BIOLOGY OF REPRODUCTION (2014) **90**(2):29, 1–10
Published online before print 3 January 2014.
DOI 10.1095/biolreprod.113.112797

The Calcium/CaMKKalpha/beta 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 Signaling and Technology of Reproduction, School of Veterinary Medicine, University of Extremadura, Caceres, Spain

ABSTRACT

Spermatozoa successfully fertilize oocytes 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 have been studied exclusively in somatic cells, our aim is to investigate the intracellular pathways that regulate AMPK phosphorylation at Thr^{172} (activity) in male germ cells. Boar spermatozoa were incubated under different conditions in the 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 spermatozoan function, Ca^{2+} and cAMP levels.

AMPK phosphorylation and activity, cAMP, Ca^{2+}, CaMKKα/β, PI3K, PKA, PKC, spermatozoa

[1]Supported by Ministerio de Ciencia e Innovacion grant AGL2010-15188 and by Government of Extremadura grants PRI09A077 and GR10156. D.M.-H. is a recipient of a Ph.D. grant from the Government of Extremadura, Spain.
[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 to this study as senior investigators.

Received: 2 August 2013.
First decision: 23 September 2013.
Accepted: 12 December 2013.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

INTRODUCTION

In order to successfully achieve a fertilization-competent status, spermatozoa must 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 bind to the zona pellucida of the oocyte, triggering the acrosome reaction and subsequently leading to egg fertilization. Biochemical changes during spermatozoan capacitation include cholesterol loss from the plasma membrane, increased plasma membrane fluidity due to phospholipid scrambling, changes in intracellular ion concentrations, hyperpolarization of plasma membrane, and increased tyrosine phosphorylation, among others [1]. The acquisition of spermatozoan functionality during capacitation is mainly dependent on 1) posttranslational modifications of pre-existing proteins, such as phosphorylation in either tyrosine [2–4] or serine/threonine [5], and/or 2) the male germ cell energetic state, determined by the ratio between cellular AMP and ATP [6].

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 Thr^{172} in somatic cells: 1) the tumor suppressor responsible for the inherited cancer disorder Peutz-Jeghers syndrome, LKB1 [13], 2) Ca^{2+}/calmodulin-dependent protein kinase kinases α and β (CaMKKα and -β) [14], and 3) transforming growth factor (TGF)-β-activated kinase-1 [15]. Although AMP binding was previously thought both to induce phosphorylation [16] and to inhibit dephosphorylation [17], more recent studies suggest that the AMP effect is exclusively mediated by an inhibition of Thr^{172} 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.

No AMPK studies had been conducted exclusively in somatic cells until 2008, when Towler et al. [21] demonstrated in male germ cells that a short splice variant of LKB1 (LKB1s), the upstream kinase of AMPK, is expressed predominantly in haploid sperm cells from testes of mammals. LKB1s knockout mice display a dramatic reduction in the number of mature spermatozoa in the epididymis, and the few spermatozoa produced are nonmotile, have an abnormal head morphology, and, as a result, are sterile [21]. These data suggest that this variant, LKB1s, has a crucial role in spermiogenesis and fertility in mice. Moreover, members of the testis-specific serine/threonine kinase (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 contributing effectively to the maintenance of mitochondrial membrane potential, spermatozoan plasma membrane fluidity and organization, and acrosomal integrity at physiological temperature [25] or during semen preservation at 17°C [26]. Supporting a relevant role of AMPK in the function of the male germ cells, Tartarin et al. [27] demonstrated that mice lacking the AMPK catalytic subunit α1 gene (α1AMPKα1knockout [KO]) show a decreased fertility in parallel with an alteration in spermatozoan morphology, as well as decreased spermatozoan motility.

As mentioned, spermatozoa need to adapt to changing external conditions, such as the transit through the female reproductive tract. The study of the signaling pathways that regulate AMPK phosphorylation (activity) in spermatozoa is crucial for the understanding of those functions of the germ cells in which this cell energy sensor kinase is involved [24–26]. Therefore, the aim of this work was to investigate the involvement of intracellular messengers, such as Ca^{2+} and cAMP, as well as different intracellular pathways CaMKKα/β, protein kinase (PK) A, PKC, and phosphatidylinositol 3-kinase (PI3K), in the activation of AMPK in male germ cells.

MATERIALS AND METHODS

Chemicals and Sources

The 8 bromoadenosine 3′5′-cyclic monophosphate sodium salt, 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, MO); LY 294002 was 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); complete, EDTA-free, protease inhibitor cocktail was purchased from Roche Diagnostics (Penzberg, Germany); Tris/Glycine/SDS buffer (10×) and Tris/glycine buffer (10×) were 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).

