence of HCO_3^- and Ca^{2+} in the incubation media of spermatozoa induces a marked time-dependent increase in the PS externalization, reaching 4 fold at 4h and 5 fold at 24h (Figure 8B, white histograms). An

increase in AMPK activity under these conditions does not modify the degree of phosphatidylserine externalization at short time (1-4h). However, a sustained increase in AMPK activity for 24 h induces a significant PS translocation to the outer leaf of spermatozoa plasma membrane (Figure 8B, black histograms), reaching 75% of spermatozoa with PS externalized.

The absence of BSA in the medium modifies the motility response of boar spermatozoa induced by an increase in AMPK activity.

As the presence of HCO_3^- and Ca^{2+} modify the intensity of the spermatozoa response in motility due to A769662, we decided to investigate the effect of other stimulus, BSA, involved in the physiological function of these male germ cells. We therefore evaluated spermatozoa motility after A769662 treatment in the presence or absence of BSA (3mg/ml) for 60 min. As seen in Figure 9, the absence of BSA in the incubation medium causes a rapid and significant reduction in boar spermatozoa motility, as the percentages of motile (Figure 9A) and rapid (Figure 9B) spermatozoa are always lower than in the presence of BSA, independently of the incubation medium. In addition, the absence of BSA clearly and significantly potentiates the inhibitory effect of A769662 in the percentage of motile (Figure 9A) and rapid (Figure 9B) spermatozoa at any time and any incubation medium. Thus, in the absence of BSA the effect of A769662 in the percentage of motile spermatozoa at 60 min under non-stimulating conditions is clear, reducing by 50% this number in TBM (Figure 9A, left), whereas in the presence of BSA the A769662 effect is not visible at this time. Same effect is observed when spermatozoa are incubated in TCM medium with the AMPK activator, where the inhibition obtained in the absence of BSA in the population of motile spermatozoa is about 75% at 60 min (Figure 9A, right). When considering only those germ cells that move with VAP>80 μm/s, rapid spermatozoa, they should be analyzed after 60 min incubation at 38.5°C (Figure 9B). At this time, the absence of BSA in the medium leads to a more dramatic effect, as under these circumstances, an increase in AMPK activity causes a full inhibition of the rapid spermatozoa population in any medium (Figure 9B).

The absence of BSA in the incubation media causes a clear and significant reduction in any spermatozoa velocity analyzed after 60 min, VCL (Fig. 10A), VSL (Fig. 10B) and VAP (Fig. 10C), which are always higher in the presence of BSA, independently of the incubation medium. Similarly to the effect described in the percentages of motile and rapid spermatozoa, the absence of BSA significantly enhances the inhibitory effect of A769662 in spermatozoa velocities, inhibiting the A769662-induced reduction in VCL (Figure 10A), VSL (Figure 10B)

and VAP (Figure 10C). Moreover, the absence of BSA does not modify other spermatozoa motility parameters analyzed: LIN, STR, WOB, ALH and BCF in any spermatozoa conditions analyzed (data not shown).

DISCUSIÓN

A proper energy balance is essential at cellular level as well as the whole body level. A relevant kinase that regulates energy homeostasis is AMPK, which is activated in response to increases in cellular AMP:ATP and ADP:ATP ratios by mechanisms that include either allosteric activation and increased net phosphorylation at conserved residue of Thr172 within its α catalytic subunit. Our group has recently demonstrated for the first time that AMPK is highly expressed in mammalian male germ cells [11] at the entire acrosome in boar spermatozoa head as well as in the midpiece of flagellum. Moreover, its active form, phospho-Thr^{172}-AMPK, is specifically localized at the apical part of acrosome, remaining also in the midpiece of flagellum of boar spermatozoa [13].

Two well known activators of AMPK in somatic cells are AICAR (5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside, Acadesine, N^1-(β-D Ribofuranosyl)-5-aminoimidazole-4-carboxamide) and metformin (1,1-Dimethylbiguanide hydrochloride**)**, which stimulate AMPK activity by different molecular mechanisms. Surprisingly, in the experimental conditions used in this work, we were unable to observe any clear stimulation of AMPK activity in boar spermatozoa after AICAR or metformin treatments. Therefore, we used a relatively novel compound that acts as a potent and selective AMPK activator in somatic cells: A769662 [3]. This compound activates AMPK both allosterically and by inhibiting its dephosphorylation on Thr172 [8,20], independently of the upstream kinase [8]. In the present work we demonstrate for the first time that this pharmacological activator of AMPK, A769662, activates AMPK in mammalian male germ cells. Our results show a clear effect of A769662 increasing spermatozoa AMPK phosphorylation on Thr172 as rapid as 60 min and maintaining this activating action for several hours. Moreover, the concentration of A769662 that we have selected from the concentration-response experiment in spermatozoa, 200μM, has been used previously in somatic cells [14].

According to the key role of AMPK in the control of energy charge in somatic cells, it is logical to assume that this kinase could be involved in those spermatozoa functions, as motility, that are dependent of energy levels, as we have recently demonstrated [11]. Regarding spermatozoa motility, we will discuss three different levels of spermatozoa AMPK activity that we have approached in this work and previously [11] by subjecting spermatozoa to three experimental situations: i) Level of AMPK activity in the physiological range. For this discussion purpose, we consider as "physiological" those AMPK activity levels observed in

spermatozoa incubated at boar physiological temperature (38.5°C) under TBM or TCM but always in the absence of AMPK activator or inhibitor. Under these physiological conditions, the level of AMPK activity is the optimal to maintain the spermatozoa motility that is adequate to different environmental conditions in which spermatozoa is involved, such as presence or absence of Ca^{2+} and/or HCO_3^-. We consider that physiological levels of AMPK activity warrant that these germ cells are able to respond to the different demands of energy required for spermatozoa function under different extracellular conditions during transit through the female reproductive tract. We have tried to mimic these conditions experimentally by the use of TBM, which will confer a spermatozoa energy status, and also by a stimulating medium with Ca^{2+} and HCO_3^-, which logically will lead to a higher energy demand to accomplish those spermatozoa functional processes induced by these or other stimuli. ii) AMPK activity below physiological level. This spermatozoa condition has been approached previously when these germ cells were incubated in the presence of AMPK inhibitor, CC [11]. Under these conditions, AMPK is mainly inactive, which implies that although AMPK is able to detect a possible fluctuation in energy charge (AMP/ATP) under TBM or TCM, however it is unable to respond to this situation by producing those metabolic adjustments necessary to control and maintain ATP cellular levels required for spermatozoa under each extracellular environment. This sustained lack of control in the maintenance of energy charge would cause, among other consequences, that one of the main ATP-dependent spermatozoa function, motility, resulted negatively affected. This explanation is supported by two previous studies using different approaches to inactivate AMPK in spermatozoa from two mammalian species, boar [11] and mice [23]. Thus, inactivation of AMPK, using transgenic mice lacking the catalytic subunit α1 gene [α1AMPK knockout (KO)] causes a great reduction in mice sperm motility as well as lower fertility [23]. In addition, we have showed in boar spermatozoa that AMPK inactivation by an inhibitor causes a reduction in the main parameters that characterize spermatozoa motility: percentage of motile spermatozoa, velocities VCL, VSL and VAP, and subsequently the number of rapid spermatozoa (VAP>80 μm/s). In summary, both studies in two different mammalian species suggested that certain level of AMPK activity is necessary for proper spermatozoa motility. iii) AMPK activity increased over physiological levels. This work, that approaches this third situation using A769662, shows that an increase over physiological levels of AMPK activity that are adequate for normal spermatozoa function, negatively affects motility. A possible explanation is based in the proposal that a sustained increase in AMPK activity over physiological levels would lead to a deregulation of spermatozoa metabolism caused by a sustained stimulation of ATP-generating catabolic

