Porcine sperm motility is regulated by serine phosphorylation of the glycogen synthase kinase-3 α

I M Aparicio, M J Bragado, M C Gil, M Garcia-Herreros, L Gonzalez-Fernandez, J A Tapia and L J Garcia-Marin

Research Group of Intracellular Signalling and Technology of Reproduction, Faculty of Veterinary, University of Extremadura, 10071 Caceres, Spain

Correspondence should be addressed to L J Garcia-Marin who is now at Departamento de Fisiologia, Facultad de Veterinaria, Universidad de Extremadura, Avenida de la Universidad, s/n, 10071 Caceres, Spain; Email: ljgarcia@unex.es

Abstract

Sperm functions are critically controlled through the phosphorylation state of specific proteins. Glycogen synthase kinase-3 (GSK3) is a serine/threonine kinase with two different isoforms (α and β), the enzyme activity of which is inhibited by serine phosphorylation. Recent studies suggest that GSK3 is involved in the control of bovine sperm motility. Our aim was to investigate whether GSK3 is present in porcine spermatozoa and its role in the function of these cells. This work shows that both isoforms of GSK3 are present in whole cell lysates of porcine sperm and are phosphorylated on serine in spermatozoa stimulated with the cAMP analog, 8Br-cAMP. A parallel increase in serine phosphorylation of the isoform GSK3 α , but not in the isoform GSK3 β , is observed after treatments that also induce a significant increase in porcine sperm velocity parameters. Therefore, a significant positive correlation among straight-line velocity, circular velocity, average velocity, rapid-speed spermatozoa, and GSK3 α serine phosphorylation levels exists. Inhibition of GSK3 activity by alsterpaullone leads to a significant increase in the percentage of rapid- and medium-speed spermatozoa as well as in all sperm velocity parameters and coefficients. Moreover, pretreatment of porcine spermatozoa with alsterpaullone significantly increased the percentage of capacitated porcine spermatozoa and presents no effect in the number of acrosome-reacted porcine spermatozoa. Our work suggests that the isoform GSK3 α plays a negative role in the regulation of porcine sperm motility and points out the possibility that sperm motile quality might be modulated according to the activity state of GSK3 α .

Reproduction (2007) **134** 435–444

Introduction

Mammalian spermatozoa released from the testis are immotile and unable to fertilize an oocyte. To reach the oocyte and acquire the competence to fertilize, they need to undergo a sequential series of complex process of activation consisting of maturation and acquisition of motility as well as capacitation and the ability to undergo the acrosome reaction (Spungin *et al.* 1995). All these processes occur in the transit through the female uterus and oviduct and are under the control of different factors. These processes can be reproduced *in vitro* by the incubation of spermatozoa in a defined medium containing bicarbonate, calcium, and BSA (Go & Wolf 1985, Choi & Toyoda 1998, Flesch *et al.* 1999, 2001, Tardif *et al.* 2004). Both bicarbonate and calcium have been demonstrated to play a pivotal role in regulating sperm motility, capacitation, and acrosome reaction (Visconti *et al.* 1995, Breitbart & Spungin 1997, Breitbart 2002, Holt & Harrison 2002, Brewis *et al.* 2005). Both ions

mediate their action through activation of a sperm adenylyl cyclase, which produces an increase in the intracellular levels of cAMP (Wuttke *et al.* 2001). Recent results demonstrate that the presence of cAMP or its analogs in the incubation medium are able to induce capacitation and to increase motility in spermatozoa from different species (Si & Okuno 1999, Baldi *et al.* 2000, Holt & Harrison 2002, Tardif *et al.* 2004). A major downstream target of cAMP in spermatozoa is the serine/threonine kinase PKA (Visconti *et al.* 1995) which might phosphorylate several target proteins resulting in the activation of a downstream, as yet unidentified, effector.

Previous results demonstrated that immotile bovine caput spermatozoa contained sixfold higher glycogen synthase kinase-3 (GSK3) serine/threonine kinase activity than motile caudal spermatozoa (Vijayaraghavan *et al.* 1996). Moreover, these authors describe the presence of both the α and β GSK3 isoforms in bovine epididymal

spermatozoa (Vijayaraghavan *et al.* 1996, 1997) and discovered a 55 kDa protein whose tyrosine phosphorylation was closely correlated to the motility state of epididymal bovine spermatozoa and the treatment of these cells with motility activators, like isobutylmethylxanthine or 8Br-cAMP, resulted in increased tyrosine phosphorylation of this protein (Vijayaraghavan *et al.* 1997). The same authors showed a correlation between the 55 kDa tyrosine phosphorylation motility-associated protein and the GSK3 and suggested that GSK3, which is also regulated by tyrosine phosphorylation, could be an important key underlying motility initiation in the epididymis (Vijayaraghavan *et al.* 2000). Finally, Somanath *et al.* (2004) showed that serine phosphorylation of GSK3 increases significantly in bovine spermatozoa during their passage through the epididymis with a parallel decrease in GSK3 activity (Somanath *et al.* 2004).

Over 20 years ago, GSK3 was discovered as one of several protein serine/threonine kinases, which phosphorylates and inactivates glycogen synthase, the final enzyme in glycogen biosynthesis (Frame & Cohen 2001). At present, GSK3 is recognized as a key component of a large number of cellular processes implicated in cell adhesion, division, survival, and apoptosis (Frame & Cohen 2001). GSK3 exists as two isoforms, GSK3 α and GSK3 β , ubiquitously expressed in mammalian tissues (Woodgett 1990). Both isoforms share 97% sequence similarity within their kinase catalytic domain, but differ significantly outside this region, with GSK3 α possessing an extended N-terminal glycine-rich tail (Woodgett 1990). Unlike other kinases, GSK3 activity is significantly reduced by phosphorylation of an N-terminal serine, Ser9 in GSK3 β and Ser21 in GSK3 α (Frame *et al.* 2001). Contrary to its inhibitory regulation by serine phosphorylation, GSK3 activity is facilitated by tyrosine phosphorylation, Tyr216 in GSK3 β and Tyr279 in GSK3 α (Tardif *et al.* 2001).

To date, there are no studies in the literature regarding the role of GSK3 in the regulation of porcine spermatozoa functions, including sperm motility. Therefore, the aims of the present study are to determine whether GSK3 is present in porcine spermatozoa and to study its involvement in the regulation of porcine spermatozoon function.

