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Protein Kinases A and C and Phosphatidylinositol 3 Kinase Regulate Glycogen Synthase Kinase-3A Serine 21 Phosphorylation in Boar Spermatozoa

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ABSTRACT

The cAMP-dependent protein kinase (PKA), protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3K) pathways control most relevant functions in male germ cells including motility. Recently we demonstrated that phosphorylation state of glycogen synthase kinase-3α (GSK3A) is also a key event in the control of boar spermatozoa motility. However, the upstream regulators of GSK3A serine phosphorylation (inhibition) in male germ cells remain largely unknown. This work investigates the involvement of PKA, PKC and PI3K pathways in GSK3A phosphorylation in boar spermatozoa. A capacitating medium (TCM) or the phosphodiesterase-resistant cell permeable cAMP analogue 8Br-cAMP cause a significant increase in Ser21 GSK3A phosphorylation associated with a simultaneous significant increase in boar spermatozoa motility. These effects are blocked after preincubation of spermatozoa with PKA inhibitor H89 or PKC inhibitor Ro-32-0432. The PI3K inhibitor LY294002 increases both spermatozoa motility parameters and the basal GSK3A phosphorylation, but does not affect either TCM- or 8Br-cAMP-stimulated GSK3A phosphorylation. PI3K inhibition effects are mediated by an increase in intracellular cAMP levels in boar spermatozoa and are suppressed by PKA inhibitor H89. In summary, we demonstrate that PKA, PKC and PI3K pathways crosstalk in porcine male germ cells to crucially regulate GSK3A phosphorylation which subsequently controls cell motility. In addition, our results suggest that PI3K is upstream of PKA which lies upstream of PKC in this regulatory cascade(s). Our findings contribute to elucidate the molecular mechanisms underlying the regulation of one of the most relevant male germ cell functions, motility. J. Cell. Biochem. 109: 65–73, 2010.

KEY WORDS: SPERMATOZOA; MOTILITY; PHOSPHORYLATION; GSK3; PKA; PKC; PI3K

Mammalian spermatozoa released from the testis are immotile an unable to fertilize. To reach the oocyte and acquire the competence to fertilize, they need to undergo a sequential series of complex process of activation that implies maturation and acquisition of motility in the epididymis, as well as development of the hyperactivated motility in the female reproductive tract, process known as capacitation [Rodriguez-Martinez, 2007]. In mammals, motility of spermatozoa is initiated and maintained by the cAMP-dependent phosphorylation of flagellar proteins [Si and Okuno, 1999]. The predominant enzyme that produces cAMP in male germ cells is an atypical soluble adenylyl cylclase now identified as SACY [Hess et al., 2005] that is activated by bicarbonate and calcium [Carlson et al., 2007]. Demonstration that SACY null mice are infertile [Hess et al., 2005] and that their spermatozoa do not activate motility in presence of bicarbonate [Xie et al., 2006] indicates that SACY translates the bicarbonate signal for spermatozoa motility activation to a pathway that is essential for fertilization. Increased cAMP levels subsequently lead to the activation of the cAMP-dependent protein kinase (PKA) [Okamura et al., 1985; Holt and Harrison, 2002; Bajpai

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and Doncel, 2003; Luconi et al., 2005], which critically controls spermatozoa motility by the phosphorylation of downstream proteins [Okamura et al., 1985; Holt and Harrison, 2002; Bajpai and Doncel, 2003; Luconi et al., 2005]. In this regard Harayama and Miyake [2006] have previously established an important role of cAMP-dependent PKC activation, in the hyperactivation of flagellar movement in boar spermatozoa. Moreover, a crucial involvement in the regulation of spermatozoa motility for the phosphoinositide 3-kinase (PI3K) has been demonstrated in human, pig and hamster [Luconi et al., 2001; NagDas et al., 2002; du Plessis et al., 2004; Aparicio et al., 2005]. Luconi et al. [2001], demonstrated that the increase in spermatozoa motility observed after inhibition of PI3K is due to an increase in both intracellular cAMP levels and tyrosine phosphorylation of AKAP3, a PKA anchoring protein (AKAP) of the fibrous sheath in spermatozoa tails. This effect subsequently leads to the recruitment of PKA in a subcellular compartment which is associated with an increase in spermatozoa motility [Luconi et al., 2004]. In addition to PKA and PI3K, Vijayaraghavan et al. [1997] demonstrated that inhibition or stimulation of bovine spermatozoa motility resulted in a corresponding decrease or increase in the tyrosine phosphorylation of a 55 kDa protein that was further identified as glycogen synthase kinase 3, GSK3 [Vijayaraghavan et al., 2000]. The GSK3 is a signaling enzyme involved in biochemical pathways mediating several actions in somatic cells which possesses two isoforms in mammalian cells, GSK3A and GSK3B encoded by two independent genes [Woodgett, 1990]. GSK3 is regulated through phosphorylation at its tyrosine 214 amino acid residue and also at its serine 21 in GSK3A and serine 9 in GSK3B. When this serine is phosphorylated by different kinases, GSK3 changes to a catalytically incompetent conformation that inactivates it [Fang et al., 2000] and can be reverted to a dephosphorylated form, enzymatically active, by a phosphatase [King et al., 2006].

In male germ cells, GSK3 was recently involved in the signaling pathway that regulates bovine [Somanath et al., 2004] and porcine [Aparicio et al., 2007b] spermatozoa motility. In the last specie we have demonstrated a close direct relationship between GSK3A serine phosphorylation state and spermatozoa motility [Aparicio et al., 2007b], being this isoform primarily active (dephosphorylated) in static spermatozoa, whereas is inactivated by ser 21 phosphorylation in motile spermatozoa [Aparicio et al., 2007]. On of the most notable qualities of GSK3 is the vast number of signaling pathways that converge on this enzyme to regulate cell physiology. Whereas in somatic cells, it is clearly established that phosphoinositide3-kinase (PI3K) [Duronio et al., 1998], Akt [Hemmings, 1997], PKA [Fang et al., 2000], and PKC [Goode et al., 1992; Cook et al., 1996] are upstream regulators of GSK3 phosphorylation, in male germ cells the signaling pathway(s) leading to GSK3 phosphorylation is currently unknown. Therefore, our aim was to investigate upstream regulators of GSK3A activity in mammalian male germ cells by studying the effects of PI3K, PKA, PKC and their biochemical crosstalk in the Ser 21 GSK3A phosphorylation in boar spermatozoa and to correlate them with germ cells motility parameters.