Incubation Media for Spermatozoa

Tyrode 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), 5 mmol/L EGTA, and 3 mg/ml BSA. A variant of TBM, which includes direct activators of spermatozoan soluble adenylyl cyclase (sAC), was made by adding 1 mmol/L $CaCl_2$ and 15 mmol/L $NaHCO_3$ and equilibrating with 95% O_2 and 5% CO_2, and is termed Tyrode complete medium (TCM). For experiments designed to study the effect of addition of Ca^{2+} 5 mM, a particular EGTA-free TBM was prepared. All Tyrode media 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 yr 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 12 h at 17°C before use. In addition, in order to minimize individual boar variation, 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 $2000 \times g$ for 4 min, washed with PBS, and placed in TBM or TCM. 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 preincubation of spermatozoa with different inhibitors (H89, Ro-32-0432, LY294002, STO-609, or BAPTA-AM) was performed for 1 h at room temperature. 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 dimethyl sulfoxide [DMSO] ≤0.1% for the rest of the 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 spermatozoan parameters analyzed.

Western Blotting

Spermatozoa under different treatments were centrifuged 20 sec at $7000 \times g$, washed with PBS supplemented with 0.2 mM Na_3VO_4, and then lysated in a lysis buffer consisting of 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 inhibitor cocktail (Complete, EDTA-free), 0.2 mmol/L Na_3VO_4, and 1 mmol/L PMSF by sonication for 5 sec at 4°C. After 20 min at 4°C, samples were centrifuged at $10\,000 \times g$ (15 min, 4°C) and the supernatant (lysate) was used for analysis of protein concentration. Proteins from porcine spermatozoa lysates were resolved by SDS-PAGE and electrotransferred to nitrocellulose membranes. Western blotting was performed as previously described [24, 29] using anti-phospho-Thr^{172}-AMPKα (1:500) and anti-GSK3β (1:2000) polyclonal antibodies as primary antibodies. The intensity of bands in the film was measured using a scanning densitometer and quantified using ImageJ software for Macintosh (Research Service Branch, NIMH, NIH, Bethesda, MD).

Statistical Analysis

The mean and SEM were calculated for descriptive statistics. The effect of treatment on the spermatozoa variables was assessed with an 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 PKA. Our recent work suggests that AMPK lies downstream of PKA in boar spermatozoa, as the nonhydrolyzable cAMP analog, 8Br-cAMP, which elevates intracellular cAMP levels, greatly increases phospho-Thr^{172}-AMPK [25]. We further investigated the role of PKA pathway in AMPK activity by using a pharmacological inhibitor of PKA, H89