Materials and Methods

Materials

Chlortetracycline (CTC), polyvinyl pyrrolidone (PVP), polyvinyl alcohol (PVA), 1,4-diazabicyclo(2.2.2)octane (DABCO), Triton X-100 and deoxycholic acid from Sigma; and A23187 from Calbiochem, La Jolla, CA, USA; FITC-PNA (*Arachis hypogaea* (peanut) agglutinin FITC-conjugated), ethidium homodimer-1, and propidium iodide from Molecular Probes, Leiden, The Netherlands; protease inhibitor cocktail Complete tablets, Mini, EDTA-free from Boehringer Mannheim; anti-GSK3 α , anti-GSK3 β , and anti-phospho GSK3 α/β polyclonal antibodies from Cell

Signaling, Beverly, CA, USA; Tris/glycine/SDS buffer (10 times concentrated) and Tris/glycine buffer (10 times concentrated) from BIO-RAD; Hyperfilm ECL from Amersham; ECL detection reagents and anti-mouse and anti-rabbit IgG-horseradish peroxidase conjugates from Pierce, Rockford, IL, USA; and nitrocellulose membranes from Schleicher & Schuell, Keene, NH, USA.

Media

Spermatozoa-capacitating medium, Tyrode's complete medium (TCM; Aparicio *et al.* 2005), which consisted of 96 mM NaCl, 4.7 mM KCl, 0.4 mM MgSO₄, 0.3 mM NaH₂PO₄, 5.5 mM glucose, 1 mM sodium pyruvate, 21.6 mM sodium lactate, 1 mM CaCl₂, 10 mM NaHCO₃, 20 mM HEPES (pH 7.45), and 3 mg/ml BSA. TCM was equilibrated with 5% CO₂. A variant of the TCM medium was made by omitting CaCl₂, NaHCO₃, and BSA and was termed Tyrode's basal medium (TBM). All Tyrode's media were prepared on the day of use and maintained at an osmolarity of 290–310 mmol/kg at pH 7.45 at 38 °C.

Collection and washing of semen

Commercial artificial insemination (AI) doses from Duroc boars of proven fertility and routinely used for AI, diluted to 30 × 10⁶ sperm cells per ml, in 80 ml commercial extender (MR-A, Kubus, Madrid, Spain) and stored for 12 h at 17 °C, were obtained from Semen Porcino Andalucia SL, Sevilla, Spain. In order to minimize individual boar variation, samples from up to four animals were pooled, using semen from at least eight boars. Semen was centrifuged once (3 min, 2300 g) and washed twice with TBM. Samples of 1.5 ml containing 1 × 10⁸ spermatozoa per ml were incubated at 38 °C in TCM or TBM for different times. When spermatozoa cells were treated with the inhibitor, a preincubation for 30 min at 38 °C was performed at the concentrations indicated with alsterpaullone. In order to minimize experimental variations, all the treatments were made for each semen pool and, when necessary, a control with the final concentration of the solvent (DMSO 0.1%) was added.

Western blotting

Samples (1 ml) were washed with PBS with 0.2 mM Na₃VO₄, and sonicated for 5 s at 4 °C in lysis buffer (50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 1 mM EGTA, 0.4 mM EDTA, protease inhibitor cocktail (1 tablet/50 ml), and 0.2 mM Na₃VO₄). The homogenate was centrifuged at 10 000 g (15 min, 4 °C) and supernatant containing soluble proteins in non-ionic and ionic detergents was used for analysis of protein content in porcine spermatozoa.

Cell lysates were resolved in duplicate by SDS-PAGE and transferred to nitrocellulose membranes. Western

blotting was performed as previously described (Aparicio *et al.* 2003) using phospho GSK3 α/β (1:1000), GSK3 α (1:500), and GSK3 β (1:1000) polyclonal antibodies as primary antibodies.

Motility analysis

Analysis was based on the examination of 25 consecutive digitalized images obtained from a single field using a 20 $\times$ negative-phase contrast objective. Images were taken with a time lapse of 1 s, the image capture speed was therefore once every 40 ms. 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 $\mu\text{m/s}$ was considered immobile, while objects with a velocity >10 $\mu\text{m/s}$ were considered motile. Objects with velocities between 10–15 and 16–35 $\mu\text{m/s}$ were considered as low- and medium-speed objects respectively; those with a velocity >45 $\mu\text{m/s}$ were considered rapid-speed objects. Spermatozoa deviated 10% from a straight line were designated linear motile (Table 1).

Evaluation of spermatozoa capacitation

CTC staining was used to determine spermatozoa maturational status (Wang *et al.* 1995). After being washed, 30 $\times$ 10⁶ spermatozoa per ml were first stained by 16 μl ethidium homodimer-1 staining solution (11.67 mM) and centrifuged at 1400 *g* for 5 min through 4 ml of 3% PVP in PBS. The pellet was resuspended in 45 μl WS-PVA solution (140 mM NaCl, 20 mM HEPES, and 0.1% PVA) and mixed thoroughly with 45 μl CTC staining solution: 750 mM CTC (Sigma), 5 mM DL-cysteine, 130 mM NaCl, and 20 mM Tris–HCl (pH 7.8). Then, 8 μl of 12.5% (w/v) paraformaldehyde in 0.5 M Tris–HCl (pH 7.4) were added. Five microliters of the fixed spermatozoa suspension were placed on a glass slide and mixed well with an equal amount of anti-fade solution: DABCO dissolved in glycerol:PBS (9:1). The evaluations were performed using an epifluorescence microscope (Nikon Instrument Europe BV, Bodhoevedorp, The Netherlands). We confirmed the viability of spermatozoa using excitation at 530–560 nm and emission at 580 nm, and we evaluated the CTC

staining patterns under blue-violet illumination (excitation at 330–380 nm, emission at 420 nm). Only live spermatozoa (ethidium homodimer-1-negative) were assessed for CTC staining patterns. We classified 200 viable spermatozoa into three patterns according to the method described by Wang *et al.* (1995). F-pattern (intact): fluorescence is detected over the whole region of the spermatozoon head. B-pattern (capacitated): fluorescence is detected in the spermatozoon head except in the post-acrosomal region. Acrosome-reacted-pattern (AR): weak fluorescence is observed over the spermatozoon head and a bright band was detected in the equatorial segment.