In summary, using porcine spermatozoa as a model of mammalian male germ cells this work demonstrates that PKA, PKC and PI3K pathways crosstalk in mammalian male germ cells to crucially downstream regulate GSK3A phosphorylation which ultimately plays an essential role in the motility in these germ cells. In addition, our results suggest that PI3K is upstream of PKA which lies upstream of PKC in this regulatory cascade(s). Our findings contribute to elucidate the molecular mechanisms underlying the regulation of one of the most relevant male germ cell functions, motility.

MATERIALS AND METHODS

CHEMICALS, EQUIPMENT, AND SOURCES

LY249002, Ro-32-0432, and H89 were from Calbiochem (La Jolla, CA). The phosphodiesterase-resistant cell permeable cAMP analogue, 8Br-cAMP was from Sigma–Aldrich (St Louis, MO). Ethidium homodimer-1 and propidium iodide from Molecular Probes (Leiden, The Netherlands). Complete, EDTA-free, protease inhibitor cocktail was from Roche Diagnostics (Penzberg, Germany). Anti phospho-GSK3A/B pAb and anti-GSK3A pAb were from Cell Signaling (Beverly, CA). Tris/Glycine/SDS buffer (10 times concentrated) and Tris/Glycine buffer (10 times concentrated) from Bio-Rad (Richmond, CA). Hyperfilm ECL and cAMP Biotrak kit (RPN225) were from Amersham GE Healthcare (Buckinghamshire, UK). 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, BioSience (Keene, NH).

MEDIA

Tyrode's complete medium (TCM) was used as spermatozoa capacitating medium [Aparicio et al., 2005] and consisted of 96 mM NaCl, 4.7 mM KCl, 0.4 mM $MgSO_4$, 0.3 mM NaH_2PO_4, 5.5 mM glucose, 1 mM sodium pyruvate, 21.6 mM sodium lactate, 1 mM $CaCl_2$, 10 mM $NaHCO_3$, 20 mM HEPES (pH 7.45), and 3 mg/ml BSA. TCM was equilibrated with 95% O_2 and 5% CO_2. A variant of the TCM medium was made by omitting $CaCl_2$, $NaHCO_3$ and BSA and was termed Tyrode's basal medium (TBM). All Tyrode's mediums were prepared on the day of use and maintained at pH 7.45 at 38°C with an osmolarity of 290–310 mOsm kg^{-1}.

COLLECTION AND WASHING OF SEMEN

Commercial doses from Duroc boars of proven fertility and routinely used for artificial insemination (AI) were used. Doses were obtained from Semen Porcino Andalucia (Seville, Spain) and consisted in 80 ml of a commercial extender (MR-A, Kubus Madrid, Spain) containing 30×10^8 spermatozoa cells per doses. All doses were stored for 12 h at 17°C before use and, in order to minimize individual boar variation, samples from up to 4 animals were pooled using semen from no less than 8 boars in different combinations. Semen was centrifuged for 3 min at 2,130*g* and washed with TBM. Samples of 1.5 ml containing 100×10^6 spermatozoa per ml were incubated at 38°C in TCM or TBM for different times. In experiments involving chemical inhibitors, cells were preincubated with different agents for 30 min at 38°C at concentration indicated in the figure legend and Results Section. When high concentration of inhibitors was used, we have ensured that the incubation of spermatozoa under those conditions does not affect at all spermatozoa viability. In order to minimize possible experimental variations, every treatment 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

Samples (1 ml) were centrifuged (7,000*g*, 5 s) and washed with phosphate buffered saline (PBS) supplemented with 0.2 mM Na_3VO_4. After washing, samples were sonicated for 5 s at 4°C in a lysis buffer consisting in 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 1 mM EGTA, 0.4 mM EDTA, a protease inhibitor cocktail (Complete, EDTA-free), and 0.2 mM Na_3VO_4. The homogenates were clarified by centrifugation at 10,000*g* (15 min, 4°C) and the supernatant, containing the proteins solubilized by the detergents of the lysis buffer, were used for analysis of protein concentration. Lysates from porcine spermatozoa were resolved in duplicate by SDS–PAGE and, finally, transferred to nitrocellulose membranes. Western blotting was performed as previously described [Aparicio et al., 2003] using anti phospho-GSK3A (1:1000) and anti GSK3A (1:500) polyclonal antibodies as primary antibodies.

SPERMATOZOA MOTILITY EVALUATION BY COMPUTER ASSISTED SPERM ANALYSIS (CASA) SYSTEM

Analysis was based on the examination of 25 consecutive digitalized images obtained from a single field using a 10× negative-phase contrast objective. Images were taken with a time lapse of 1 s, the image capture speed was therefore one every 40 ms. The number of objects incorrectly identified as spermatozoa was minimized on the monitor by using the playback function. With respect to the setting parameters for the ISAS® program (Projectes i Serveis R + D, SL; Buñol, Spain), an object with an average path velocity (VAP), <10 μm/s was considered immobile, while objects with a velocity >10 μm/s were considered motile. Objects with velocities between 10–15 μm/s and 16–35 μm/s were considered as low and medium speed objects, respectively; those with a velocity >45 μm/s were considered rapid speed objects. Spermatozoa deviated 10% from a straight line were designated linear motile.

MEASUREMENT OF SPERMATOZOA INTRACELLULAR cAMP LEVELS

Evaluation of intracellular cAMP levels was performed in spermatozoa lysates by a non-acetylation enzymeimmunoassay technique according to the manufacturer instructions. Briefly, it combines the use of a peroxidase-labeled cAMP conjugate, a specific antiserum anti-cAMP which can be immobilized onto pre-coated microplates and peroxidase substrate. The D.O. results from a microplate reader (450 nm) were extrapolated in the cAMP standard curve to obtain the amount of cAMP (pmol) and normalized by the amount of protein (μg) measured in each sample.