pathways and by a sustained inhibition of ATP-consuming anabolic pathways. This cannot be considered as physiological situation as is triggered by a permanent upactivation of a kinase (AMPK) that in physiological conditions, as any other intracellular signaling cascade, would be inactivated after a minimum time of activation. This deregulation of metabolic pathways is not likely adequate for the maintenance of proper spermatozoa motility under any extracellular conditions. In fact, under these conditions, we observe in general a reduction in the number of motile spermatozoa, a decrease in the sperm velocities VCL, VSL and VAP, and, subsequently, a lower percentage of rapid spermatozoa (VAP>80μm/s). Exceptionally, the percentage of motile spermatozoa in a medium without stimulus added is not affected by AMPK overactivity, which suggests that the effect of an increase in AMPK activity in spermatozoa motility is influenced by the presence or the absence of stimuli in the extracellular medium. In this sense, it is logical to assume that spermatozoa energy charge in a non-stimulating medium is very different to the cell germ energy charge under a stimulating medium. Therefore, it is plausible to assume that under different extracellular conditions, experimentally approached in this study by the presence or absence of stimuli, such as Ca^{2+}, HCO_3^- and/or BSA, the sustained increase in AMPK over physiological levels might cause a different effect in some motility parameter. In fact, this idea is supported by results showing a different effect of A769662 in the presence and in the absence of another spermatozoa physiological stimulus, BSA.

Summarizing results about the role of AMPK in mammalian spermatozoa motility, our present study as well as previous work in boar [11] or mice [23] allow the conclusion that either up or down fluctuations in the physiological activity level of AMPK play a negative function in spermatozoa motility. Our studies suggest that physiological AMPK activity is necessary to maintain optimal boar spermatozoa motility, adequate to the changing extracellular conditions, as the presence or absence of HCO_3^-, Ca^{2+} and/or BSA. As these stimuli are present, among others, in different concentrations in the female reproductive tract, we propose that this relevant function of AMPK in spermatozoa motility might likely also occur *in vivo* in boar. This role of AMPK in motility is further supported by the localization of AMPK, expressed in important levels at the midpiece of flagellum, where one of the main ATP-generating organelles, mitochondria, are exclusively located in spermatozoa. Moreover, as AMPK activity is also involved in the regulation of motility in boar spermatozoa preserved during several days at 17ºC [18], we extend our proposal that AMPK activity is essential also

to maintain optimal motility under those spermatozoa conditions that can be considered as cell stress, which might also occur during their transit through the female reproductive tract.

Regarding cell viability, data from this work confirm our previous proposal [11] that AMPK activity might be involved in the control of spermatozoa viability under some live-compromising conditions, as AMP activity prevents at least in part the loss in germ cell viability induced by temperature, HCO_3^- and/or Ca^{2+} at the short time. Thus, under those extracellular conditions reported to lead to a loss in spermatozoa viability, such as medium containing high concentrations of Ca^{2+} [6,16] or those that would represent germ cell stresses, the activity of AMPK at the short term would be crucial to contribute to the maintenance of cell viability.

Recently, AMPK activity has been also involved in the control of the mitochondrial membrane potential, ΔΨm, in spermatozoa from boar [13] and mice [23]. Thus, inactivation of AMPK by two different experimental approaches causes a decrease in ΔΨm, although in the case of boar spermatozoa the effect of AMPK activity in ΔΨm is dependent on the extracellular stimuli such as HCO_3^- and Ca^{2+} [13]. Tartarin et al., (2012) also described in parallel reduced basal oxygen consumption in mice lacking catalytic subunit α of AMPK. In this work we show that increased AMPK activity prevents the fall in the high ΔΨm occurring in boar spermatozoa under HCO_3^- and Ca^{2+} conditions at the short time. The present work supports mentioned recent studies that describe that AMPK activity regulates spermatozoa ΔΨm [13,23], and additionally suggest that a level of AMPK activity is essential to maintain a proper mitochondrial membrane potential in spermatozoa under certain conditions. As occurs in the sperm viability, the consequences of an increased AMPK activity in the mitochondrial membrane potential totally depend on the spermatozoa extracellular conditions, supporting the idea that AMPK serves as a metabolic checkpoint by integrating extracellular stimuli - triggered signalling with germ cell metabolism.

Moreover, our group, by using an AMPK inhibitor has proposed recently that AMPK signalling plays a relevant role in the maintenance of the lipid organization of plasma membrane, as well as in the integrity of acrosome membrane in boar spermatozoa [13,18]. In this work we demonstrate that an increase in the spermatozoa activity level of AMPK in the short term does not modify either the lipid organization in the plasma membrane or the PS translocation at the outer leaf. However, a sustained (24h) increase in AMPK activity caused by A769662 leads to higher lipid disorganization in plasma membrane, which correlates with an increase in PS externalization, independently of the incubation media. These results

suggest that a sustained increase in AMPK signalling pathway likely controls the fluidity and organization of spermatozoa plasma membrane under any extracellular conditions. In addition to the dependence of stimuli, our data clearly support the idea that the AMPK effects on spermatozoa membranes are also dependent on the time of AMPK up-activation over basal levels. This time-dependent regulatory function of AMPK activity in spermatozoa membranes also includes the acrosome region where a majority of AMPK active (phosphorylated at Thr172) is localized at physiological conditions [13]. Thus, the present study shows that in parallel to changes occurring at the spermatozoa plasma membrane, a sustained increase in AMPK activity for 24h causes a higher destabilization of the acrosome membrane integrity.

In summary, in light of the remarkable effects caused by short and sustained increase in AMPK activity over the physiological range in spermatozoa, this study suggest that AMPK exerts relevant functional roles at several levels in male germ cells physiology, probably by its action as a metabolic checkpoint by integrating cellular metabolism with signalling triggered by stimuli and/or stresses present in the extracellular medium. As all above mentioned AMPK-regulated spermatozoa processes (motility, viability, mitochondrial membrane potential, organization and fluidity of plasma membrane, acrosome membrane integrity) are required under different environmental conditions of male germ cells when transiting through the female reproductive tract to achieve fertilization, this work concludes that AMPK protein plays an important and necessary regulatory role in the mammalian spermatozoa function.

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FIGURE LEGENDS

Figure 1. Phosphorylation of AMPK at Thr172 is increased by A766962 in boar spermatozoa in a time (B) and concentration (A) dependent manner. Spermatozoa from several boars were pooled and incubated in TBM medium at 17°C (0 min) or at 38.5°C for different times (B) in the presence or absence of different concentrations of A766962 from 25-300µM (A) and then lysed. Proteins (20µg) from spermatozoa lysates were analyzed by western blotting using anti-phospho-Thr^{172}-AMPKα as primary antibody. A representative film of each experiment, that was performed at least 4 times, is shown. Arrow in upper film indicates the cross-reactive band of phospho-Thr^{172} AMPK. Protein loading controls for each experiment using anti-GSK3β antibody are showed in lower film.