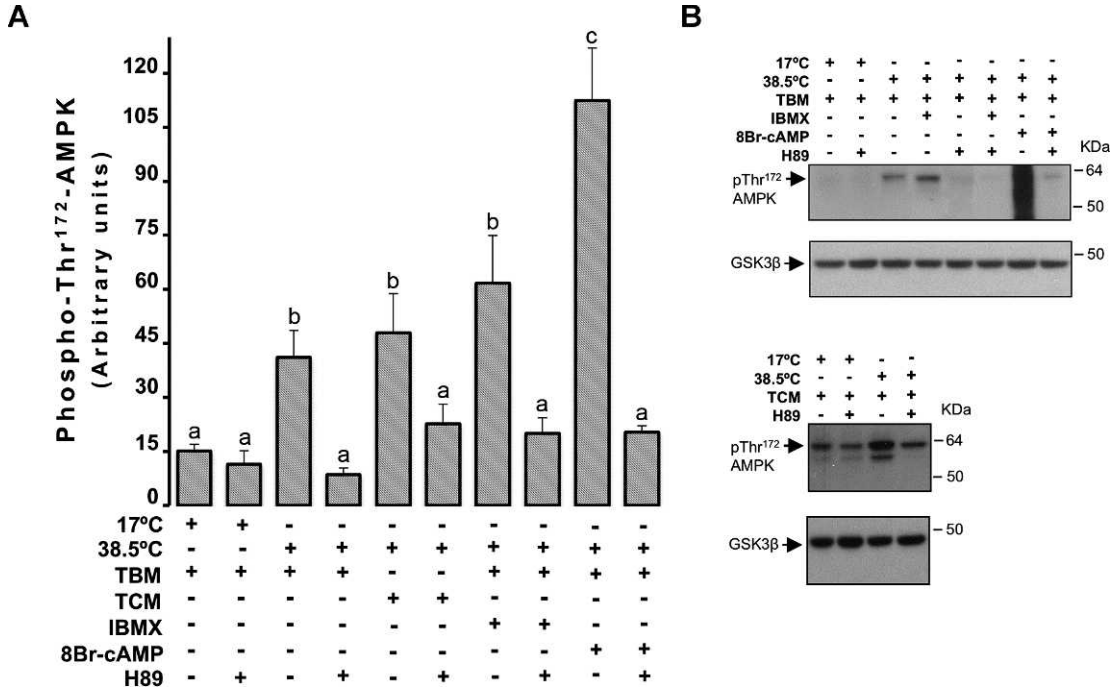

FIG. 1. Phosphorylation of AMPK at Thr^{172} is greatly increased by an elevation of cAMP levels, due to IBMX or 8 bromoadenosine 3′5′-cyclic monophosphate (8Br-cAMP), and is blocked by PKA inhibitor H89 in boar spermatozoa. Male germ cells from several boars were pooled and incubated in TBM (**B**, upper films) or TCM (**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 8Br-cAMP (1 mM) or IBMX (100 μM), and then lysed. Sperm samples treated with PKA inhibitor were previously preincubated for 1 h at room temperature (RT) with H89 (100 μM). Proteins (20 μg) from spermatozoan 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 six times. A representative film of each experiment is shown at the right. Protein loading controls for each experiment using anti-GSK3β antibody are shown in the lower films. **A**) Analysis of phospho-Thr^{172}-AMPK bands in the films by densitometry is shown at the left. Results are expressed as the mean ± SEM. Statistical differences between treatments are shown with different letters when $P < 0.05$.

(half maximal inhibitory concentration [IC_{50}] = 48 nM), at a concentration of 100 μM, which possesses demonstrated effects on boar spermatozoa [30]. As seen in Figure 1, phospho-Thr^{172}-AMPK (activity) in boar spermatozoa is minimal at 17°C in TBM. Interestingly, under these nonstimulated conditions of spermatozoa, AMPK activity is not dependent on PKA, as pretreatment 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 (38.5°C), 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 spermatozoan incubation medium, although slight differences between media can be cited (Fig. 1). Whereas, in TCM, a stimulating (capacitating) medium for spermatozoa, a clear inhibition of AMPK activity is observed by H89, in TBM, the PKA inhibitor totally abolishes the temperature-induced AMPK phosphorylation, reaching minimal values comparable to those at 17°C or even lower.

In order to further demonstrate the involvement of PKA in AMPK activity, we incubated spermatozoa in TBM with IBMX (100 μM), a phosphodiesterase 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 (Fig. 1). Although, in both cases, a potent increase in phospho-Thr^{172}-AMPK levels was achieved, the induction of the AMPK activity due to the cAMP analog was almost twice that of IBMX (densitometry analysis in Fig. 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 shown that preincubation 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 the nonstimulating medium, TBM (slightly modified, as EGTA was omitted), with the addition of different concentrations (mM) of extracellular Ca^{2+}, although only the effect of 5 mM Ca^{2+} is shown (Fig. 2). The phosphorylation of AMPK potently increased by 2-fold in spermatozoa incubated at 38.5°C in a medium with 5 mM Ca^{2+} as unique stimulus. This enhanced AMPK activity due to Ca^{2+} was 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 the absence of Ca^{2+}.

Two known kinase members of the Ca^{2+}-activated pathways are CaMKKα and -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; K_i = 15 ng/ml for CaMKKβ). Results in Figure 2 show that the Ca^{2+}-induced AMPK activity under physiological conditions (38.5°C) is mediated by the activity of CaMKKα or β, as STO-609 (40