STATISTICAL ANALYSIS

The mean and standard error of the mean were calculated for descriptive statistics. Data were first tested using a Kolmogorov–Smirnov test to determine the normality of the data distribution. When appropriate for multiple comparisons we have used an analysis of variance (ANOVA) with 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

THE INHIBITION OF PKA BY H89 MODIFIES THE MOTILITY PATTERN OF PORCINE SPERMATOZOA

Although it is widely accepted that the cAMP/PKA intracellular pathway is an important regulator of spermatozoa motility [Okamura et al., 1985; Holt and Harrison, 2002; Bajpai and Doncel, 2003; Luconi et al., 2005], few experiments have been conducted in boar spermatozoa using chemical compounds capable to specifically inhibit PKA activity. Therefore, we first investigated the effect of PKA inhibitor H89 on porcine spermatozoa in order to determine the minimal concentration of this compound able to significantly inhibit motility parameters in our experimental conditions. Boar spermatozoa were incubated for 60 min in a non-capacitating medium (TBM) with increasing concentrations of H89 (10, 30, and 100 μM) and motility was estimated by using a CASA system (Table I). Preincubation of spermatozoa with the lowest concentration tested of H89, 10 μM, has little or no effect in any on the motility parameters evaluated (Table I). Treatment with 30 μM H89 causes a modest but significant decrease in the percentage of motile spermatozoa, whereas does not significantly affect the percentage of rapid spermatozoa, curvilinear velocity (VCL), linear velocity (VSL), mean velocity (VAP), linearity coefficient (LIN), straightness coefficient (STR) or the wobble coefficient (WOB). However, incubation of boar spermatozoa with 100 μM H89 modifies most of the motility parameters evaluated, excepting STR coefficient. This concentration causes a significant decrease in the percentage of both motile spermatozoa (75% vs. 37%) and rapid speed spermatozoa (8%

TABLE I. Effect of Different Concentrations of H89, a Specific Inhibitor of PKA, in Boar Spermatozoa Motility Parameters

Parameters	TBM	TBM + H89 (10 μM)	TBM + H89 (30 μM)	TBM + H89 (100 μM)
Motile spermatozoa (%)	75 ± 5.5^{a}	70 ± 7.0^{a}	52 ± 6.5^{b}	37 ± 1.4^{b}
Rapid spermatozoa (>45 μm/s) (%)	8 ± 2.4^{a}	15 ± 3.5^{a}	5 ± 1.3^{a}	3 ± 0.6^{b}
VCL (μm/s)	48.9 ± 0.9^{a}	56.8 ± 3.3^{a}	45.0 ± 2.1^{a}	36.7 ± 1.7^{b}
VSL (μm/s)	30.4 ± 1.5^{a}	35.0 ± 3.0^{a}	25.6 ± 2.8^{a}	17.2 ± 1.3^{b}
VAP (μm/s)	36.1 ± 1.2^{a}	42.6 ± 3.0^{a}	31.9 ± 2.7^{a}	23.1 ± 1.5^{b}
LIN (%)	61.9 ± 1.9^{a}	61.5 ± 2.3^{a}	56.4 ± 3.9^{a}	46.7 ± 1.8^{b}
STR (%)	84.0 ± 1.3	82.0 ± 1.6	79.7 ± 2.3	74.5 ± 1.2
WOB (%)	73.7 ± 1.1^{a}	74.9 ± 1.4^{a}	70.5 ± 3.1^{a}	62.7 ± 1.6^{b}

VCL, curvilinear velocity; VSL, linear velocity; VAP, mean velocity; LIN, linearity coefficient; STR, straightness coefficient; WOB, wobble coefficient.
Spermatozoa were washed, incubated in a non-capacitating medium (TBM) at 38°C for 1 h with several concentrations of PKA inhibitor, H89 (10, 30, and 100 μM) and motility parameters were measured as described in Materials and Methods Section. Values shown in the table are mean ± SEM of five independent experiments in duplicate. Within a row, values with different superscripts are statistically different from each other, so that for $^{a,b,c}P < 0.05$.

TABLE II. Effect of the PKA Inhibitor H89 in Presence of 8Br-cAMP on Boar Spermatozoa Motility Parameters

Parameters	TBM	TBM + 8Br-cAMP (1 mM)	TBM + H89 (100 μM)	TBM + 8Br-cAMP + H89
% Motile spermatozoa	75 ± 5.5^{a}	$64 \pm 3.6^{a,b}$	37 ± 1.4^{c}	50 ± 4.9^{b}
Rapid spermatozoa (>45 μm/s) (%)	8 ± 2.4^{a}	34 ± 5.5^{b}	$3 \pm 0.6^{a,c}$	$13 \pm 1.7^{a,d}$
VCL (μm/s)	48.9 ± 0.9^{a}	83.4 ± 7.1^{b}	36.7 ± 3.3^{c}	58.2 ± 3.1^{d}
VSL (μm/s)	30.4 ± 1.5^{a}	61.4 ± 6.3^{b}	17.2 ± 2.6^{c}	38.3 ± 6.1^{a}
VAP (μm/s)	36.1 ± 1.2^{a}	70.9 ± 6.9^{b}	23.1 ± 2.9^{c}	45.3 ± 5.3^{a}
LIN (%)	61.9 ± 1.9^{a}	73.7 ± 1.8^{a}	46.7 ± 3.5^{b}	65.6 ± 7.9^{a}
STR (%)	84.0 ± 1.3^{a}	86.6 ± 0.6^{a}	74.4 ± 2.5^{b}	84.2 ± 4.1^{a}
WOB (%)	73.7 ± 1.1^{a}	85.1 ± 1.6^{a}	62.7 ± 3.2^{b}	77.7 ± 5.7^{a}

VCL, curvilinear velocity; VSL, linear velocity; VAP, mean velocity; LIN, linearity coefficient; STR, straightness coefficient; WOB, wobble coefficient.
Spermatozoa were washed, incubated in a non-capacitating medium (TBM) with no addition or in presence of 1 mM 8Br-cAMP, 100 μM H89 or a combination of both. Motility parameters were measured as described in Materials and Methods Section. Values shown in the table are mean ± SEM of five independent experiments in duplicate. Within a row, values with different superscripts are statistically different from each other, so that for $^{a,b,c,d}P < 0.05$.

vs. 3%) and a concomitant inhibition of VCL, VSL, VAP, LIN, and WOB motility parameters.