Figure 2. Time-course effect of the A769662-induced increase in AMPK activity in the percentages of motile (A) and rapid (B) spermatozoa. Spermatozoa were incubated in non-stimulating medium TBM (left) or HCO_3^- and Ca^{2+}-stimulating medium TCM (right) at 38.5°C in the absence (white histograms) or presence (black histograms) of the AMPK activator A769662 (200µM) during indicated times. Samples at 17°C were considered as time 0. The percentages of motile (A) and rapid (B) spermatozoa were measured by the ISAS system as described. Each experiment was performed at least 5 times and the results express: (A) the percentage of motile spermatozoa from the total analyzed (4.000-5.000) and (B) the percentage of those motile spermatozoa with VAP > 80µm/s, defined as rapid spermatozoa. Statistical differences were considered when $p<0.05$ and showed with an asterisk.

Figure 3. Time-course effect of the A769662-induced increase in AMPK activity in spermatozoa velocities: curvilinear VCL (A), straight-line VSL (B) and average path VAP (C). Spermatozoa were incubated in non-stimulating medium TBM (left) or HCO_3^- and Ca^{2+}-stimulating medium TCM (right) at 38.5°C in the absence (white histograms) or presence (black histograms) of the AMPK activator A769662 (200µM) during indicated times. Samples at 17°C were considered as time 0. Spermatozoa velocities were measured as described and expressed as µm/s. This experiment was performed at least 6 times and results indicate the mean ± standard error of the mean. Statistical differences were considered when $p<0.05$ and showed with an asterisk.

Figure 4. Time-course effect of the A769662-induced increase in AMPK activity in spermatozoa viability. Spermatozoa were incubated in non-stimulating medium TBM (A) or

HCO_3^- and Ca^{2+}-stimulating medium TCM (B) at 38.5°C in the absence (white histograms) or presence (black histograms) of the AMPK activator A769662 (200µM) during indicated times. Samples at 17°C were considered as time 0. Spermatozoa viability was measured by flow cytometry using SYBR-14/IP as probes. This experiment was performed at least 4 times and results expressed as percentage of viable cells are shown as mean ± standard error of the mean. Statistical differences were considered when $p<0.05$ and showed with an asterisk.

Figure 5. Time-course effect of the A769662-induced increase in AMPK activity in the mitochondrial membrane potential of boar spermatozoa. Spermatozoa were incubated in non-stimulating medium TBM (A) or HCO_3^- and Ca^{2+}-stimulating medium TCM (B) at 38.5°C in the absence (white histograms) or presence (black histograms) of the AMPK activator A769662 (200µM) during indicated times. Samples at 17°C were considered as time 0. Mitochondrial membrane potential was measured by flow cytometry as described in material and methods using JC-1 as a probe. Spermatozoa population exhibiting high $\Delta\Psi m$ are 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 $p<0.05$.

Figure 6. Time-course effect of the A769662-induced increase in AMPK activity in the degree of lipid organization of spermatozoa plasma membrane. Spermatozoa were incubated in non-stimulating medium TBM (A) or HCO_3^- and Ca^{2+}-stimulating medium TCM (B) at 38.5°C in the absence (white histograms) or presence (black histograms) of the AMPK activator A769662 (200µM) during indicated times. Samples at 17°C were considered as time 0. 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 one asterisk when $p<0.05$.

Figure 7. Time-course effect of the A769662-induced increase in AMPK activity in the integrity of the spermatozoa acrosome membrane. Spermatozoa were incubated in non-stimulating medium TBM (A) or HCO_3^- and Ca^{2+}-stimulating medium TCM (B) at 38.5°C in the absence (white histograms) or presence (black histograms) of the AMPK activator A769662 (200µM) during indicated times. Samples at 17°C were considered as time 0.

Acrosome destabilization was measured by flow cytometry as described using PNA-FITC as a probe. Spermatozoa population with unstable acrosomes (lower acrosome integrity) 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 $p<0.05$.

Figure 8. Time-course effect of the A769662-induced increase in AMPK activity in the phosphatidylserine externalization at the plasma membrane of boar spermatozoa. Spermatozoa were incubated in non-stimulating medium TBM (A) or HCO_3^- and Ca^{2+}-stimulating medium TCM (B) at 38.5°C in the absence (white histograms) or presence (black histograms) of the AMPK activator A769662 (200µM) during indicated times. Samples at 17°C were considered as time 0. The level of phosphatidylserine (PS) 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 one asterisk when $p<0.05$.

Figure 9. BSA modulates the effect of the A769662-induced increase in AMPK activity in the percentages of motile (A) and rapid (B) spermatozoa. Boar spermatozoa were incubated in the presence or absence of BSA (3mg/ml) in non-stimulating medium TBM (left) or HCO_3^- and Ca^{2+}-stimulating medium TCM (right), in a CO_2 incubator at 38.5°C in the absence (white histograms) or presence (black histograms) of the AMPK activator A769662 (200µM) for 60 min. Samples at 17°C were considered as time 0. The percentages of motile (A) and rapid (B) spermatozoa were measured by the ISAS system as described. Each experiment was performed at least 5 times and the results express: (A) the percentage of motile spermatozoa from the total analyzed and (B) the percentage of those rapid spermatozoa (VAP > 80µm/s). For each time (0, 60 min), statistical differences were considered when $p<0.05$ and indicted with different letters (a-d).

Figure 10. BSA modulates the effect of A769662-induced increase in AMPK activity in spermatozoa velocities: curvilinear VCL (A), straight-line VSL (B) and average path VAP (C). Spermatozoa were incubated in the presence or absence of BSA (3mg/ml) in non-stimulating medium TBM (left) or HCO_3^- and Ca^{2+}-stimulating medium TCM (right), in a

CO_2 incubator at 38.5°C in the absence (white histograms) or presence (black histograms) of the AMPK activator A769662 (200µM) for 60 min. Samples at 17°C were considered as time 0. Spermatozoa velocities were measured as described and expressed as µm/s. This experiment was performed at least 5 times and results indicate the mean ± standard error of the mean. For each time (0, 60 min), statistical differences were considered when $p<0.05$ and indicated with different letters (a-d).