Flow cytometry

For flow cytometric purposes, sperm cells were incubated in a TCM plus ionophore A23187 (1 μM) at 38 °C at times indicated. For the determination of the acrosomal integrity, samples, each containing 5 $\times$ 10⁷ spermatozoa per ml, were labeled with FITC-PNA (0.5 $\mu\text{g/ml}$) and propidium iodide (1.2 μM) for 5 min at room temperature and were analyzed on a FACScan (Becton Dickinson, San Jose, CA, USA). The system collects fluorescence data in logarithmic mode and light-scatter data in linear mode. Only sperm-specific events, which appeared in a typically L-shape scatter profile, were positively gated. For each file, 10 000 events were stored in the computer and only live spermatozoa (propidium iodide-negative) were further analyzed with Cell-Quest software, Becton Dickinson.

Statistical analysis

The mean and S.E.M. 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 ANOVA with the Scheffe test for comparisons between treatments. The Spearman non-parametric test was used to study the correlation between the serine phosphorylation of both isoforms of GSK3 and the motility parameters determined by the ISAS system. All analyses were performed by SPSS v11.0 for MacOs X software (SPSS Inc., Chicago, IL, USA). The level of significance was set at $P < 0.05$.

Table 1 Definition of motility descriptors.

Name	Units	Description
Curvilinear velocity (VCL)	$\mu\text{m/s}$	Measures the sequential progression along the true trajectory
Straight linear velocity (VSL)	$\mu\text{m/s}$	Measures the straight trajectory of the spermatozoa per unit of time
Average path velocity (VAP)	$\mu\text{m/s}$	Measures the mean trajectory of the spermatozoa per unit of time
Linearity (LIN)	%	$\text{VSL/VCL} \times 100$
Straightness (STR)	%	$\text{VSL/VAP} \times 100$
Wobble coefficient (WOB)	%	$\text{VAP/VCL} \times 100$
Amplitude of lateral head displacement (ALH)	μm	Measures the mean head displacement along the curvilinear trajectory
Beat cross frequency (BCF)	Hz	Measures the time average rate at which the curvilinear sperm tract crosses its average path trajectory

Figure 1 Identification of both GSK3 isoforms in porcine spermatozoa. Fifteen micrograms of proteins from precleared whole cell lysates were loaded in each lane, resolved by SDS-PAGE and transferred to nitrocellulose membrane. Western blotting was performed as described in Materials and Methods. Lanes 1 and 2, whole cell lysates from porcine spermatozoa; lanes 3–5, whole cell lysates from porcine pancreas, spleen, and brain; and lane 6, whole cell lysates from A431 cells in which the presence of GSK3 isoforms has previously been identified. (A) Proteins were analyzed by Western blotting using anti-GSK3 α/β polyclonal antibody. (B) Proteins were analyzed by western blotting using anti-GSK3 β polyclonal antibody. Results shown are representative from three independent experiments. Molecular weight marked positions are indicated on the left.

Results

Identification of GSK3 protein in porcine spermatozoa

We performed a Western blot analysis using two specific antibodies against both isoforms of GSK3 in whole cell lysates of porcine spermatozoa. Control lysates from

different tissues were also used to ensure that both protein bands correspond to GSK3 α and GSK3 β isoforms. The antibody against isoform α recognized a single protein band of ~ 51 kDa in porcine spermatozoa as well as in control lanes (Fig. 1A). The antibody against isoform β also recognized a single band of ~ 47 kDa in porcine spermatozoa (Fig. 1B). These results show that both the α and β GSK3 isoforms are present in porcine spermatozoa.

cAMP/PKA pathway stimulation and GSK3 serine phosphorylation

Porcine spermatozoa were incubated in TBM for 1 h in the presence or the absence of 8Br-cAMP. After incubation, samples were taken to measure the motility parameters by ISAS (Table 2) and Western blotting was performed with two phosphospecific antibodies directed against Ser21 and Ser9 of GSK3 α and GSK3 β respectively (Fig. 2). As previously described (Aparicio *et al.* 2005), our results showed that incubation with 8Br-cAMP induced, in porcine spermatozoa, a significant increase in the percentage of rapid- and medium-speed spermatozoa and in the curvilinear velocity (VCL), straight linear velocity (VSL), average path velocity (VAP), linearity coefficient (LIN), and wobble coefficient (WOB). In these conditions, no significant effects were detected in the percentage of static and low-speed spermatozoa and in the straightness (STR), amplitude of lateral head displacement (ALH) and frequency of head displacement (or beat cross-frequency, BCF), when compared with porcine spermatozoa incubated at 38 °C in TBM alone (Table 2). On the other hand, treatment with 8Br-cAMP induced a clear and significant increase in the phosphorylation of both isoforms of GSK3, GSK3 α (Fig. 2A, lane 2)

Table 2 Porcine spermatozoa velocity and motility patterns under different treatments including capacitating and non-capacitating incubation conditions, temperature decrease, glycogen synthase kinase-3 inhibition, and addition of a cAMP analog.

Motility parameters	Treatment				
	TBM 17 °C	TBM 38 °C	TBM 38 °C + 8Br-cAMP (1 mM)	TCM 38 °C	TBM 38 °C + alsterpaullone (30 μ M)
Static (%)	78 $\pm$ 4.4 ^a	21 $\pm$ 3.7 ^b	23 $\pm$ 2.5 ^b	18 $\pm$ 3.2 ^b	23 $\pm$ 4.5 ^b
Rapid speed (%)	2 $\pm$ 1.1 ^a	13 $\pm$ 3.0 ^b	39 $\pm$ 2.2 ^d	42 $\pm$ 5.1 ^{d,e}	53 $\pm$ 2.7 ^e
Medium speed (%)	10 $\pm$ 2.4 ^a	59 $\pm$ 2.8 ^c	30 $\pm$ 3.2 ^b	33 $\pm$ 3.0 ^b	19 $\pm$ 1.7 ^{a,b}
Low speed (%)	10 $\pm$ 1.6	7 $\pm$ 1.1	8 $\pm$ 0.4	7 $\pm$ 0.2	5 $\pm$ 0.7
VCL (μ /s)	23.6 $\pm$ 3.7 ^a	56.2 $\pm$ 6.3 ^b	83.8 $\pm$ 8.4 ^c	85.5 $\pm$ 14.4 ^c	101.7 $\pm$ 3.4 ^c
VSL (μ /s)	7.1 $\pm$ 1.2 ^a	29.0 $\pm$ 3.2 ^b	51.9 $\pm$ 7.2 ^c	52.6 $\pm$ 10.1 ^{c,d}	68.2 $\pm$ 6.9 ^e
VAP (μ /s)	11.9 $\pm$ 1.9 ^a	37.1 $\pm$ 4.2 ^b	62.9 $\pm$ 8.3 ^c	64.5 $\pm$ 12.8 ^c	80.1 $\pm$ 4.1 ^d
LIN (%)	28.1 $\pm$ 1 ^a	51.7 $\pm$ 3.6 ^b	61.9 $\pm$ 4.2 ^c	61.3 $\pm$ 3.0 ^c	67.0 $\pm$ 6.8 ^c
STR (%)	57.5 $\pm$ 1.7 ^a	78.3 $\pm$ 2.4 ^b	82.6 $\pm$ 1.4 ^b	81.6 $\pm$ 2 ^b	85.1 $\pm$ 4.9 ^b
WOB (%)	48.9 $\pm$ 0.9 ^a	66.0 $\pm$ 3.2 ^b	74.9 $\pm$ 4.1 ^c	75.1 $\pm$ 2.8 ^c	78.7 $\pm$ 4.1 ^c
ALH (%)	1.4 $\pm$ 0.3 ^a	2.6 $\pm$ 0.32 ^{a,b}	3.27 $\pm$ 0.4 ^b	3.2 $\pm$ 0.3 ^b	3.6 $\pm$ 0.4 ^b
BCF (%)	3.6 $\pm$ 0.6 ^a	7.3 $\pm$ 3.2 ^{a,b}	6.60 $\pm$ 0.6 ^b	7.2 $\pm$ 0.3 ^b	6.6 $\pm$ 0.7 ^b