We next investigated the effect of H89 inhibitor on the porcine spermatozoa motility stimulated by direct activation of the cAMP/PKA pathway with 8Br-cAMP, a phosphodiesterase-resistant cell-permeable cAMP analogue (Table II). Boar spermatozoa were incubated in a TBM medium in presence or absence of 1mM 8Br-cAMP, 100 μM H89 or a combination of both. Results show that the direct activation of PKA increases spermatozoa motility parameters, although only the percentage of rapid spermatozoa, the VCL, VSL, and VAP coefficients result statistically significant. As shown in Table II, H89 inhibits the increase in motility parameters induced by 8Br-cAMP on boar spermatozoa. The effect of H89 on spermatozoa motility parameters was further studied under experimental conditions where these germ cells undergo the physiological process of capacitation, using TCM, a well described capacitating medium, in presence or absence of H89 100 μM (Table III). Results show that H89 is able to suppress the TCM-induced increase in the motility parameters evaluated (Table III).

The inhibition of PKA by H89 blocks GSK3A serine phosphorylation induced by either 8Br-cAMP or capacitating conditions in boar spermatozoa.

We have previously demonstrated a close correlation between the phosphorylation of GSK3A at Ser21 and the motility parameters of boar spermatozoa [Aparicio et al., 2007b]. Therefore, we aimed to investigate whether cAMP/PKA pathway, in addition to its established role in the control of spermatozoa motility might also regulate GSK3A serine phosphorylation (Fig. 1, upper panel). The treatment of boar spermatozoa with 1 mM 8Br-cAMP in TBM for 60 min causes a significant increase in the phosphorylation at Ser21 of the A isoform of GSK3, compared with TBM alone (Fig. 1). Moreover, GSK3A serine phosphorylation is inhibited by H89 in a concentration-dependent manner, causing a full inhibition at 100 μM H89 (Fig. 1). We next studied whether the cAMP/PKA pathway might also regulate the Ser21 phosphorylation of GSK3A detected in a capacitating medium TCM. As seen in Figure 2, TCM-stimulated GSK3A phosphorylation (12 ± 3-fold over basal levels) is completely suppressed by 100 μM H89. As a loading control, the total GSK3A, phosphorylated and non-phosphorylated, is analyzed (Figs. 1 and 2, lower panels).

THE INHIBITION OF PKC BY Ro-32-0432 BLOCKS BOTH GSK3A SERINE PHOSPHORYLATIONAND GERM CELL MOTILITY INDUCED BY EITHER 8BR-CAMP OR CAPACITATING CONDITIONS IN BOAR SPERMATOZOA

As it was previously shown the involvement of PKCs in both the hyperactivation of flagellar movement in boar spermatozoa [Harayama and Miyake, 2006] and in the activity state of GSK3 [Goode et al., 1992; Cook et al., 1996] we next studied whether PKC cascade plays a role in the phosphorylation of GSK3A in spermatozoa and if so, the biochemical relationship between PKA and PKC in these germ cells. Preincubation of spermatozoa with a specific PKC inhibitor Ro-32-4032 (50 μM) totally suppresses the GSK3A phosphorylation induced by either 8Br-cAMP or a capacitating medium, as seen in Figure 3A. Simultaneously, 8Br-cAMP- or TCM-stimulated germ cells motility, evaluated in same

TABLE III. Effect of the PKA Inhibitor H89 in Boar Spermatozoa Motility Parameters Evaluated Under Capacitating Conditions (TCM) or in Presence of the PI3K Inhibitor LY294002

Parameters	TBM	H89 (100 μM)	TCM	TCM + H89	LY294002 (100 μM)	LY294002 + H89
% Rapid spermatozoa (>45 μm/s)	3.3 ± 3.0^{a}	0.8 ± 2.2^{b}	27.1 ± 3.2^{c}	0.8 ± 0.7^{b}	34.1 ± 2.6^{c}	$1.7 \pm 1.5^{a,b}$
VCL (μm/s)	41.9 ± 0.5^{a}	36.8 ± 0.6^{a}	70.2 ± 1.3^{b}	38.8 ± 0.7^{a}	77.3 ± 1.1^{b}	42.1 ± 0.8^{a}
VSL (μm/s)	23.1 ± 0.4^{a}	18.6 ± 0.3^{a}	49.4 ± 1.2^{a}	16.1 ± 0.3^{a}	60.2 ± 1.1^{b}	19.3 ± 0.5^{a}
VAP (μm/s)	27.5 ± 0.3^{a}	22.9 ± 0.4^{a}	56.1 ± 1.2^{b}	22.2 ± 0.4^{a}	65.0 ± 1.1^{b}	24.9 ± 0.5^{a}
LIN (%)	56.2 ± 0.59^{a}	52.6 ± 1.1^{a}	66.6 ± 0.6^{b}	46.1 ± 1.6^{c}	75.2 ± 0.7^{b}	48.4 ± 1.2^{c}
STR (%)	81.7 ± 0.4^{a}	78.8 ± 0.9^{a}	84.0 ± 0.5^{b}	71.8 ± 1.4^{c}	88.9 ± 0.5^{b}	73.4 ± 1.1^{c}
WOB (%)	67.5 ± 0.5^{a}	64.9 ± 0.9^{a}	77.6 ± 0.5^{b}	61.9 ± 1.3^{c}	82.9 ± 0.5^{b}	62.7 ± 1.1^{c}

VCL, curvilinear velocity; VSL, linear velocity; VAP, mean velocity; LIN, linearity coefficient; STR, straightness coefficient; WOB, wobble coefficient.
Spermatozoa were washed, incubated in TBM, or with the PI3K inhibitor LY294002 (100 μM), or in a capacitating medium (TCM) in absence or presence of H89 (100 μM). Motility parameters were measured as described in Materials and Methods Section. Values shown in the table are mean ± SEM of four independent experiments in duplicate. Within a row, values with different superscripts are statistically different from each other, so that for $^{a,b,c}P < 0.01$.