Figure 1

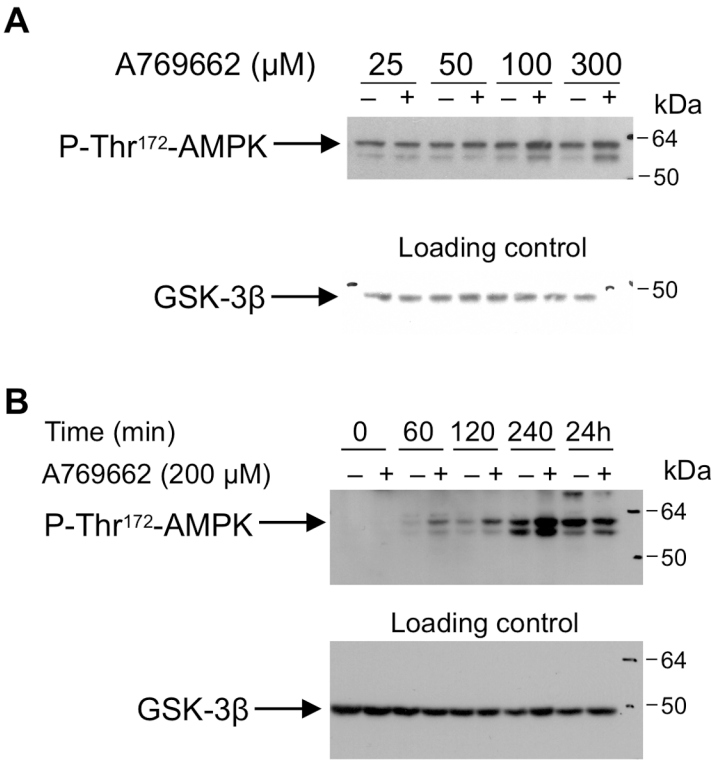

A
A769662 (μM)
25
50
100
300
– + – + – + – +
kDa
P-Thr172-AMPK
64
50
Loading control
GSK-3β
50
B
Time (min)
0
60
120
240
24h
A769662 (200 μM)
– + – + – + – + – +
kDa
P-Thr172-AMPK
64
50
Loading control
GSK-3β
64
50

Figure 2

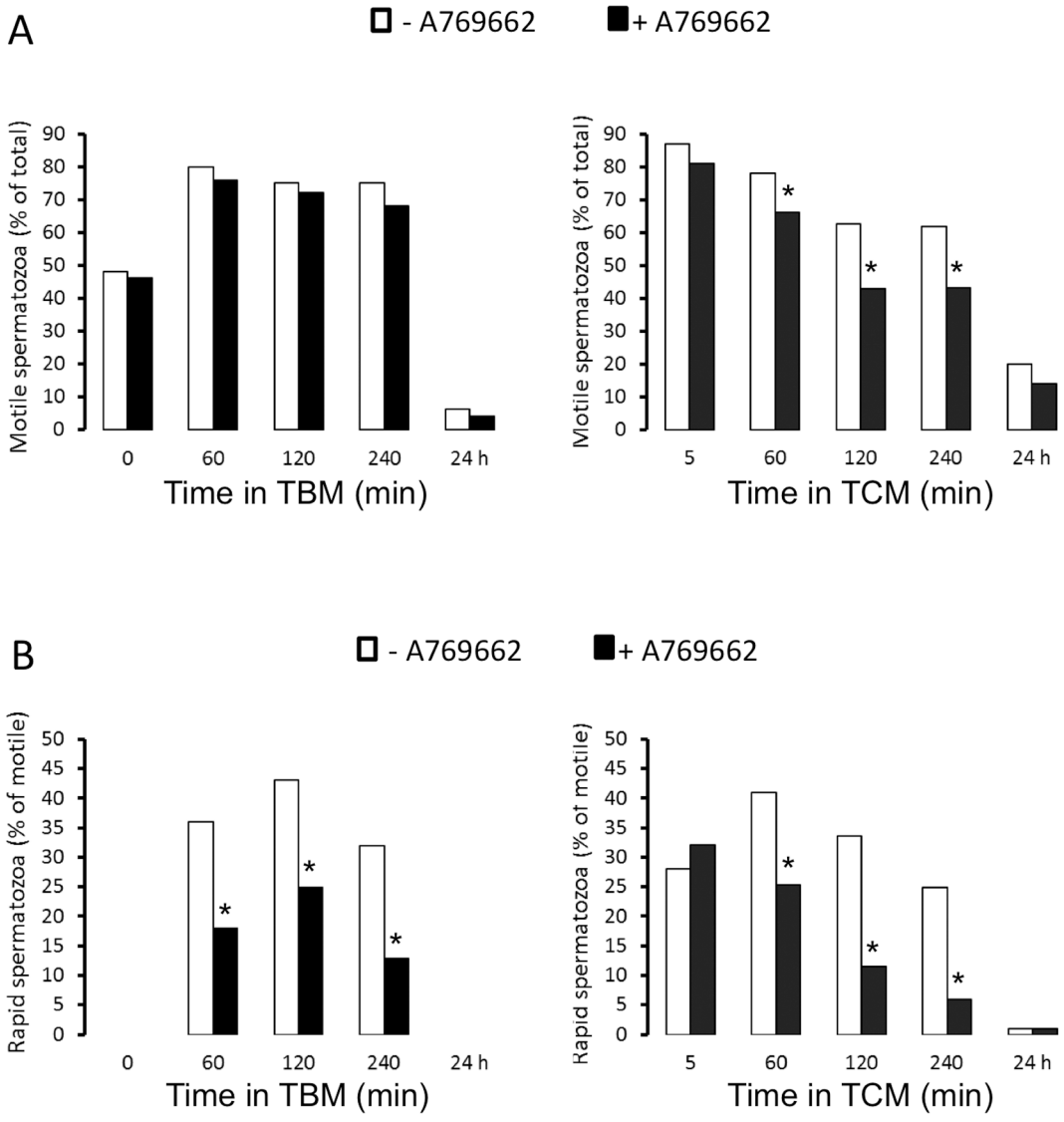

A
- A769662
+ A769662
Motile spermatozoa (% of total)
0
10
20
30
40
50
60
70
80
90
0
60
120
240
24 h
Time in TBM (min)
Motile spermatozoa (% of total)
5
60
120
240
24 h
Time in TCM (min)
*
B
- A769662
+ A769662
Rapid spermatozoa (% of motile)
0
5
10
15
20
25
30
35
40
45
50
0
60
120
240
24 h
Time in TBM (min)
Rapid spermatozoa (% of motile)
5
60
120
240
24 h
Time in TCM (min)
*