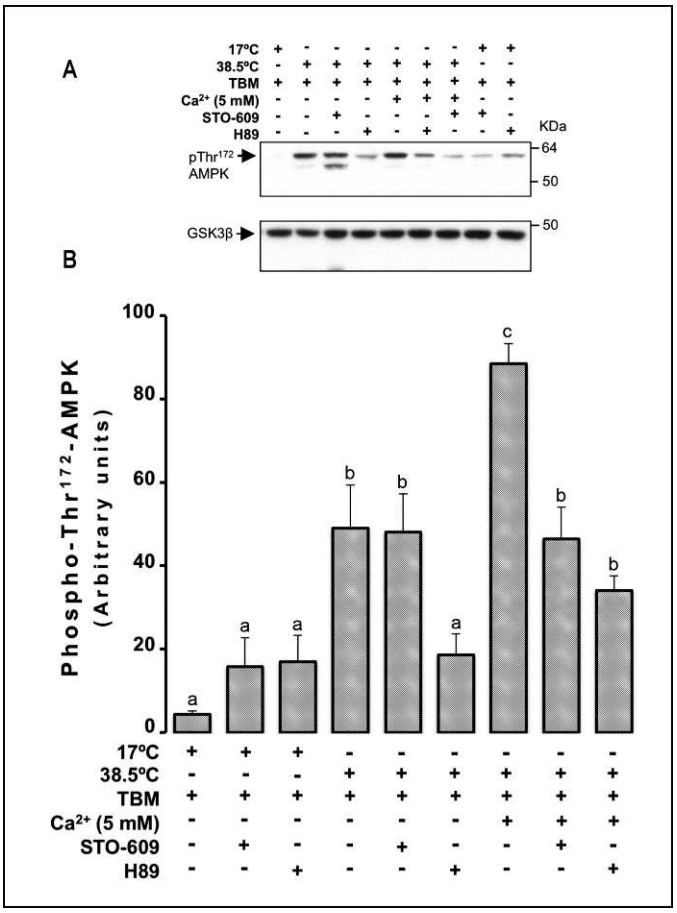

FIG. 2. Phospho-Thr^{172}-AMPK levels are potently stimulated by an increase in intracellular calcium via CaMKKα/β and PKA pathways in boar spermatozoa. Male germ cells from several boars were pooled and incubated in TBM at 17°C or in a CO_2 incubator at 38.5°C for 60 min in the absence or presence of extracellular Cl_2Ca (5 mM), which was added to a modified TBM, 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 (20 μg) from spermatozoan 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 six times, and a representative film is shown in the upper part. Protein loading control using anti-GSK3β antibody is shown in the lower film. **B**) Analysis of phospho-Thr^{172}-AMPK bands in the films by densitometry is shown at the bottom. Results are expressed as the mean ± SEM. Statistical differences between treatments are shown with different letters when $P < 0.05$.

μM) abolishes the increase in phosphorylation induced by 5 mM 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 PKC in AMPK activity in spermatozoa was studied by incubating these germ cells in TCM, 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 increased 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 ($IC_{50} = 9$ nM for PKC_α primary target; $IC_{50} = 28$ nM for $PKC_{\beta1}$; $IC_{50} = 108$ nM for PKC_ε secondary targets) at a 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 stimuli of spermatozoa, such as Ca^{2+} and HCO_3^- present in TCM.

Effect of the Inhibitor LY294002 in AMPK Activity in Spermatozoa

The possible involvement of PI3K in the AMPK activation pathway was studied by incubating male germ cells in TCM in the presence of LY294002 (IC_{50} for PI3K = 1.4 μM) at 100 μM (Fig. 4), a concentration with a demonstrated effect on the motility of boar spermatozoa [30]. The AMPK activity due to physiological stimuli of spermatozoa (temperature of 38.5°C and activators of sAC, Ca^{2+}, and HCO_3^-) does not seem to be mediated by the activation of PI3K, 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 signaling pathways leading to AMPK activity in boar spermatozoa by using a novel, direct, and potent AMPK activator, A769662 [31], in a TCM (Fig. 5). It has been shown 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 (Fig. 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 (10 μM for 5 min), which inhibits NADH:ubiquinone oxidoreductase, and cyanide (2 mM 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 for 60 min under hyperosmotic stress caused by the addition of 500 mM sorbitol. Interestingly, a specific cellular stress for male germ cells, which is the total absence of intra- and extracellular calcium due to preincubation with the permeable Ca^{2+}-quelator BAPTA-AM (50 μM) in a Ca^{2+}-free medium (TBM contains EGTA), potently activates AMPK in a time-dependent manner.

DISCUSSION

Maintaining energy balance is an essential process at the 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