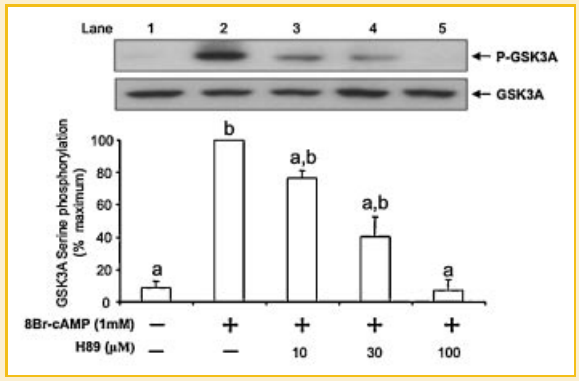

Fig. 1. Effect of PKA inhibition in Ser21 GSK3A phosphorylation. Spermatozoa were incubated in a non-capacitating medium for 1 h at 38°C either in absence or in presence of 8Br-cAMP (1 mM) and different concentrations of the PKA inhibitor H89 (10, 30, and 100 μM). Spermatozoa proteins (10 μg) were resolved by SDS–PAGE, transferred to nitrocellulose membranes and analyzed by Western blotting using anti-GSK3A and anti-phospho-GSK3A/B specific antibodies. Quantification of bands was performed by scanning densitometry. Values shown in the graph are mean ± SEM of five independent experiments represented as percentage of maximum value. Columns with different letters are statistically different from each other, so that for $^{a,b}P < 0.01$.

samples as % of rapid spermatozoa, is also significantly suppressed by the addition of Ro-32-4032 (Fig. 3B). Moreover, male germ cells motility was also evaluated with additional parameters such as the % of total motile spermatozoa (Fig. 3B), which is also reduced by the incubation with the PKC inhibitor (decrease of 57% in germ cells

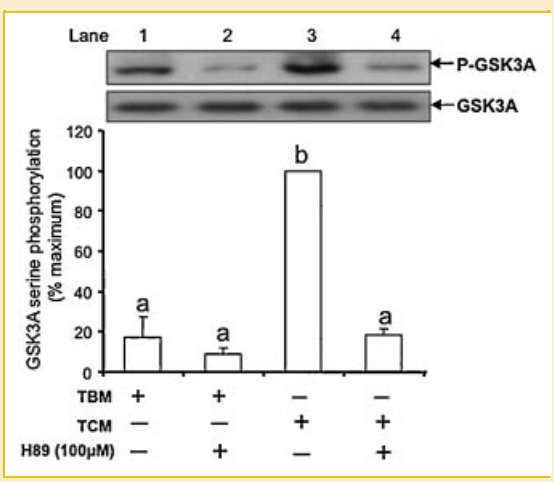

Fig. 2. Effect of PKA inhibition in the Ser21 phosphorylation of GSK3A under capacitating and non-capacitating conditions. Spermatozoa were incubated in a non-capacitating (TBM) or capacitating (TCM) medium in absence or presence of the PKA inhibitor H89 (100 μM) for 1 h at 38°C. Spermatozoa proteins (10 μg) were resolved by SDS–PAGE followed by Western blotting using anti-GSK3A and anti-phospho-GSK3A/B specific antibodies. Quantification of bands was performed by scanning densitometry. Values shown in the graph are mean ± SEM of five independent experiments represented as percentage of the maximum value. Columns with different letters are statistically different from each other, so that for $^{a,b}P < 0.01$.

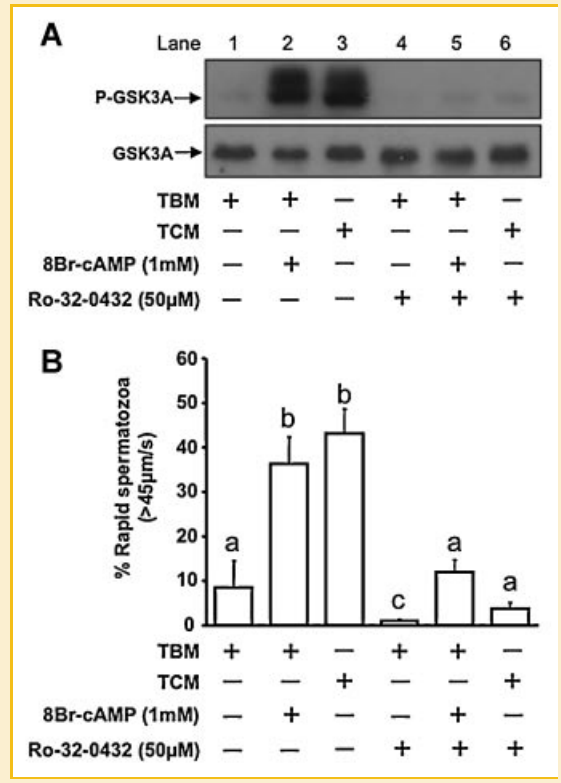

Fig. 3. Effect of PKC inhibition in the Ser21 phosphorylation GSK3A and male germ cells motility. Spermatozoa were incubated in a capacitating (TCM) or non-capacitating (TBM) medium for 1 h at 38°C either in absence or in presence of 8 Br-cAMP or the PKC inhibitor Ro-32-0432 (50 μM). A: Spermatozoa proteins (10 μg) were analyzed by Western blotting using anti-GSK3A and anti-phospho-GSK3A/B specific antibodies. A representative film of five independent experiments is shown. B: Male germ cell motility was evaluated in same conditions as described. Values shown in the graph of % rapid spermatozoa (<45 μm/s) are mean ± SEM of five independent experiments. Columns with different letters are statistically different from each other, so that for $^{a,b}P < 0.05$.

incubated in TBM, 49% in 8Br-cAMP-treated spermatozoa and 76% in TCM-treated spermatozoa).