Porcine spermatozoa were washed and incubated for 1 h at 38 °C in a non-capacitating medium (TBM) in the presence or the absence of 8Br-cAMP (1 mM) and alsterpaullone (30 μ M) or in a capacitating medium (TCM). After incubation, samples were taken to assess spermatozoa motility parameters by ISAS. Within a row, values with different superscripts are statistically different from each other, so that for (a–b–c–d), $P < 0.05$.

Figure 2 Effect of 8Br-cAMP and the incubation of porcine spermatozoa in a capacitating medium in GSK3 α/β phosphorylation. Sperm were washed and incubated in a non-capacitating medium (TBM) in the absence or the presence of 8Br-cAMP (1 mM) and in a capacitating medium (TCM) for 1 h at 38 °C. After incubation, proteins were extracted and 10 μ g proteins were loaded and resolved by SDS-PAGE. Western blotting was performed as described in Materials and Methods. (A) Proteins were analyzed using anti-phospho GSK3 α/β polyclonal and anti-GSK3 α polyclonal antibodies. (B) Proteins were analyzed using anti-phospho GSK3 α/β polyclonal and anti-GSK3 β polyclonal antibodies. Quantification of bands was performed by scanning densitometry. Values shown in the graph are mean $\pm$ S.E.M. of eight independent experiments represented as experimental fold increase versus control. Columns with different superscripts are statistically different from each other, so that for (a–b–c) $P < 0.05$.

and β (Fig. 2B, lane 2), with a 12.5- and 3.7-fold increase respectively. Our results indicate that PKA could be modulating GSK3 serine phosphorylation and subsequently its activity either directly or by the activation of an additional intermediary kinase.

Are both isoforms of GSK3 implicated in porcine sperm motility?

Incubation of porcine spermatozoa in a medium containing bicarbonate and calcium (TCM) for 1 h at 38 °C resulted in a clear and significant increase in the percentage of rapid- and medium-speed spermatozoa and in VCL, VSL, VAP, LIN, and WOB. However, no significant effects were detected in the percentage of static- and low-speed spermatozoa and in STR, ALH and BCF, when compared with porcine spermatozoa incubated at 38 °C in TBM (Table 2). From the same pool, samples were taken to perform a Western blotting analysis to study the phosphorylation of Ser21 of GSK3 α and of Ser9 of GSK3 β under capacitating conditions. Our results showed a significant increase in the phosphorylation of the isoform α at Ser21 (4.7-fold increase; Fig. 2A, lane 3). Interestingly, the phosphorylation of GSK3 β at Ser9 was not significantly modified after incubation of porcine spermatozoa in TCM (Fig. 2B, lane 3).

To test whether the different effect in the serine phosphorylation of GSK3 α and β under capacitating conditions could be due to the incubation time, porcine spermatozoa were incubated in TCM for different times and the serine phosphorylation of GSK3 α and β were determined (Fig. 3). Our results clearly showed a significant time-dependent increase in the serine

Figure 3 Time-course of GSK3 α/β phosphorylation of porcine spermatozoa incubated under capacitating conditions. Spermatozoa were washed in a non-capacitating medium and incubated in a capacitating medium at 38 °C for 15–30–60 min. After incubation, proteins were extracted and 10 μ g proteins were loaded and resolved by SDS-PAGE. Separate proteins were transferred to nitrocellulose membranes and analyzed by Western blotting using anti-phospho GSK3 α/β polyclonal antibody. Quantification of bands was performed by scanning densitometry. Values shown in the graph are mean $\pm$ S.E.M. of four independent experiments represented as experimental fold increase versus control.

phosphorylation of GSK3 α but not in the phosphorylation of Ser9 of GSK3 β (Fig. 3).

We next investigated the effect of the temperature on porcine sperm motility and GSK3 phosphorylation (Fig. 4). Motility was measured in spermatozoa kept 1 h in two conditions: at 17 °C in TBM and at 38 °C in TBM (Table 2). Our results showed a clear and significant increase in all motility parameters when porcine spermatozoa were incubated at 38 °C, except in ALH and BCF (Table 2). When spermatozoa were incubated at 38 °C, a significant increase in the serine phosphorylation of Ser21

of GSK3 α was observed (Fig. 4A). However, the phosphorylation of GSK3 β was not modified after incubation of porcine spermatozoa at 38 °C for 1 h (Fig. 4B).

Effect of GSK3 inhibition on porcine spermatozoa motility, capacitation, and acrosome reaction

To establish whether GSK3 plays a role in the control of the porcine sperm function, we used alsterpaullone, a specific inhibitor of its kinase activity, and studied its effects on the kinetic parameters of spermatozoa. The treatment of porcine spermatozoa in a non-capacitating medium with 30 μ M alsterpaullone for 60 min produced a significant increase in the percentage of rapid-speed spermatozoa and a decrease in the percentage of medium-speed spermatozoa when compared with TBM alone (Table 2). Moreover, the pretreatment with alsterpaullone also caused a significant increase in VCL, VSL, VAP, LIN, and WOB, whereas no significant effects were detected in the percentage of static- and low-speed spermatozoa, STR, ALH, and BCF, when compared with TBM alone (Table 2).