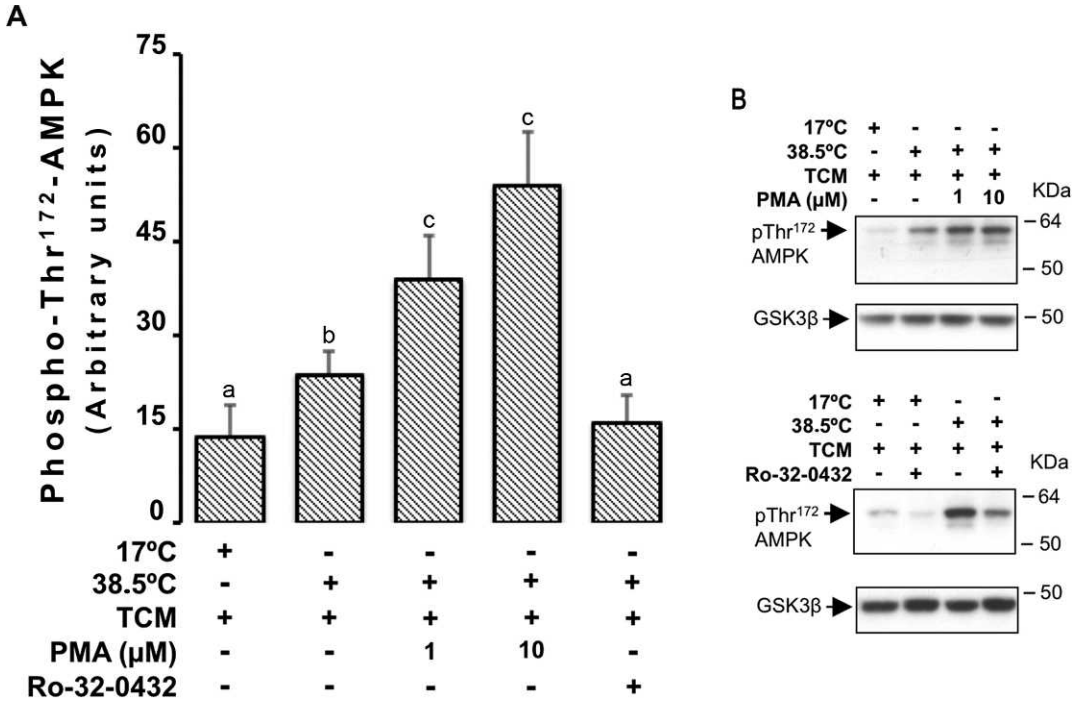

FIG. 3. Phosphorylation of Thr^{172}-AMPK is markedly increased by direct stimulation of PKC with PMA and blocked by PKC inhibitor Ro-32-0432 in boar spermatozoa. Male germ cells were incubated in TCM at 17°C or in a CO_2 incubator at 38.5°C for 60 min in the absence or presence of indicated concentrations 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 (20 μg) from spermatozoan 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 five times; a representative film of each is shown, and protein loading controls for each experiment using anti-GSK3β antibody are shown in the lower films. **A**) Analysis of phospho-Thr^{172}-AMPK bands in the films was performed by densitometry, and data are expressed as arbitrary units (mean ± SEM). Statistical differences between treatments are shown with different letters when $P < 0.05$.

cells until recently. In 2012, our group demonstrated, for the first time, that AMPK is highly expressed in male germ cells [24] at the entire acrosome in the spermatozoan head, as well as in the midpiece of the flagellum, whereas its active form, phospho-Thr^{172}-AMPK, is specifically localized at the apical part of the acrosome, also remaining in the midpiece of the flagellum [25]. The physiological role(s) of AMPK in mammalian male germ cells has been demonstrated under different spermatozoan conditions (basal, capacitating, and/or preservation at 17°C), and includes an important function in the regulation of spermatozoan 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 1) its lipid organization and fluidity and 2) the suitable outward translocation of phosphatidylserine. The role of AMPK in spermatozoan membrane reaches the acrosomal region, where AMPK activity is localized and controls the integrity of acrosomal membrane [25, 26]. As these AMPK-regulated spermatozoan 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 17°C boar semen preservation [26], these previous studies clearly point to AMPK as an essential regulator of spermatozoan function. However, to date, signaling pathways involved in the control of AMPK activity in male germ cells are unknown.

As previously described [34, 35], activation of AMPK requires phosphorylation on Thr^{172} within the α catalytic subunit. Our recent work [25] suggests that cAMP-mediated pathway, likely through PKA, is an 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 nonhydrolyzable analog 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 known downstream target of cAMP is PKA, which plays a central and essential role in spermatozoan 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 signaling 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 Ser431 by PKA in response to activation of adenylate cyclase by forskolin [39, 44] or

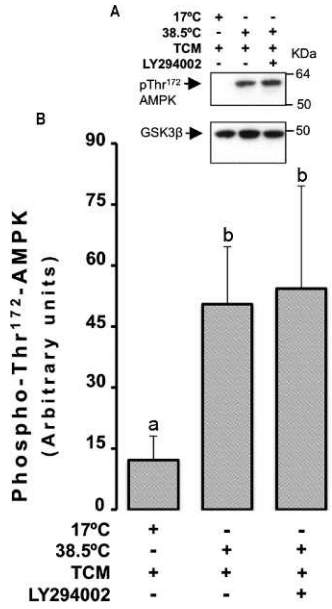

FIG. 4. Effect of PI3K inhibitor LY294002 in the TCM-induced AMPK phosphorylation at Thr^{172} in spermatozoa. Male germ cells were incubated in TCM at 17°C or in a CO_2 incubator at 38.5°C for 60 min in the absence or presence of PI3K inhibitor LY294002 (100 μM). Sperm samples treated with PI3K inhibitor LY294002 were previously preincubated for 1 h at RT. Proteins (20 μg) from spermatozoan 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 five times, and a representative film is shown. Protein loading control for each experiment using anti-GSK3β antibody is shown in the lower film. **B**) Analysis of phospho-Thr^{172}-AMPK bands in the films by densitometry is shown. Results are expressed as the mean ± SEM. Statistical differences between treatments are shown with different letters when $P < 0.05$.