Figure 3

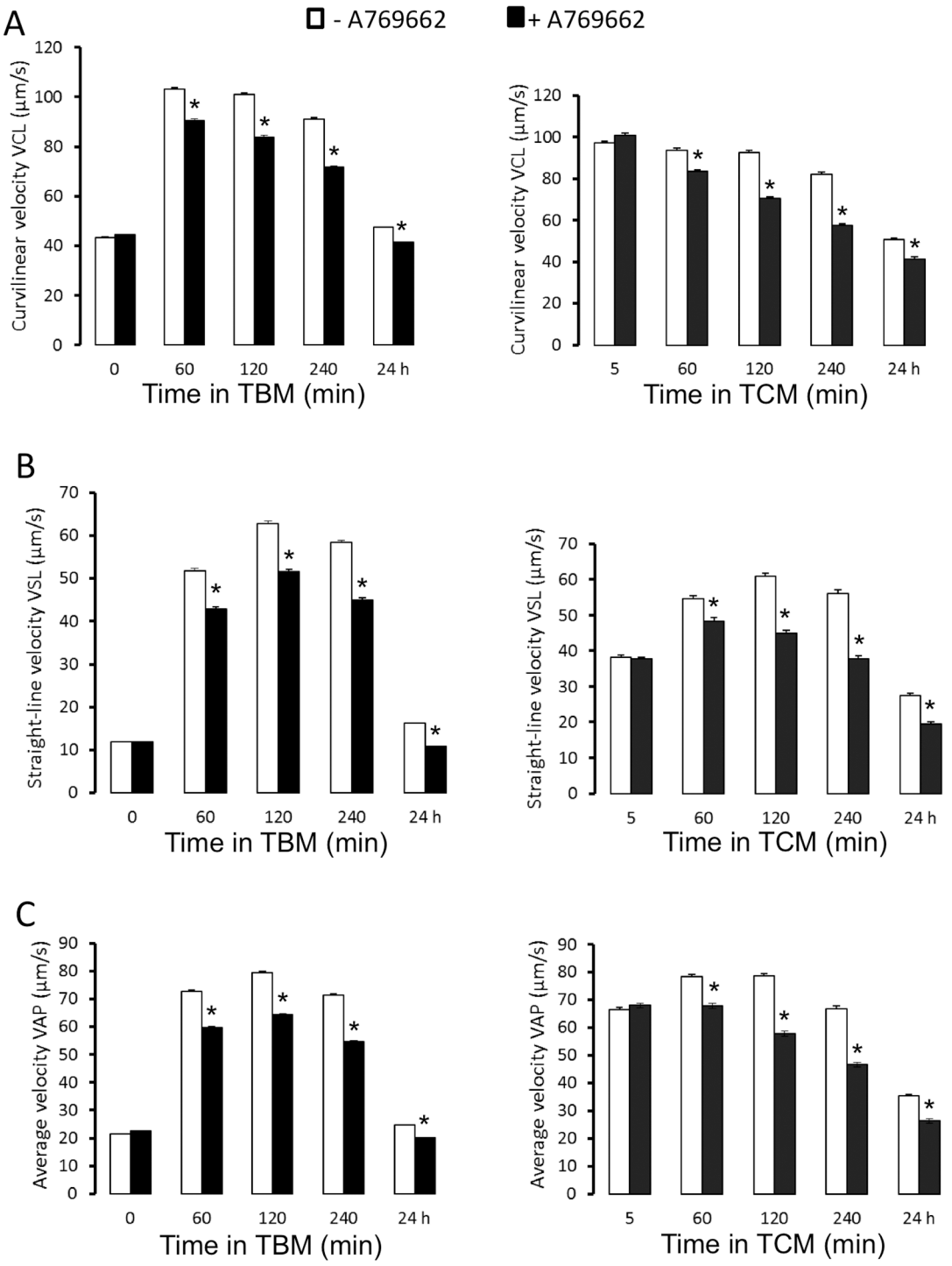

A
- A769662
+ A769662
Curvilinear velocity VCL (µm/s)
Time in TBM (min)
Time in TCM (min)
B
Straight-line velocity VSL (µm/s)
C
Average velocity VAP (µm/s)
0
60
120
240
24 h
5

Figure 4

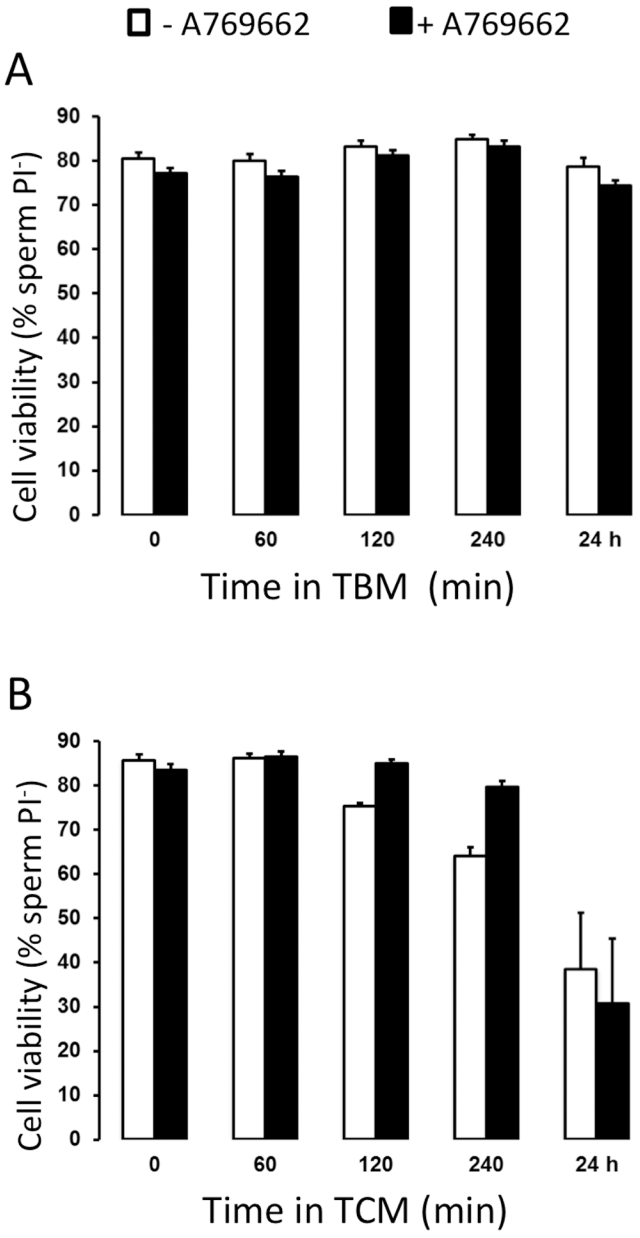

- A769662
+ A769662
A
Cell viability (% sperm PI-)
90
80
70
60
50
40
30
20
10
0
0
60
120
240
24 h
Time in TBM (min)
B
Cell viability (% sperm PI-)
90
80
70
60
50
40
30
20
10
0
0
60
120
240
24 h
Time in TCM (min)