Next, we wanted to know whether GSK3 could have a role in the regulation of porcine spermatozoa capacitation and acrosome reaction. With this aim, porcine spermatozoa were incubated in TBM or TCM either in the presence or the absence of alsterpaullone (30 μ M) and the percentage of capacitated porcine spermatozoa were assessed by CTC staining. As expected, when porcine spermatozoa were incubated in a capacitating medium, a significant increase in the percentage of capacitated and acrosome-reacted spermatozoa was observed (Table 3). The incubation of porcine spermatozoa in TBM in the presence of alsterpaullone caused a significant increase in the percentage of capacitated spermatozoa when compared with TBM alone (Table 3). However, the addition of alsterpaullone in TCM had no effect on the percentage of capacitated spermatozoa when compared with TCM alone (Table 3).

Finally, we evaluated whether GSK3 could be playing a role in the acrosome reaction, which was induced *in vitro* by incubation of porcine spermatozoa in a capacitating medium in the presence of the calcium ionophore A23187 (1 μ M). Acrosomal status was evaluated by flow cytometry using a double staining with FITC-PNA and propidium iodide. The treatment of porcine spermatozoa with A23187, with or without alsterpaullone, resulted in a significant increase in the percentage of acrosome-reacted spermatozoa after 60 min of incubation in TCM (Fig. 5). Similar values were obtained either in the presence or the absence of alsterpaullone at any time (Fig. 5), indicating that GSK3 was not likely involved in the regulation of the calcium-induced acrosome reaction in porcine spermatozoa.

Figure 4 Effect of temperature on GSK3 α/β phosphorylation of porcine spermatozoa. Spermatozoa were washed and incubated in a non-capacitating medium at 17 and 38 °C for 60 min. After incubation, proteins were extracted and 10 μ g proteins were loaded and resolved by SDS-PAGE. Separate proteins were transferred to nitrocellulose membranes and analyzed by Western blotting as described in Materials and Methods. (A) Proteins were analyzed using anti-phospho GSK3 α/β polyclonal and anti-GSK3 α polyclonal antibodies. (B) Proteins were analyzed using anti-phospho GSK3 α/β polyclonal and anti-GSK3 β polyclonal antibodies. Quantification of bands was performed by scanning densitometry. Values shown in the graph are mean $\pm$ S.E.M. of eight independent experiments represented as experimental fold increase versus control.

Table 3 Evaluation of the capacitation state of porcine spermatozoa by the chlortetracycline staining.

Treatment	Percentage of live spermatozoa classified as:		
	Non-capacitated (F)	Capacitated with intact acrosome (B)	Acrosome-reacted (AR)
TBM	67 $\pm$ 2.0 ^a	33 $\pm$ 2.0 ^a	0 $\pm$ 0 ^a
TBM + alsterpaullone (30 μ M)	57 $\pm$ 3.9 ^b	42 $\pm$ 4.7 ^b	1 $\pm$ 2.0 ^a
TCM	50 $\pm$ 4.5 ^c	40 $\pm$ 4.1 ^b	10 $\pm$ 5.8 ^b
TCM + alsterpaullone (30 μ M)	44 $\pm$ 4.3 ^d	42 $\pm$ 6.1 ^b	14 $\pm$ 4.1 ^b

Porcine sperm was washed, incubated in a non-capacitating medium (TBM) for 30 min at 38 °C in the absence or the presence of alsterpaullone (30 μ M) followed by incubation on TBM or in a capacitating medium (TCM) for 60 min at 38 °C in the absence or the presence of alsterpaullone. Values are means $\pm$ S.E.M. of five independent experiments in duplicate. Within a column, values with different superscript are statistically different from each other, so that for (a–b–c), $P < 0.05$.

Relationship between GSK3 α serine phosphorylation and motility on porcine spermatozoa

Our results have shown that a close relationship between motility and GSK3 α serine phosphorylation likely exists, since incubation with decreased temperature or 8Br-cAMP in TBM or in a capacitating medium showed a parallel decrease or increase both in motility parameters and GSK3 α serine phosphorylation. Therefore, these results encouraged us to study the statistic correlation between GSK3 α phosphorylation and some motility parameters. Table 4 demonstrates a clear and significant level of correlation between the densitometric quantification of GSK3 α serine phosphorylation and the percentage of rapid-speed spermatozoa, the VCL, VSL, and VAP velocities and WOB. However, a statistically significant correlation was not observed between the serine phosphorylation of GSK3 β and the motility parameters studied.

Discussion

This work demonstrated for the first time that both isoforms of the GSK3 protein, α and β , are expressed in spermatozoa lysates from porcine ejaculates. We performed a Western blot using two different antibodies that specifically recognize each isoform of the protein. Our data show a unique band for each isoform with a molecular mass of ~ 51 kDa for the α -isoform and ~ 47 kDa for the β -isoform. The presence of both GSK3 isoforms in spermatozoa was previously demonstrated by Somanath *et al.* (2004) in bovine epididymal spermatozoa. However, in human spermatozoa, Aquila *et al.* (2004) only detected the GSK3 β isoform. The molecular mass of both GSK3 isoforms observed in this work is in agreement with those found in the above-mentioned studies. Additionally, we used cellular lysates from several tissues of porcine: brain, spleen, and pancreas as well as from A431 cells as positive controls to confirm that both bands correspond with the described in the literature as GSK3 α and β . In these positive control samples, each antibody recognized a protein band with molecular mass similar to that found

in porcine spermatozoa, confirming the identification of GSK3 isoforms in these cells.

In order to study the plausible regulatory role of GSK3 in porcine spermatozoa, we have used different experimental approaches: (a) treatment of spermatozoa with the specific inhibitor of GSK3, alsterpaullone; (b) the phosphorylation state in a serine residue of GSK3 α and β by western blotting with phosphospecific antibodies; and (c) incubation of spermatozoa in different conditions varying media, temperature, and the presence or the absence of an analog of cAMP.