REGULATION OF GSK3A SERINE PHOSPHORYLATION AND MALE GERM CELL MOTILITY BY THE PHOSPHATIDYLINOSITOL 3-KINASE (PI3K) PATHWAY

We have previously demonstrated that the inhibition of both kinases, PI3K and GSK3, produces an increase in the motility of porcine spermatozoa [Aparicio et al., 2005, 2007b]. Thus, we next studied the biochemical relationship between both kinases pathways. Boar spermatozoa were incubated in presence or absence of the specific inhibitor of PI3K activity, LY294002 (100 μM), under non-capacitating and capacitating conditions and the phosphorylation state of GSK3A in Ser21was analyzed. As observed in Figure 4A (upper panel), treatment with the PI3K inhibitor in a non-capacitating medium TBM significantly increases GSK3A phosphorylation at Ser21 (9 ± 2-fold increase). Interestingly, PI3K inhibitor does not affect at all the increase in TCM-stimulated GSK3A phosphorylation detected in spermatozoa. As GSK3A serine

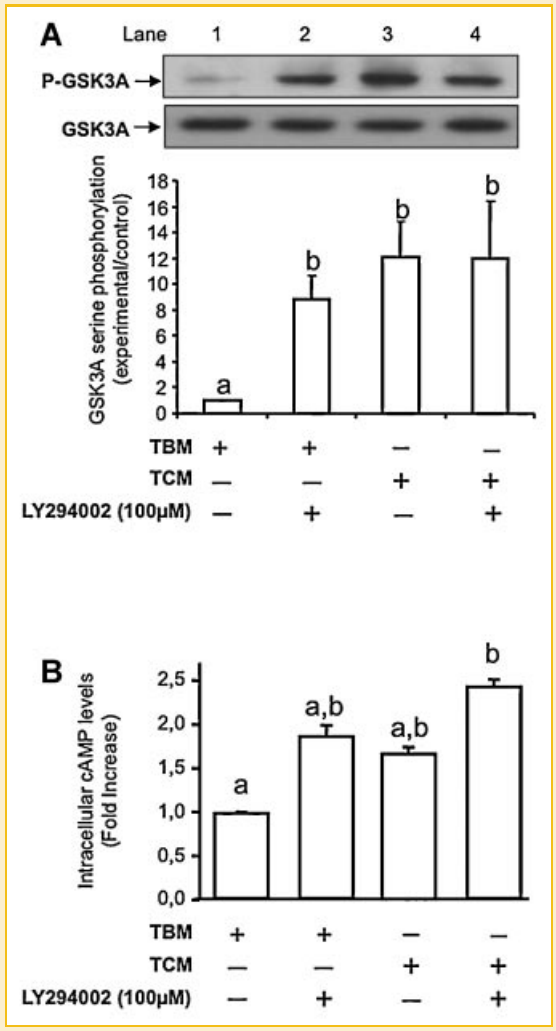

Fig. 4. Effects of the PI3K inhibition in the Ser21 phosphorylation GSK3A and in the intracellular cAMP levels. Spermatozoa were incubated in a non-capacitating (TBM) or capacitating (TCM) medium for 1 h at 38°C either in absence or in presence of PI3K inhibitor LY294002 (100 μM). A: Spermatozoa proteins (10 μg) were analyzed by Western blotting using anti-GSK3A and anti-phospho-GSK3A/B specific antibodies. Quantification of bands was performed by scanning densitometry. Values shown in the graph are mean ± SEM of five independent experiments represented as fold increase (experimental/control). Columns with different letters are statistically different from each other, so that for $^{a,b}P < 0.01$. B: Intracellular cAMP levels were measured in spermatozoa under same conditions and results expressed as pmol/μg protein were represented as fold increase (experimental/control). Values shown are the mean ± SEM of three independent experiments. The average cAMP level measured in basal/control conditions (TBM) is 0.19 pmol/μg protein ±0.49 (SEM). Columns with different letters are statistically different from each other, so that for $^{a,b}P < 0.05$.

phosphorylation is totally dependent on PKA in our conditions in boar spermatozoa, we next studied whether PI3K inhibition-induced GSK3A phosphorylation is mediated by an increase in cAMP levels. As observed in Figure 4B, PI3K inhibition leads to a significant increase in the intracellular cAMP levels, which reach values comparable to those measured during capacitation of spermatozoa (TCM). Moreover, the simultaneous addition of LY294002 in TCM medium does not have a synergistic effect on the cAMP levels of spermatozoa.

We further investigate the role of PI3K in the regulation of the PKA-dependent GSK3A serine phosphorylation, and therefore we evaluated the effect of PI3K inhibition in the GSK3A phosphorylation stimulated by 1mM 8Br-cAMP (Fig. 5A) or inhibited by H89 (Fig. 5B). As mentioned, 8Br-cAMP treatment induces a significant increase in serine GSK3A phosphorylation in spermatozoa (37 ± 8-fold increase). Incubation of porcine spermatozoa with the PI3K inhibitor caused also a significant increase in GSK3A phosphorylation (Fig. 5A), although smaller than 8Br-cAMP (9 ± 2-fold increase), whereas LY294002 did not modify at all the increase in 8Br-cAMP-induced GSK3A phosphorylation (Fig. 5A). Interestingly, pretreatment of spermatozoa with the PKA inhibitor H89 significantly suppresses the increase in LY294002-induced GSK3A phosphorylation (Fig. 5B).