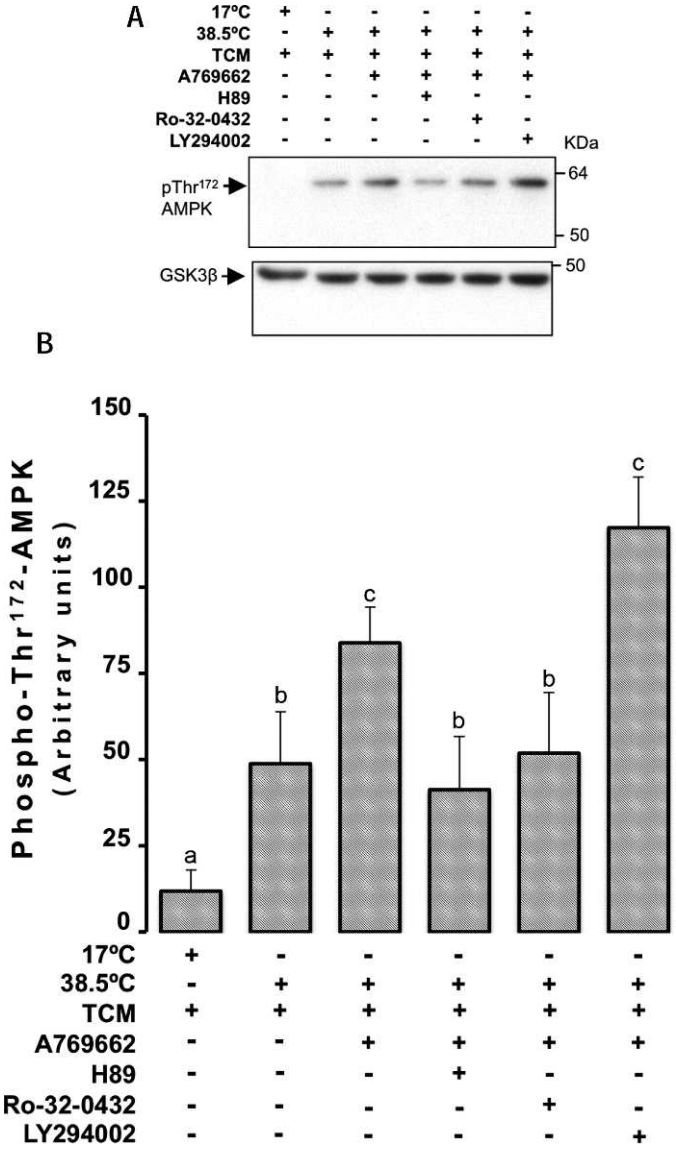

FIG. 5. AMPK phosphorylation at Thr^{172} is potently enhanced by its pharmacological activator A769662 via PKA and PKC pathways in spermatozoa. Male germ cells were incubated in TCM at 17°C or in a CO_2 incubator at 38.5°C for 60 min in the presence or absence of AMPK activator A769662 (200 μM), and then lysed. Sperm samples treated with kinase inhibitors H89 (100 μM), Ro-32-0432 (50 μM), or LY294002 (100 μM) were previously preincubated for 1 h at RT. Proteins (20 μg) from spermatozoan 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 six times, and a representative film is shown. Protein loading control using anti-GSK3β antibody is shown in the lower film. **B**) Analysis of phospho-Thr^{172}-AMPK bands in the films by densitometry is shown. Results are expressed as the mean ± SEM. Statistical differences between treatments are shown with different letters when $P < 0.05$.

IBMX [39]. Regarding this upstream kinase, LKB1, it has been shown that its short splice variant, $LKB1_S$, is highly expressed in haploid spermatids in mouse 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 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 by direct activation of PKA, by an phosphodiesterases-induced increase in AMP levels that allosterically activate AMPK, or by both mechanisms.