Our results clearly showed that the inhibition of GSK3 in a non-capacitating medium significantly increased the percentage of capacitated spermatozoa, reaching similar levels to those obtained after incubation in a capacitating medium in the presence or the absence of GSK3 inhibitor. These results are in agreement with the fact that the pretreatment of porcine spermatozoa in a

Figure 5 Effect of GSK3 inhibition on the percentage of acrosome-reacted porcine spermatozoa. Spermatozoa were washed and incubated in a capacitating medium (TCM) in presence of the calcium ionophore A23187 (1 μ M) and in the absence or the presence of alsterpaullone (30 μ M) for 15–30–60 min at 38 °C. Acrosomal status was evaluated by flow cytometry using a double staining with FITC-labeled peanut agglutinin (FITC-PNA) and propidium iodide (IP). For each file, 10 000 events, in duplicate, were stored and analyzed with Cell-Quest software.

Table 4 Correlation between the phosphorylation of both isoforms of glycogen synthase kinase-3 (GSK3) and the motility parameters of porcine spermatozoa.

Phosphorylation	Motility parameters				
	Rapid speed	VCL	VSL	VAP	WOB
GSK3 α	$r=0.46^*$	$r=0.62^*$	$r=0.75^+$	$r=0.70^+$	$r=0.70^+$
GSK3 β	$r=0.07$	$r=0.39$	$r=0.36$	$r=0.31$	$r=0.40$

* $P<0.05$, $^+P<0.01$. The Spearman non-parametric test was used to test the correlation among the densitometric quantification of both GSK3 isoforms and the assessment of motility parameters ($n=14$). All analyses were performed by SPSS v11.0 for MacOs X software (SPSS Inc., Chicago, IL, USA).

capacitating medium leads to a clear and significant increase in the serine phosphorylation state of GSK3 α isoform, which subsequently leads to an inhibition of its kinase activity. Initial studies described GSK3 protein as a regulator of glycogen synthesis (Cohen *et al.* 1978). Subsequent studies show that this regulation could involve the signaling pathway through PI3K-Akt/PKB (Cross *et al.* 1995). Insulin, a key regulator of glycogen metabolism, binds to a tyrosine kinase receptor that leads to the activation of PI3K and subsequently Akt/PKB. Active Akt/PKB phosphorylates GSK3 and subsequently inhibits its kinase activity, which leads to dephosphorylation and activation of glycogen synthase and subsequently to the glycogen synthesis (Cross *et al.* 1995). Thus, a plausible explanation for our observed effect of GSK3 in capacitation would be related to the control of metabolism of glycogen during this physiological process in porcine spermatozoa. In this regard, Aquila *et al.* (2005) showed an increase in the secretion of both leptin and insulin during the capacitating process in human spermatozoa. Consequently, higher levels of both hormones in the incubation medium during capacitation would contribute to the modulation of the availability of energetic substrates during this process (Aquila *et al.* 2005). In addition, Albarracin *et al.* (2004) demonstrated changes in glycogen storage induced during the capacitating process in canine spermatozoa, which reaches a maximum at 2-h incubation (Albarracin *et al.* 2004). In any case, by modulation of glycogen synthase and therefore the glycogen metabolism or by regulation of any other effector, our results show for the first time a possible regulatory role of GSK3 α in the capacitation of porcine spermatozoa, although future experiments are necessary to clarify the role of GSK3 inhibition in this relevant function of spermatozoa.

After capacitation, the subsequent physiological event of spermatozoa in the process of oocyte fertilization is the acrosome reaction, which seems to be regulated by different signaling pathways (Luconi *et al.* 1998, Baldi *et al.* 2002). In this regard, the acrosome reaction of mammalian spermatozoa is defined as an exocytotic process, which is mainly regulated by the calcium ion (Publicover & Barratt 1999, Darszon *et al.* 2001). Our results in porcine spermatozoa experimentally induced to acrosome reaction showed that GSK3 inhibition did not modify at all the percentage of spermatozoa that undergo acrosome reaction after 1-h

incubation. Then, our data would suggest that GSK3 activity is not involved in the regulation of the acrosome reaction process in porcine spermatozoa.

Regarding another physiological function of spermatozoa, cellular motility, Somanath *et al.* (2004) showed that GSK3 activity is significantly lower in motile spermatozoa from the epididymal tail than in those immotile obtained from the epididymal head. In addition, it has been demonstrated that the stimulation of spermatozoa motility with calyculin A, an inhibitor of serine/threonine phosphatases (Smith *et al.* 1996), induces a dose-dependent increase in both the serine phosphorylation of GSK3 and the motility of bovine epididymal spermatozoa (Somanath *et al.* 2004). On the contrary, the inhibition of spermatozoa motility with sHT31 results in a decrease in serine phosphorylation of GSK3, which demonstrates a relationship between the inhibition of GSK3 by serine phosphorylation and an increase in the motility of bovine epididymal spermatozoa (Somanath *et al.* 2004). Our results are in accordance with this relationship, as we have observed a clear involvement of GSK3 in the regulation of sperm motility: the inhibition of GSK3 produced a significant increase in the motility of porcine spermatozoa that was comparable to that obtained when they were incubated with either a cAMP analog or in a capacitating medium.

Both GSK3 isoforms present a high structural similarity, although recent works in somatic cells postulate different GSK3 functions that are differentially regulated for each one of the GSK3 isoforms (Goode *et al.* 1992, Hoeflich *et al.* 2000). For instance, GSK3 β is more efficient in the phosphorylation of the inhibitor 2, regulatory subunit of PP1 (protein phosphatase 1), than GSK3 α (Wang *et al.* 1994). Goode *et al.* (1992) demonstrate that GSK3 β , but not GSK3 α , mediates the transcription factor activator protein 1 activation by PKC (Goode *et al.* 1992). In addition, it has been shown that the disruption of the mouse GSK3 β gene leads to embryonic death and in this situation GSK3 α is not able to reverse the effect (Hoeflich *et al.* 2000).

Our work showed a significant correlation between the serine phosphorylation levels of GSK3 α and the motility of porcine spermatozoa. An increase in the temperature or the incubation of spermatozoa with either a cAMP analog or a capacitating medium produced a clear increase in the different velocities

and coefficients that define the motility of porcine spermatozoa. This increase in the motility parameters was statistically correlated with a significant increase in serine phosphorylation of GSK3 α . However, there was no correlation between serine phosphorylation of GSK3 β and porcine spermatozoa motility. Previously, a study pointed to GSK3 α as the isoform involved in the regulation of bovine epididymal spermatozoa motility, although the potential involvement of GSK3 β was not analyzed (Vijayaraghavan *et al.* 2000). In a later study performed in spermatozoa from the same species (Somanath *et al.* 2004), it is postulated that both GSK3 isoforms are involved in the regulation of spermatozoa motility, which disagrees with our results. The differences found with results from Vijayaraghavan *et al.* (2000) could be explained by the existence of differences between species or by the fact that these authors use epididymal spermatozoa, whereas in this work we have used ejaculated spermatozoa.