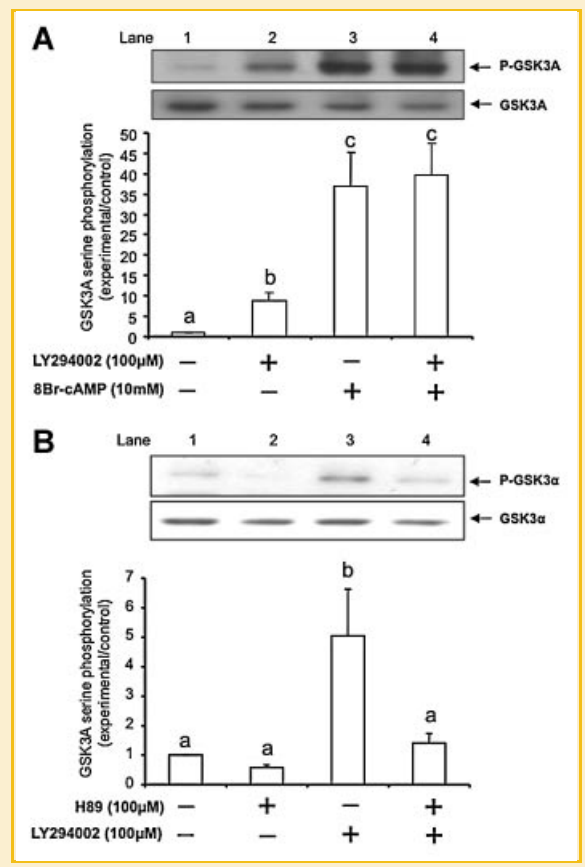

Fig. 5. Effect of PI3K and PKA inhibition in the Ser21 phosphorylation of GSK3A. Spermatozoa were incubated in a non-capacitating medium (TBM) for 1h at 38°C either in absence or in presence of LY294002 (100 μM) without or with 8Br-cAMP (1mM) (A) or H89 (100 μM) (B). Spermatozoa proteins (10 μg) were analyzed by Western blotting using anti-GSK3A and anti-phospho-GSK3A/B specific antibodies. Quantification of bands was performed by scanning densitometry. Values shown in both graphs are mean ± SEM of five independent experiments represented as fold increase (experimental/control). Columns with different letters are statistically different from each other, so that for $^{a,b,c}P < 0.01$.

As described before [Aparicio et al., 2005], and similarly to the effect observed in a capacitating medium TCM, incubation of boar spermatozoa with LY294002 significantly increases all motility parameters evaluated (Table III). Interestingly, H89 significantly suppresses the LY294002-induced increase in boar spermatozoa motility parameters (Table III).

DISCUSSION

Male germ cell motility which mainly contributes to its fertilizing ability, is tightly regulated by the phosphorylation state of GSK3A isoform in boar [Aparicio et al., 2007b], however the intracellular signaling pathways underlying the control of the phosphorylation state (activity) of this motility key enzyme in spermatozoa are unknown. Mammalian spermatozoa motility is mainly controlled by the cAMP-PKA cascade [Okamura et al., 1985; Holt and Harrison, 2002; Bajpai and Doncel, 2003; Luconi et al., 2005; Harayama and Miyake, 2006; Aparicio et al., 2007b; Harayama and Nakamura, 2008]. This work clearly shows that the cAMP-PKA pathway that regulates male germ cells motility also includes GSK3A, as the phosphorylation of GSK3A is totally dependent of the PKA in boar spermatozoa (Fig. 2). It is interesting to notice that the PKA cascade is involved on GSK3A phosphorylation independently of the stimulus that causes spermatozoa motility (Fig. 2). Thus, PKA is mediating the GSK3A phosphorylation stimulated either by a pharmacological agent, cAMP analogue, or by a more physiological conditions using a capacitating medium (TCM) which includes main components that lead to spermatozoa capacitation or hyperactivation in vivo. The idea that PKA and GSK3 lie in the same intracellular pathway is supported by previous studies in somatic cells where the protein AKAP220, which has been also identified in spermatozoa [Reinton et al., 2000], binds to both the regulatory subunit of PKA and GSK3 and this closer cellular localization allows PKA to phosphorylate GSK3 [Tanji et al., 2002]. In this regard, AKAP proteins have been identified and classified in several types in mammalian male germ cells [Carrera et al., 1994; Vijayaraghavan et al., 1999; Reinton et al., 2000; Luconi et al., 2005], where they drive PKA subcellular localization to spermatozoa specific compartments.

Mammalian germ cells motility is also dependent of PKC in boar [Harayama and Miyake, 2006]. Our data showing that PKA and PKC regulates mammalian spermatozoa motility confirm the previous work of Harayama and Miyake [2006] which concludes that the cAMP pathway may have a unique role as the up-regulator of PKCs activity during the hyperactivation of boar spermatozoa. Moreover, we show that PKC inhibition, besides the blockade in all spermatozoa motility parameters induced by 8Br-cAMP, leads to a simultaneous blockade of the GSK3A phosphorylation in male germ cells (Fig. 3). As observed with the PKA pathway, the PKC-mediated GSK3A phosphorylation is independent of the stimulus that induces spermatozoa motility. Thus, PKC is mediating the GSK3A phosphorylation stimulated either by cAMP analogue or by a capacitating medium (TCM). This surprising effect of PKC-dependent GSK3A phosphorylation in germ cells contrasts with studies in somatic cells where only the GSK3B isoform is phosphorylated in Ser9 by PKC [Goode et al., 1992; Cook et al., 1996; Chen et al., 2000; Tsujio et al., 2000; Espada et al., 2009]. The PKC dependence in the phosphorylation of GSK3A can be achieved by a kinase reaction (i) directly catalyzed by PKC, as it has been demonstrated for GSK3B in somatic cells [Goode et al., 1992] or (ii) by a PKC-dependent intermediary kinase. Our results do not allow us to elucidate which one of these mechanisms is underlying GSK3A phosphorylation in male germ cells. However, to our knowledge, this is the first work reporting that PKC lies upstream of the GSK3A isoform phosphorylation in male germ cells. Our data allow us to conclude that, in addition to PKA, the intracellular cascade that controls GSK3A activity, and subsequently germ cell motility, also involves PKC which is located downstream of PKA.