An essential regulator of any spermatozoan functional process is the intracellular messenger Ca^{2+}, which activates the specific sAC, expressed in these germ cells. Our results now provide strong evidence that intracellular Ca^{2+} acts as an additional signaling 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 demonstrates that the Ca^{2+}-induced signaling 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 spermatozoan sAC that catalyzes the synthesis of cAMP, which, in turn, allosterically activates PKA. Besides working through the PKA pathway, Ca^{2+} might also lead to the phosphorylation of AMPK through the activation of CaMKKα/β, which lie upstream of AMPK in somatic cells [14, 47]. In this work, we have used STO-609, which acts a specific inhibitor of both CaMKKα and -β [48]. Although the expression of both CaMKK isoforms has been shown in spermatozoa from different mammalian species, such as rat [49] and mouse [50], to date, the expression level of each CaMKK isoform in boar spermatozoa is unknown; therefore, we cautiously refer in this work to both isoforms, using CaMKKα/β. The CaMKK inhibitor STO-609 caused a complete inhibition of Ca^{2+}-induced AMPK activity in

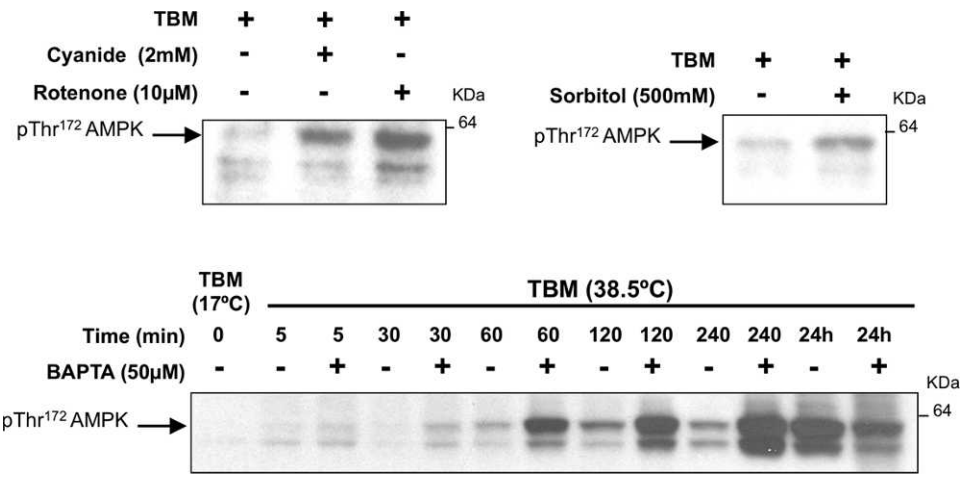

FIG. 6. AMPK activation is increased by different cellular stresses in spermatozoa. Male germ cells were incubated in TBM at 17°C or in a CO_2 incubator at 38.5°C for different times in the presence or absence of inhibitors of mitochondrial activity, rotenone (10 μM, 5 min) and cyanide (2 mM, 5 min), inducer of hyperosmotic stress, sorbitol (500 mM, 60 min), or a Ca^{2+} absence-induced sperm stress, BAPTA-AM (50 μM, time-course), and then lysed. Proteins (20 μg) from spermatozoan 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 four to six times. A representative film of each experiment is shown.

spermatozoa, in agreement with previous studies in somatic cells [14, 47]. As expected, in the absence of any extracellular Ca^{2+} (neutralized by EGTA present in TBM), phospho-Thr^{172}-AMPK levels due to spermatozoan 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 spermatozoan basal conditions.

Additionally, this work describes that AMPK phosphorylation is dose-dependently stimulated by direct activation of PKC with 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 also been demonstrated that AMPK is not a substrate for PKC. Instead, Xie et al. [52] first demonstrated that the atypical PKCζ is a kinase that phosphorylates LKB1 at Ser428, and that, later, 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 $LKB1_L$ and Ser399 in $LKB1_S$, 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 bull sperm [58], PKCζ in hamster [59], and mouse sperm [60]. It is therefore plausible that PKCζ might play a similar role in spermatozoa as 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 et al. [30] demonstrated 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 signaling can induce the activation of calcium-sensitive PKCs, which are responsible for hyperactivation of spermatozoa. Thus, we propose that another PKC isoform(s) besides PKCζ, which is not calcium sensitive, could likely be mediating AMPK activation, at least in response to an elevation of cAMP levels, on boar spermatozoa.

A different signaling pathway that regulates several processes in spermatozoan 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 signaling pathway that controls spermatozoan 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-Thr^{172}-AMPK levels than the increase due to A769662 alone is explained through the activation of PKA pathway due to the LY294002-induced elevation in cAMP levels, which we demonstrated to occur in boar spermatozoa [30]. Despite this, and supporting the idea that PI3K may regulate cAMP levels, a crosstalk between PKA and PKC has been demonstrated 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.