Figure 5

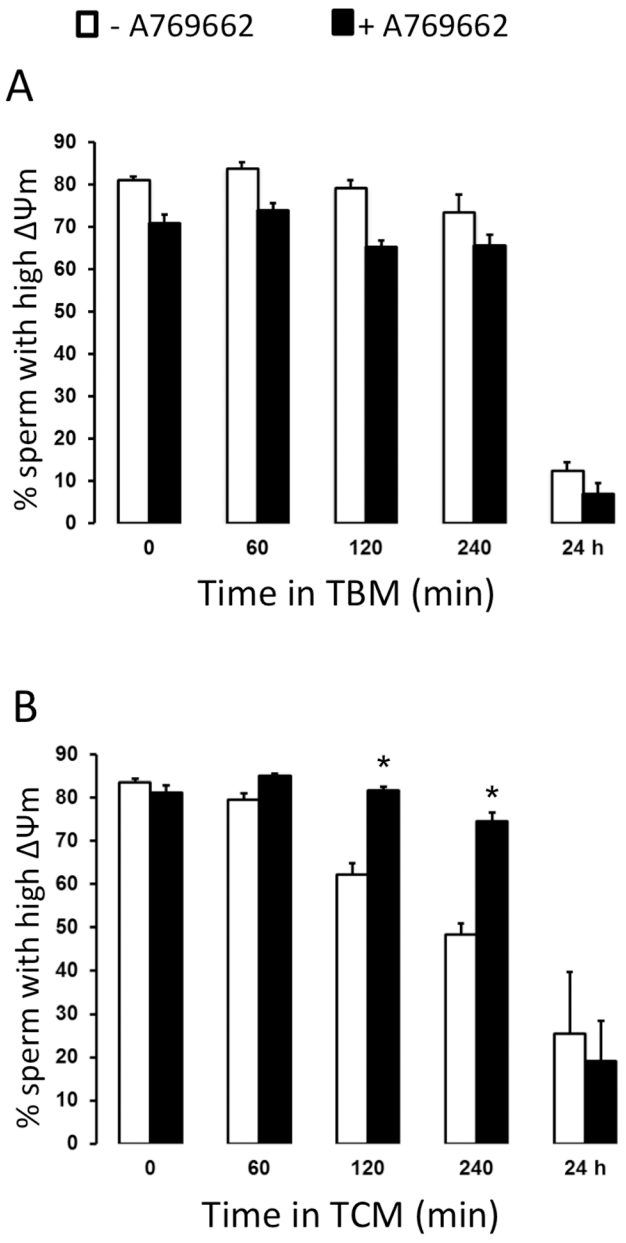

- A769662
+ A769662
A
% sperm with high ΔΨm
90
80
70
60
50
40
30
20
10
0
0
60
120
240
24 h
Time in TBM (min)
B
% sperm with high ΔΨm
90
80
70
60
50
40
30
20
10
0
*
*
0
60
120
240
24 h
Time in TCM (min)

Figure 6

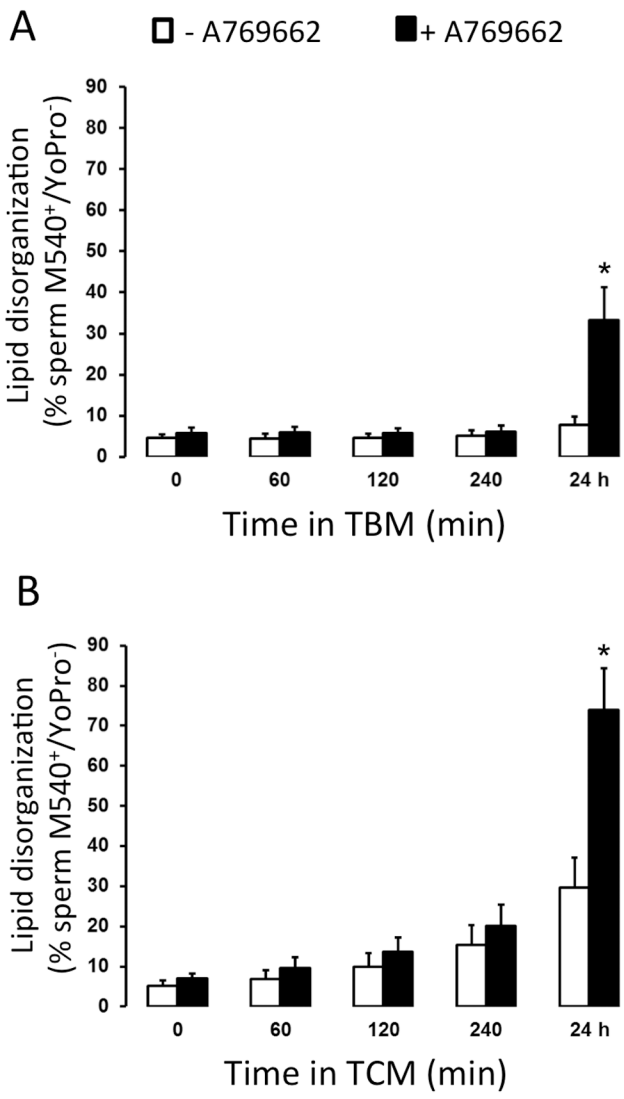

A
- A769662
+ A769662
Lipid disorganization
(% sperm M540+/YoPro-)
90
80
70
60
50
40
30
20
10
0
*
0
60
120
240
24 h
Time in TBM (min)
B
Lipid disorganization
(% sperm M540+/YoPro-)
90
80
70
60
50
40
30
20
10
0
*
0
60
120
240
24 h
Time in TCM (min)

Figure 7

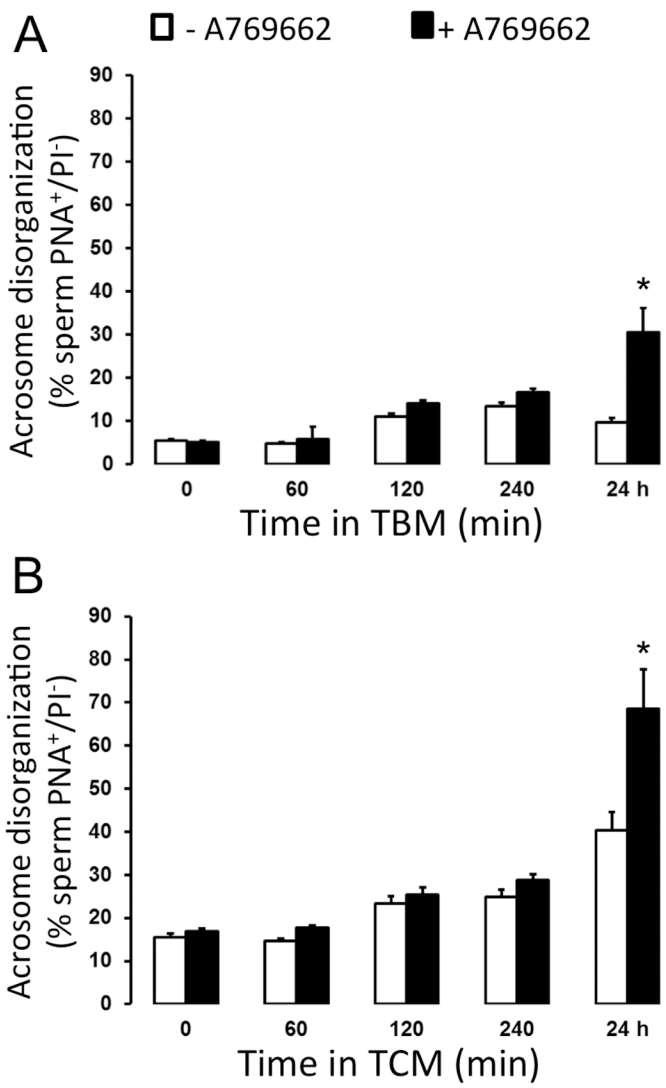

A
- A769662
+ A769662
Acrosome disorganization
(% sperm PNA+/PI-)
90
80
70
60
50
40
30
20
10
0
*
0
60
120
240
24 h
Time in TBM (min)
B
Acrosome disorganization
(% sperm PNA+/PI-)
90
80
70
60
50
40
30
20
10
0
*
0
60
120
240
24 h
Time in TCM (min)

