Calcium–calmodulin and pH regulate protein tyrosine phosphorylation in stallion sperm

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Abstract

The mechanisms leading to capacitation in stallion sperm are poorly understood. The objective of our study was to define factors associated with regulation of protein tyrosine phosphorylation in stallion sperm. Stallion sperm were incubated for 4 h in modified Whitten's media with or without bicarbonate, calcium, or BSA. When sperm were incubated in air at 30×10^6 /ml at initial pH 7.25, protein tyrosine phosphorylation was detected only in medium containing 25 mM bicarbonate alone; calcium and BSA inhibited phosphorylation. Surprisingly, this inhibition did not occur when sperm were incubated at 10×10^6 /ml. The final pH values after incubation at 30×10^6 and 10×10^6 sperm/ml were 7.43 ± 0.04 and 7.83 ± 0.07 (mean $\pm$ S.E.M.) respectively. Sperm were then incubated at initial pH values of 7.25, 7.90, or 8.50 in either air or 5% CO₂. Protein tyrosine phosphorylation increased with increasing final medium pH, regardless of the addition of bicarbonate or BSA. An increase in environmental pH was observed when raw semen was instilled into the uteri of estrous mares and retrieved after 30 min (from 7.47 ± 0.10 to 7.85 ± 0.08), demonstrating a potential physiological role for pH regulation of capacitation. Sperm incubated in the presence of the calmodulin (CaM) inhibitor W-7 exhibited a dose-dependent increase in protein tyrosine phosphorylation, suggesting that the inhibitory effect of calcium was CaM mediated. These results show for the first time a major regulatory role of external pH, calcium, and CaM in stallion sperm protein tyrosine phosphorylation.

Reproduction (2012) **144** 411–422

Introduction

Immediately after ejaculation, mammalian sperm are unable to fertilize oocytes. This ability is acquired in the female reproductive tract, where sperm undergo a maturational process known as capacitation (Austin 1951, Chang 1951). Capacitation is still not completely understood. In part, it involves plasma membrane reorganization and increased membrane permeability due to cholesterol loss, an increase in intracellular calcium and HCO₃[−], and activation of secondary messenger systems, including soluble adenylyl cyclase (sAC; Osheroff *et al.* 1999, Visconti *et al.* 1999, Demarco *et al.* 2003, Wennemuth *et al.* 2003, Hess *et al.* 2005). Specifically, type 10 sAC has been shown to induce protein tyrosine phosphorylation in stallion sperm (McPartlin *et al.* 2011). Increase in cAMP initiates activation of protein kinase A (PKA), which eventually results in the phosphorylation of tyrosine residues on many proteins. Protein tyrosine phosphorylation is considered to be one of the molecular hallmarks of sperm capacitation (Visconti *et al.* 1995, Galantino-Homer *et al.* 1997, Visconti & Kopf 1998,

Naz & Rajesh 2004) and has been shown to result in activation of proteins related to the acrosome reaction in boar sperm (Kalab *et al.* 1998), and in hyperactivated motility in human sperm (Nassar *et al.* 1999), *in toto* allowing the spermatozoa to fertilize the oocyte. However, the relationship between protein tyrosine phosphorylation and these processes is not clear and may be species-specific. Changes resulting in functional capacitation (ability of the sperm to fertilize oocytes) may be induced *in vitro* in many species by incubating sperm in media containing BSA as a cholesterol acceptor (Boerke *et al.* 2008), bicarbonate, and Ca²⁺ (simple capacitating media).