Aside from the demonstration of which signaling pathways lead to AMPK activation in boar spermatozoa under both physiological and pharmacological (A769662) conditions, this work also provides clear evidence that AMPK becomes activated in male germ cells under conditions that cause some type of cellular stress. Thus, inhibition of spermatozoan mitochondrial activity by blocking of the electron transport chain, as well as hyperosmotic 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 1) an increase in AMP levels, 2) the generation of reactive oxygen species (ROS), which act as signaling molecules to activate AMPK [62] through LKB1- and CaMKK-dependent pathways, or 3) both. Surprisingly, the absence of intracellular Ca^{2+} in boar

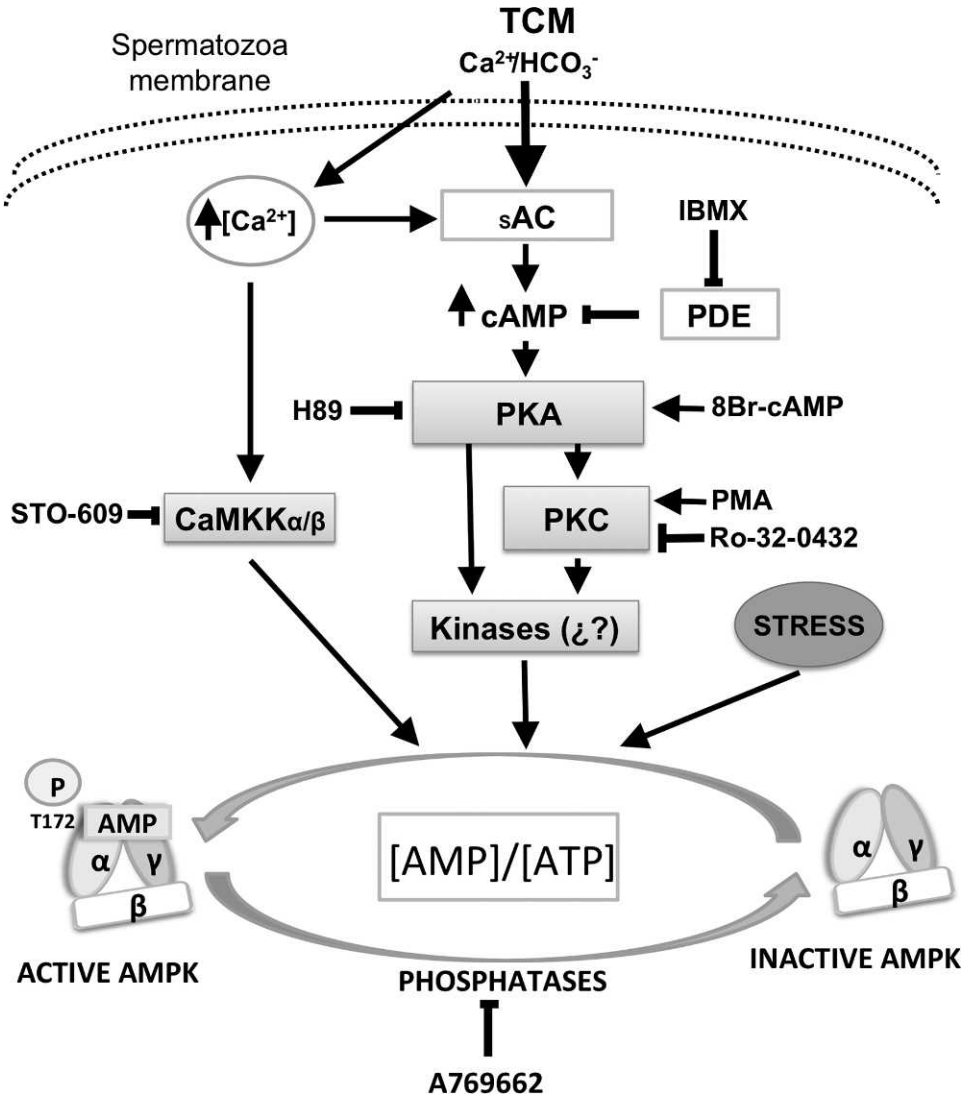

FIG. 7. Proposed scheme of intracellular signaling pathways leading to regulation of AMPK activity in mammalian spermatozoa. Intracellular mechanisms involved in AMPK activity in boar spermatozoa that have been investigated in this work are shown. Activators, such as 8Br-cAMP, Ca^{2+}, HCO_3^-, PMA, 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.

spermatozoa by incubation with BAPTA-AM in a Ca^{2+}-free medium 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 an ROS (NO·). In this regard, it has recently 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 interact with the cAMP pathway as occurs in humans [65], leading to the AMPK activation as mentioned previously here.

In summary, the results presented here provide mechanistic insight into the signaling pathways leading to AMPK activation in boar spermatozoa under physiological and pharmacological conditions (Fig. 7). Thus, AMPK phosphorylation is regulated upstream by intracellular messengers Ca^{2+} and cAMP, as well as by PKA, PKC, and CaMKKα/β signaling 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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