Previous works have demonstrated a relevant specie-specific role for PI3K in the regulation of spermatozoa motility in different mammalian species although the effect appears to be dependent on the specie under study [Luconi et al., 2001; NagDas et al., 2002; Nauc et al., 2004; du Plessis et al., 2004; Aparicio et al., 2005]. This work demonstrates that GSK3A activity is also regulated by the PI3K cascade in spermatozoa, as the inhibition of PI3K by LY294002 clearly leads to an increase in Ser21 GSK3A phosphorylation (Figs. 4 and 5), which correlates with an increase in spermatozoa motility under these conditions (Table III), as expected. This effect of PI3K inhibition is explained by the fact that treatment of male germ cells with LY294002 leads to a marked increased in the intracellular cAMP levels, reaching values comparable to those obtained in spermatozoa under capacitating conditions (Fig. 4). Furthermore, the inhibition of PI3K in a capacitating medium has not a synergistic effect in the increase of spermatozoa cAMP levels, which suggest that both experimental conditions (TCM and PI3K inhibition) are working through the same intracellular pathway that controls male germ cells cAMP levels. The PI3K inhibition-induced increase in cAMP levels directly activates PKA, which in turn leads to GSK3A phosphorylation and the subsequent described effects in spermatozoa motility. These results agree with a previous study by Luconi et al. [2004] which propose a biochemical interaction between PI3K and cAMP/PKA pathways in human spermatozoa where PI3K inhibition also resulted in an increase of intracellular cAMP levels [Luconi et al., 2004]. However we cannot rule out an additional effect of PI3K inhibition in male germ cells, the blockade of phosphodiesterases activity such as PDE3A [Lefievre et al., 2002], present in human spermatozoa, which also would contribute to increase cAMP levels. Interestingly, spermatozoa PI3K inhibition does not affect at all the GSK3A phosphorylation levels induced by either cAMP/PKA pathway or capacitating conditions, suggesting that PI3K and PKA pathways crosstalk to downstream regulate GSK3A phosphorylation in boar spermatozoa. Moreover, the fact that PKA inhibitor is able to prevent the effect of PI3K inhibition on both GSK3A phosphorylation and male germ cell motility, suggests that PI3K lies upstream of PKA in this motility regulatory pathway.

Based in our results and others, we propose the following model (Fig. 6) of intracellular mechanisms that regulate GSK3A phosphorylation which ultimately leads to the control of spermatozoa motility. Briefly, bicarbonate and calcium, components included in a capacitating medium, physiologically activate spermatozoa adenylyl cyclase SACY [Wuttke et al., 2001; Xie et al., 2006;

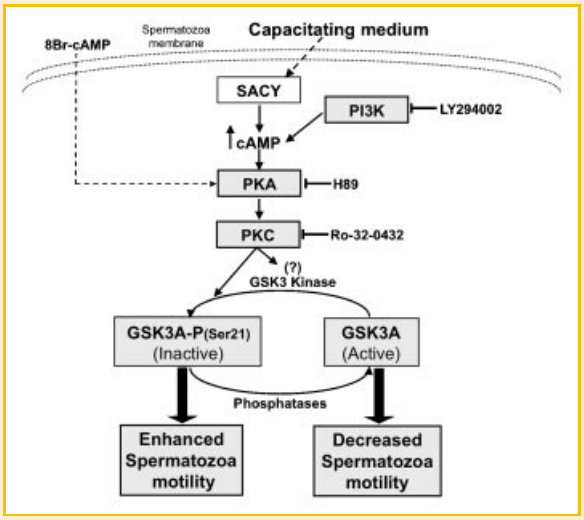

Fig. 6. Proposed model of intracellular mechanisms that regulate GSK3A serine phosphorylation and ultimately control boar germ cells motility. Spermatozoa protein kinase A (PKA) can be activated either by physiological stimulation of adenylyl cyclase (SACY) with a capacitating medium or directly by a permeable cAMP analog, 8Br-cAMP. The PKA cascade includes PKC downstream which directly or by an intermediary GSK3A-kinase(s), leads to GSK3A phosporylation at Ser21 and subsequently induces an enhancement of spermatozoa motility. Inhibition of PKA (H89) or PKC (Ro-32-0432) blocks both GSK3A phosphorylation and simultaneously inhibits spermatozoa motility. An additional kinase that regulates spermatozoa motility is PI3K, which leads to inhibition of GSK3A phosphorylation and decreases boar spermatozoa motility. This is supported by the fact that inhibition of PI3K activity (LY294002) leads to both stimulation of GSK3A phosphorylation and a concomitant enhancement of germ cell motility. A full description of this model is included in the discussion.

Carlson et al., 2007], which in turn increases cAMP levels and subsequently activates PKA [Okamura et al., 1985; Holt and Harrison, 2002; Breitbart, 2003]. PKA activity in male germ cells might lead to either (i) direct phosphorylation of GSK3A in Ser 21 in boar spermatozoa as demonstrated in somatic cells [Fang et al., 2000] or (ii) activation of PKC [Harayama and Miyake, 2006] which subsequently increases GSK3A phosphorylation either directly or indirectly by an intermediary kinase. A different signaling pathway involved in the regulation of GSK3A phosphorylation and boar spermatozoa motility is PI3K, which lies upstream of PKA in these male germ cells. The inhibition of PI3K causes an increase in intracellular cAMP levels in spermatozoa and subsequent increases in both GSK3A phosphorylation and germ cell motility. The phosphorylation of GSK3A in Ser 21 decreases its enzymatic activity, as shown in somatic [Goode et al., 1992; Cook et al., 1996] and male germ cells [Somanath et al., 2004] and leads to an increase in the motility of boar spermatozoa [Aparicio et al., 2007b]. The link between GSK3A phosphorylation and germ cells motility is further supported by the fact that any experimental conditions that block GSK3A phosphorylation also simultaneously block spermatozoa motility.

In summary, we have demonstrated that PI3K, PKA and PKC signaling pathways crosstalk and crucially converge downstream at the level of GSK3A which plays an essential role in the motility in mammalian male germ cells. We believe that the identity of GSK3A downstream target(s) that ultimately control spermatozoa motility are very interesting and deserve further studies.

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