IVF in the horse is still largely unsuccessful, despite decades of work in this area. This appears to be due to the failure of equine sperm to penetrate the zona pellucida (Choi *et al.* 1994, Li *et al.* 1995), suggesting that effective capacitation of equine spermatozoa is not being achieved *in vitro*. Only one laboratory has reported the induction of protein tyrosine phosphorylation in equine sperm in simple capacitating medium. McPartlin *et al.* (2008, 2011) observed protein tyrosine phosphorylation when stallion sperm were incubated in

modified Whitten's (MW) medium. The phosphorylation increased over time to a maximum after 4 h incubation. The 'defined incubation conditions' (presence of BSA, HCO_3^- , and Ca^{2+}) described by these authors as necessary for induction of protein tyrosine phosphorylation in stallion sperm present a puzzle, as these conditions have been met in many previous apparently unsuccessful attempts at equine sperm capacitation. Specifically, Pommer *et al.* (2003) and Thomas *et al.* (2006) did not find a meaningful increase in protein tyrosine phosphorylation in stallion sperm unless the phosphodiesterase (PDE) inhibitors caffeine and 3-isobutyl-1-methylxanthine or the PKA activator cAMP were added to simple capacitating medium. Similarly, Baumber *et al.* (2003) described an increase in protein tyrosine phosphorylation in equine sperm when exposed to reactive oxygen species. One major difference between these latter studies and those of McPartlin *et al.* (2008, 2009) was that the McPartlin group incubated the sperm, which was in medium containing 25 mM bicarbonate, in air rather than in CO_2 . Incubation of bicarbonate-containing medium in air would result in gassing-off of CO_2 from the medium, thus driving use of H^+ ions ($\text{H}^+ + \text{HCO}_3^- \leftrightarrow \text{CO}_2 + \text{H}_2\text{O}$), resulting in an increase in medium pH via the equation $\text{pH} = 6.1 + \log ([\text{HCO}_3^-]/(0.03 \times \text{pCO}_2))$. Increasing medium pH has been shown in other species to increase protein tyrosine phosphorylation (Galantino-Homer *et al.* 2004).

To the best of our knowledge, no additional information on successful induction of protein tyrosine phosphorylation in equine sperm is available. The paucity of information in this area, especially as 4 years have passed since the successful report of McPartlin *et al.* (2008), indicates that more research is needed to identify factors affecting capacitation of equine sperm. This study was performed to define factors modulating protein tyrosine phosphorylation in stallion sperm.

Results

Experiment 1: effect of calcium, bicarbonate, and BSA on protein tyrosine phosphorylation

Stallion sperm were processed ($n=3$, one ejaculate per stallion) and the final pellet was diluted to 30×10^6 sperm/ml in 1 ml MW media at pH 7.25, with or without Ca^{2+} , bicarbonate, and BSA, and incubated in air at 37°C for 4 h. For western blotting assays, 15 μg protein were loaded into each well; film exposure length was 5 min. Significant protein tyrosine phosphorylation was found only in medium containing 25 mM bicarbonate alone (MW+Bic). Addition of Ca^{2+} or BSA, or Ca^{2+} and BSA together, inhibited the phosphorylation seen in MW+Bic (Fig. 1). At the end of the 4-h incubation period, the pH of the four media containing bicarbonate

Figure 1 Effect of combinations of calcium, bicarbonate, and BSA on protein tyrosine phosphorylation in stallion sperm incubated at $30 \times 10^6/\text{ml}$ at initial pH 7.25 for 4 h at 37°C in air. The immunoblot is representative of three independent experiments (three stallions and one ejaculate per stallion).

(mean $\pm$ s.e.m. of all three replicates) was 7.41 ± 0.01 ; the pH of all media lacking bicarbonate decreased to pH 7.03 ± 0.01 .

Experiment 2: effect of BSA preparation on protein tyrosine phosphorylation

To determine whether the unexpected results of Experiment 1 (the suppression of protein tyrosine phosphorylation by BSA) might be related to the specific BSA used, stallion sperm at $30 \times 10^6/\text{ml}$ in a volume of 1 ml were incubated with six different BSA preparations (A0281, A7030, A8806, A6003, A2153, and A3803) at 7 mg/ml in the presence or absence of 25 mM bicarbonate in MW without Ca^{2+} at initial pH 7.25 for 4 h at 37°C in air ($n=3$, one ejaculate per stallion). Lysates containing 15 μg protein were loaded into each well; films were exposed for 5 min. Protein tyrosine phosphorylation was seen in sperm incubated in MW+Bic and to a lesser extent in sperm incubated in MW alone (Fig. 2). All BSA preparations tested inhibited the protein tyrosine phosphorylation seen in the non-BSA-containing medium.