How GSK3 α regulates spermatozoa motility or how the phosphorylation/dephosphorylation cycle and the subsequent activation/inactivation cycle of GSK3 are regulated are the questions open to be elucidated in the future. However, regarding the first question, we propose the following hypothesis based on different studies performed in somatic and germinal cells. It has been described that Tctex-2 is a protein directly related with the motile structure of spermatozoa, specifically with the outer arms of dynein, and when activated by phosphorylation results in an increase in sperm motility (Inaba *et al.* 1999, Itoh *et al.* 2003). This protein is dephosphorylated by action of the PP2A phosphatase, which has also been identified in spermatozoa (Smith *et al.* 1996). This phosphatase becomes activated by phosphorylation, and it has been described as a direct substrate of GSK3 (Aitken *et al.* 1984). Thus, we can propose a plausible model of GSK3 action in spermatozoa motility in which this kinase would phosphorylate and activate the phosphatase PP2A that in turn would dephosphorylate and inactivate protein Tctex2. The subsequent Tctex2 inactivation would lead to the inhibition of the spermatozoa motility. It is important to mention that we cannot rule out other plausible explanations for the inhibitory effect of GSK3 on the spermatozoa motility or other intracellular mediators involved in the regulatory action of GSK3 in these cells.

In summary, we have shown that both α and β GSK3 isoforms are present in porcine spermatozoa and are phosphorylated on Ser21 and Ser9 respectively in response to PKA activation on porcine spermatozoa. Moreover, treatments that induced a significant increase or decrease in porcine sperm velocity parameters produced, in parallel, a clear increase or decrease in serine phosphorylation of GSK3 α but not in the isoform GSK3 β . Therefore, our results demonstrated a significant positive correlation between GSK3 α inactivation by serine phosphorylation and increases in the percentage

of rapid-speed sperm and in the velocities VCL, VSL, and VAP in porcine spermatozoa. In addition, our data supported a negative role of GSK3 in the regulation of porcine sperm motility, since its enzymatic blockade by alsterpaullone, a specific inhibitor of GSK3, produced a significant increase in the percentage of rapid- and medium-speed spermatozoa and in the straight-line velocity, circular velocity, and average velocity. Our results also showed that the pretreatment of porcine spermatozoa with alsterpaullone resulted in a significant increase in the percentage of capacitated spermatozoa with no effect on the percentage of acrosome-reacted spermatozoa. Finally, our results suggest a relevant function of GSK3 in the regulation of porcine spermatozoa physiology and point out GSK3 α as the isoform involved in the control of sperm motility.

Acknowledgements

Inés M Aparicio and Gonzalez-Fernandez L were supported by PhD fellowships from Consejería de Educación, Ciencia y Tecnología, Junta de Extremadura, Spain, and Garcia-Herreros M was supported by a PhD fellowship from Departamento de Educación, Universidades e Investigación del Gobierno Vasco, Spain. This work received financial support from Ministerio de Educación y Ciencia, Spain, AGL 2005-01205 and RZ2004-00012. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

- Aitken A, Holmes CF, Campbell DG, Resink TJ, Cohen P, Leung CT & Williams DH 1984 Amino acid sequence at the site on protein phosphatase inhibitor-2, phosphorylated by glycogen synthase kinase-3. *Biochimica et Biophysica Acta* **790** 288–291.
- Albarracín JL, Fernandez-Novell JM, Ballester J, Rauch MC, Quintero-Moreno A, Pena A, Mogas T, Rigau T, Yanez A, Guinovart JJ, Slebe JC, Concha II & Rodríguez-Gil JE 2004 Gluconeogenesis-linked glycogen metabolism is important in the achievement of *in vitro* capacitation of dog spermatozoa in a medium without glucose. *Biology of Reproduction* **71** 1437–1445.
- Aparicio IM, García-Marin LJ, Andreolotti AG, Bodega G, Jensen RT & Bragado MJ 2003 Hepatocyte growth factor activates several transduction pathways in rat pancreatic acini. *Biochimica et Biophysica Acta* **1643** 37–46.
- Aparicio IM, Gil MC, García-Herreros M, Pena FJ & García-Marin LJ 2005 Inhibition of phosphatidylinositol 3-kinase modifies porcine sperm motion parameters. *Reproduction* **129** 283–289.
- Aquila S, Sisci D, Gentile M, Middea E, Catalano S, Carpio A, Rago V & Ando S 2004 Estrogen receptor (ER) α and ER β are both expressed in human ejaculated spermatozoa: evidence of their direct interaction with phosphatidylinositol-3-OH kinase/Akt pathway. *Journal of Clinical Endocrinology and Metabolism* **89** 1443–1451.
- Aquila S, Gentile M, Middea E, Catalano S, Morelli C, Pezzi V & Ando S 2005 Leptin secretion by human ejaculated spermatozoa. *Journal of Clinical Endocrinology and Metabolism* **90** 4753–4761.
- Baldi E, Luconi M, Bonaccorsi L, Muratori M & Forti G 2000 Intracellular events and signaling pathways involved in sperm acquisition of fertilizing capacity and acrosome reaction. *Frontiers in Bioscience* **5** E110–E123.