Figure 8

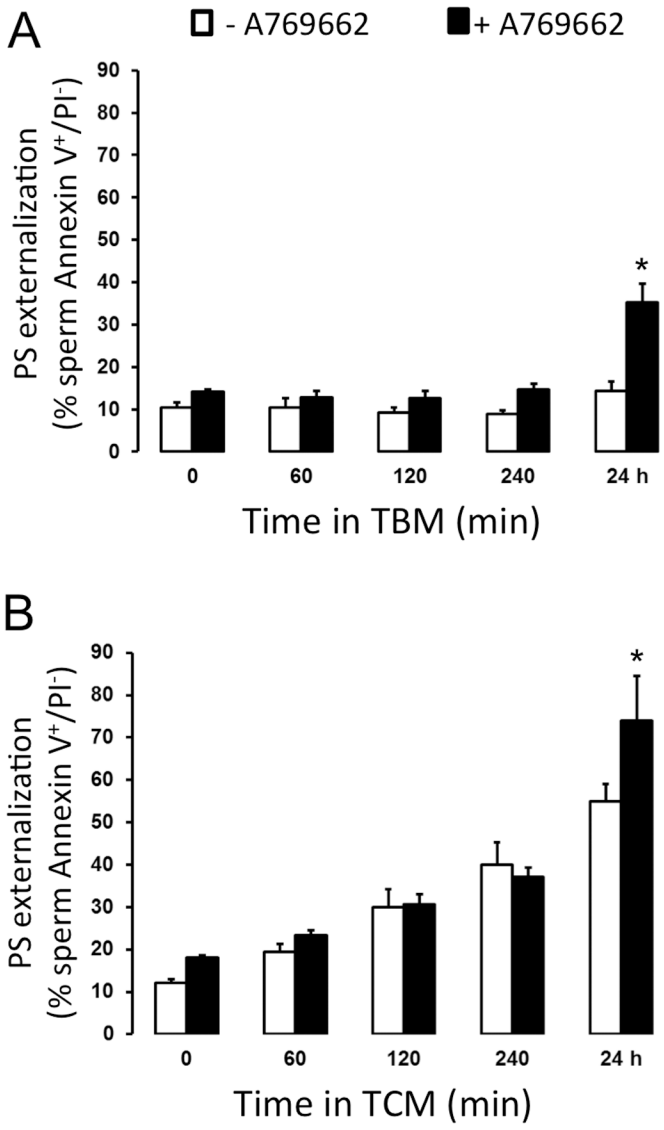

A
- A769662
+ A769662
PS externalization
(% sperm Annexin V+/PI-)
90
80
70
60
50
40
30
20
10
0
*
0
60
120
240
24 h
Time in TBM (min)
B
PS externalization
(% sperm Annexin V+/PI-)
90
80
70
60
50
40
30
20
10
0
*
0
60
120
240
24 h
Time in TCM (min)

Figure 9

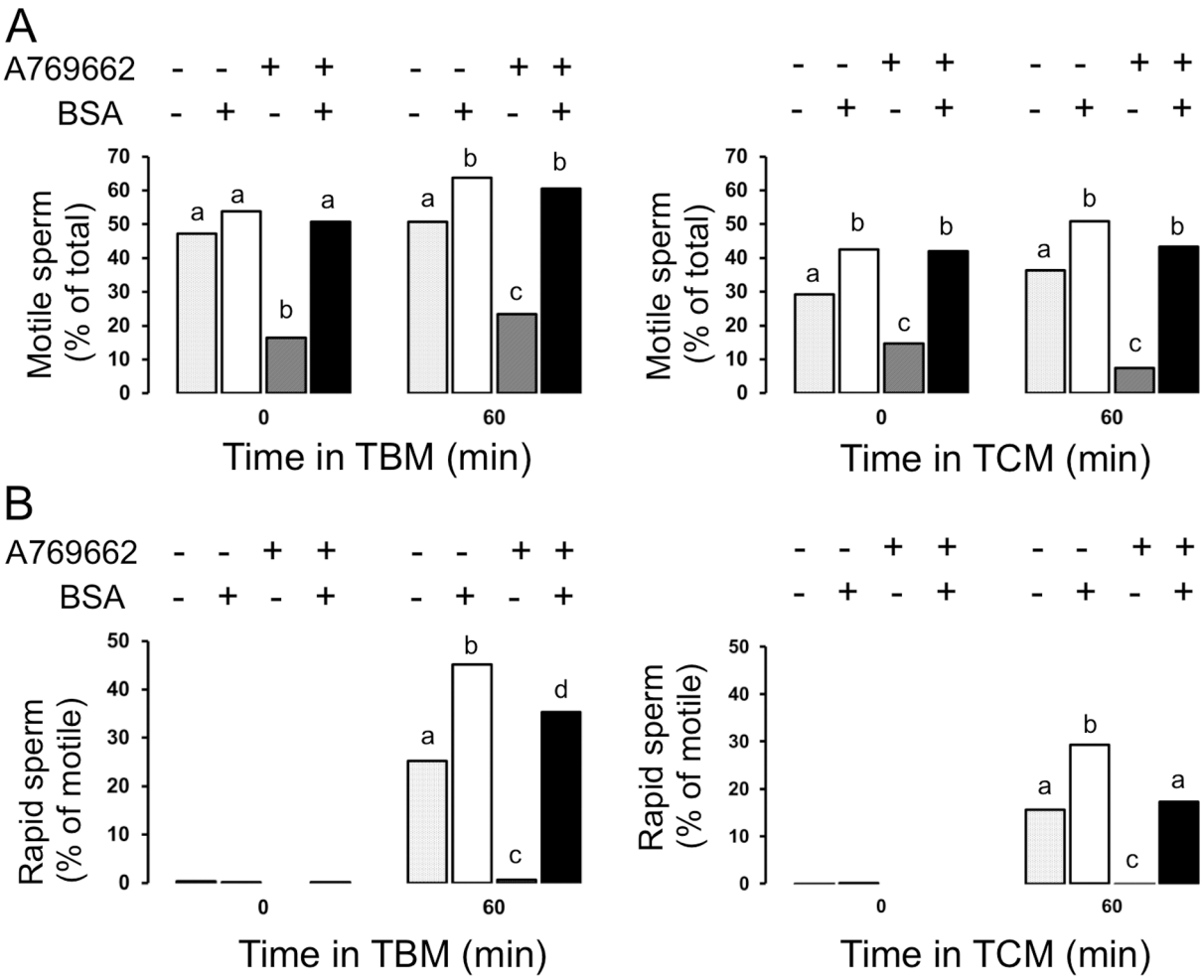
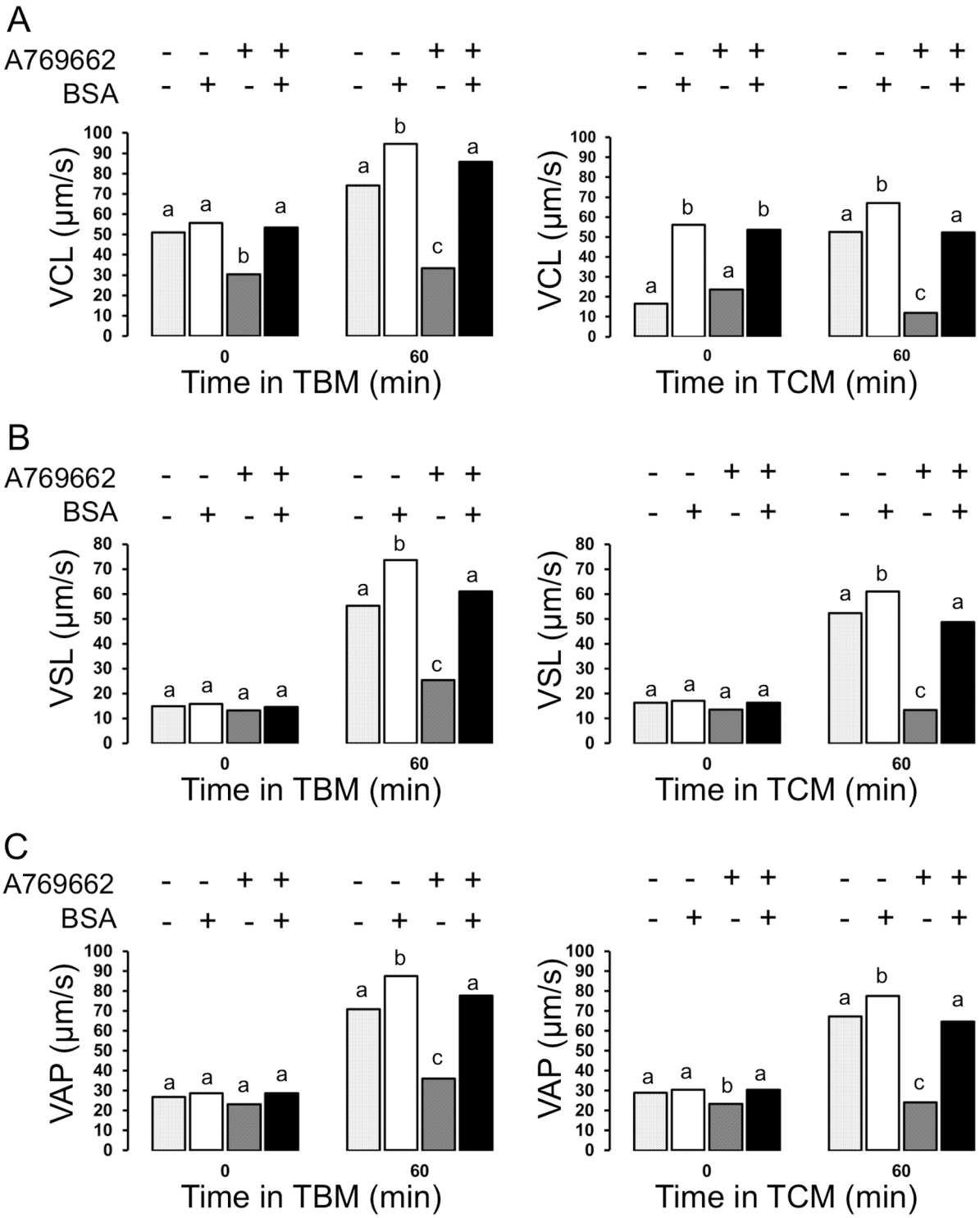