- Baldi E, Luconi M, Bonaccorsi L & Forti G 2002 Signal transduction pathways in human spermatozoa. *Journal of Reproductive Immunology* **53** 121–131.
- Breitbart H 2002 Role and regulation of intracellular calcium in acrosomal exocytosis. *Journal of Reproductive Immunology* **53** 151–159.
- Breitbart H & Spungin B 1997 The biochemistry of the acrosome reaction. *Molecular Human Reproduction* **3** 195–202.
- Brewis IA, Moore HD, Fraser LR, Holt WV, Baldi E, Luconi M, Gadella BM, Ford WC & Harrison RA 2005 Molecular mechanisms during sperm capacitation. *Human Fertility* **8** 253–261.
- Choi YH & Toyoda Y 1998 Cyclodextrin removes cholesterol from mouse sperm and induces capacitation in a protein-free medium. *Biology of Reproduction* **59** 1328–1333.
- Cohen P, Nimmo HG & Proud CG 1978 How does insulin stimulate glycogen synthesis? *Biochemical Society Symposium* **43** 69–95.
- Cross DA, Alessi DR, Cohen P, Andjelkovich M & Hemmings BA 1995 Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* **378** 785–789.
- Darszon A, Beltran C, Felix R, Nishigaki T & Trevino CL 2001 Ion transport in sperm signaling. *Developmental Biology* **240** 1–14.
- Flesch FM, Colenbrander B, van Golde LM & Gadella BM 1999 Capacitation induces tyrosine phosphorylation of proteins in the porcine sperm plasma membrane. *Biochemical and Biophysical Research Communications* **262** 787–792.
- Flesch FM, Brouwers JF, Nievelstein PF, Verkleij AJ, van Golde LM, Colenbrander B & Gadella BM 2001 Bicarbonate stimulated phospholipid scrambling induces cholesterol redistribution and enables cholesterol depletion in the sperm plasma membrane. *Journal of Cell Science* **114** 3543–3555.
- Frame S & Cohen P 2001 GSK3 takes centre stage more than 20 years after its discovery. *Biochemical Journal* **359** 1–16.
- Frame S, Cohen P & Biondi RM 2001 A common phosphate binding site explains the unique substrate specificity of GSK3 and its inactivation by phosphorylation. *Molecular Cell* **7** 1321–1327.
- Go KJ & Wolf DP 1985 Albumin-mediated changes in sperm sterol content during capacitation. *Biology of Reproduction* **32** 145–153.
- Goode N, Hughes K, Woodgett JR & Parker PJ 1992 Differential regulation of glycogen synthase kinase-3 beta by protein kinase C isoforms. *Journal of Biological Chemistry* **267** 16878–16882.
- Hoeflich KP, Luo J, Rubie EA, Tsao MS, Jin O & Woodgett JR 2000 Requirement for glycogen synthase kinase-3 β in cell survival and NF- κ B activation. *Nature* **406** 86–90.
- Holt WV & Harrison RA 2002 Bicarbonate stimulation of porcine sperm motility via a protein kinase A-dependent pathway: between-cell and between-ejaculate differences are not due to deficiencies in protein kinase A activation. *Journal of Andrology* **23** 557–565.
- Inaba K, Kagami O & Ogawa K 1999 Tctex2-related outer arm dynein light chain is phosphorylated at activation of sperm motility. *Biochemical and Biophysical Research Communications* **256** 177–183.
- Itoh A, Inaba K, Ohtake H, Fujinoki M & Morisawa M 2003 Characterization of a cAMP-dependent protein kinase catalytic subunit from rainbow trout spermatozoa. *Biochemical and Biophysical Research Communications* **305** 855–861.
- Luconi M, Krausz C, Barni T, Vannelli GB, Forti G & Baldi E 1998 Progesterone stimulates p42 extracellular signal-regulated kinase (p42erk) in human spermatozoa. *Molecular Human Reproduction* **4** 251–258.
- Publicover SJ & Barratt CL 1999 Voltage-operated Ca²⁺ channels and the acrosome reaction: which channels are present and what do they do? *Human Reproduction* **14** 873–879.
- Si Y & Okuno M 1999 Role of tyrosine phosphorylation of flagellar proteins in hamster sperm hyperactivation. *Biology of Reproduction* **61** 240–246.
- Smith GD, Wolf DP, Trautman KC, da Cruz e Silva EF, Greengard P & Vijayaraghavan S 1996 Primate sperm contain protein phosphatase 1, a biochemical mediator of motility. *Biology of Reproduction* **54** 719–727.
- Somanath PR, Jack SL & Vijayaraghavan S 2004 Changes in sperm glycogen synthase kinase-3 serine phosphorylation and activity accompany motility initiation and stimulation. *Journal of Andrology* **25** 605–617.
- Spungin B, Margalit I & Breitbart H 1995 A 70 kDa protein is transferred from the outer acrosomal to the plasma membrane during capacitation. *FEBS Letters* **357** 98–102.
- Tardif S, Dube C, Chevalier S & Bailey JL 2001 Capacitation is associated with tyrosine phosphorylation and tyrosine kinase-like activity of pig sperm proteins. *Biology of Reproduction* **65** 784–792.
- Tardif S, Lefievre L, Gagnon C & Bailey JL 2004 Implication of cAMP during porcine sperm capacitation and protein tyrosine phosphorylation. *Molecular Reproduction and Development* **69** 428–435.
- Vijayaraghavan S, Stephens DT, Trautman K, Smith GD, Khatra B, da Cruz e Silva EF & Greengard P 1996 Sperm motility development in the epididymis is associated with decreased glycogen synthase kinase-3 and protein phosphatase 1 activity. *Biology of Reproduction* **54** 709–718.
- Vijayaraghavan S, Trautman KD, Goueli SA & Carr DW 1997 A tyrosine-phosphorylated 55-kilodalton motility-associated bovine sperm protein is regulated by cyclic adenosine 3',5'-monophosphates and calcium. *Biology of Reproduction* **56** 1450–1457.
- Vijayaraghavan S, Mohan J, Gray H, Khatra B & Carr DW 2000 A role for phosphorylation of glycogen synthase kinase-3 α in bovine sperm motility regulation. *Biology of Reproduction* **62** 1647–1654.
- Visconti PE, Moore GD, Bailey JL, Leclerc P, Connors SA, Pan D, Olds-Clarke P & Kopf GS 1995 Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. *Development* **121** 1138–1150.
- Wang QM, Park IK, Fiol CJ, Roach PJ & Paoli-Roach AA 1994 Isoform differences in substrate recognition by glycogen synthase kinases 3 α and 3 β in the phosphorylation of phosphatase inhibitor 2. *Biochemistry* **33** 143–147.
- Wang WH, Abeydeera LR, Fraser LR & Niwa K 1995 Functional analysis using chlortetracycline fluorescence and *in vitro* fertilization of frozen-thawed ejaculated porcine spermatozoa incubated in a protein-free chemically defined medium. *Journal of Reproduction and Fertility* **104** 305–313.
- Woodgett JR 1990 Molecular cloning and expression of glycogen synthase kinase-3/factor A. *EMBO Journal* **9** 2431–2438.
- Wuttke MS, Buck J & Levin LR 2001 Bicarbonate-regulated soluble adenylyl cyclase. *Journal of the Pancreas* **2** 154–158.

Received 22 December 2006

First decision 6 February 2007

Revised manuscript received 15 March 2007

Accepted 2 May 2007