A
A769662 - - + + - - + +
BSA - + - + - + - +
Motile sperm (% of total)
70
60
50
40
30
20
10
0
a a b a a b c b
0 60
Time in TBM (min)
- - + + - - + +
- + - + - + - +
a b c b a b c b
0 60
Time in TCM (min)
B
A769662 - - + + - - + +
BSA - + - + - + - +
Rapid sperm (% of motile)
50
40
30
20
10
0
a b c d
0 60
Time in TBM (min)
- - + + - - + +
- + - + - + - +
a b c a
0 60
Time in TCM (min)

Figure 10


A
A769662
BSA
VCL (μm/s)
Time in TBM (min)
Time in TCM (min)
B
VSL (μm/s)
C
VAP (μm/s)

	LIN (%)		STR (%)		WOB (%)		ALH (µm)		BCF (Hz)	
	A769662		A769662		A769662		A769662		A769662	
TIME(min)	-	+	-	+	-	+	-	+	-	+
0	28.3±0.2	27.5±0.2	56.3±0.3	53.6±0.4	50.0±0.3	51.1±0.2	2.4±0.0	2.5±0.0	5.7±0.0	5.3±0.0
60	48.7± 0.3	47.1±0.3	68.1±0.3	69.1±0.4	69.4±0.3	65.9±0.2	4.1±0.0	3.7±0.0	7.1±0.0	7.3±0.0
120	59.2±0.4	59.1±0.4	74.9±0.4	76.6±0.4	76.4±0.4	75.0±0.2	3.5±0.0	3.1±0.0	7.8±0.0	7.9±0.0
240	62.3±0.4	60.7±0.4	78.7±0.4	78.4±0.4	76.9±0.4	75.0±0.2	3.2±0.0	2.7±0.0	8.9±0.0	8.0±0.0
24h	36.4±1.5	35.0±2.0	59.2±1.8	55.3±2.3	56.6±1.8	58.4±1.6	2.4±0.1	2.3±0.1	5.0±0.2	3.3±0.3

Table 1. Time-course effect of the A769662-induced increase in AMPK activity under non-stimulating conditions in some boar spermatozoa motility parameters. Spermatozoa were incubated in TBM in the absence of CO_2 at 38.5°C in the presence or absence of the AMPK activator, A769662 (200µM) for different times until 24 hours. Samples measured at 17°C were considered as time 0. Spermatozoa motility parameters including coefficients LIN (Linearity coefficient in %), STR (Straightness coefficient in %), WOB (Wobble coefficient in %), ALH (Amplitude of lateral head displacement in µm) and BCF (Beat cross frequency in Hz) were measured by the ISAS system. This experiment was performed at least 6 times and results express the mean ± standard error of the mean. No statistical differences were found.

	LIN (%)		STR (%)		WOB (%)		ALH (µm)		BCF (Hz)	
	A769662		A769662		A769662		A769662		A769662	
TIME(min)	-	+	-	+	-	+	-	+	-	+
5	39.9±0.5	37.5±0.5	56.3±0.5	54.0±0.6	68.6±0.3	67.2±0.3	3.8±0.0	3.9±0.0	7.8±0.1	8.1±0.1
60	56.0± 0.7	56.1± 0.9	66.5± 0.7	68.5± 0.9	82.2± 0.4	79.7± 0.5	2.9±0.0	2.8±0.0	7.4±0.1	7.7±0.1
120	62.1±0.9	61.0±1.2	73.1±0.8	73.6±1.2	82.4±0.5	80.1±0.8	2.8±0.0	2.4±0.0	7.2±0.1	7.2±0.2
240	65.7±0.8	64.6±1.2	79.6.±0.8	77.8±1.1	80.4±0.5	81.0±0.8	2.8±0.0	2.2±0.1	7.8±0.1	6.7±0.2
24h	51.3±1.7	48.2±2.2	69.2±1.7	66.0±2.2	68.9±1.2	66.0±1.7	2.2±0.1	2.1±0.1	6.1±0.2	4.9±0.2

Table 2. Time-course effect of the A769662-induced increase in AMPK activity under HCO_3^- and Ca^{2+}-stimulating conditions in some boar spermatozoa motility parameters. Spermatozoa were incubated in TCM at 38.5°C in the presence or absence of the AMPK activator, A769662 (200µM) for different times until 24 hours. Spermatozoa motility parameters including coefficients LIN (Linearity coefficient in %), STR (Straightness coefficient in %), WOB (Wobble coefficient in %), ALH (Amplitude of lateral head displacement in µm) and BCF (Beat cross frequency in Hz) were measured by the ISAS system. This experiment was performed at least 6 times and results express the mean ± standard error of the mean. No statistical differences were found.