Experiment 3: effect of calcium concentrations on protein tyrosine phosphorylation

To determine whether the protein tyrosine phosphorylation inhibition observed in the presence of calcium in Experiment 1 was dose-dependent, sperm were incubated at $30 \times 10^6/\text{ml}$ in 1 ml MW+Bic at initial pH 7.25 with 2, 1, 0.5, 0.2, 0.1, 0.02, or 0 mM added Ca^{2+} or 0 mM Ca^{2+} + 2 mM EGTA. Lysates containing 15 μg

Figure 2 Effect of six different preparations of BSA on protein tyrosine phosphorylation in stallion sperm incubated in MW without Ca^{2+} in the presence or in the absence of 25 mM bicarbonate at $30 \times 10^6/\text{ml}$ at initial pH 7.25 for 4 h at 37°C in air. Immunoblot shown is representative of three independent experiments (three stallions, one ejaculate per stallion).

protein were loaded into each well; films were exposed for 5 min ($n=3$, one ejaculate per stallion). Addition of Ca^{2+} inhibited protein tyrosine phosphorylation in a dose-dependent manner; slight phosphorylation was seen at the lowest dose (0.02 mM), dependent upon stallion (Fig. 3). Incubation with 0 mM Ca^{2+} + EGTA increased protein tyrosine phosphorylation 18 times (average of data for all three stallions) over that for 0 mM Ca^{2+} without EGTA.

Experiment 4: effect of BSA in the presence of EGTA on protein tyrosine phosphorylation

The results of Experiments 1 and 2 indicated an inhibitory effect of BSA on protein tyrosine phosphorylation. The present experiment was conducted to determine whether this inhibitory effect was due to calcium bound to the BSA. Sperm were incubated at 30×10^6 sperm/ml in 1 ml MW+Bic with BSA and/or EGTA at initial pH 7.25 for 4 h at 37°C in air ($n=3$, one ejaculate per stallion). The results showed that 2 mM EGTA in the presence of BSA partially recovered protein tyrosine phosphorylation (Fig. 4); however, even in the presence of EGTA, BSA significantly inhibited protein tyrosine phosphorylation compared with non-BSA-containing medium, indicating that the major suppressive effect of BSA was not calcium related.

Experiment 5: effect of sperm concentration during incubation on protein tyrosine phosphorylation

The results of Experiments 1, 2, 3 and 4, showing inhibition of protein tyrosine phosphorylation in the presence of BSA and Ca^{2+} , conflicted with the results reported by McPartlin *et al.* (2008) in the same medium

at the same initial pH. On review of that report, one apparent methodological difference was that the sperm concentration used during incubation by McPartlin *et al.* (2008) was 10×10^6 sperm/ml in a final volume of 500 μl , whereas in Experiments 1, 2, 3 and 4 of this study we used 30×10^6 sperm/ml in 1 ml. This sperm concentration is used in our laboratory to enable optimum motility evaluation via computer-assisted sperm motility analysis (CASMA). To test whether sperm concentration during incubation could affect protein tyrosine phosphorylation, sperm were processed and diluted to a final concentration of 10×10^6 or 30×10^6 sperm/ml, then aliquots of 500 μl or 1 ml respectively were incubated in MW+Ca or in MW+++ at initial pH 7.25 for 4 h at 37°C in air. Owing to the lower sperm concentration incubated, 5 μg protein/well was used for western blotting, as this was the maximum repeatedly available in all treatments. Membranes were exposed for 5 min.

When sperm were incubated at $30 \times 10^6/\text{ml}$, protein tyrosine phosphorylation was not obtained in either MW+Ca or in MW+++ , consistent with the results of

Figure 3 Effect of calcium concentration on protein tyrosine phosphorylation in equine sperm. Sperm were incubated in MW+Bic (25 mM HCO_3^-) at $30 \times 10^6/\text{ml}$ at initial pH 7.25 for 4 h at 37°C in air. The immunoblot shown is representative of three independent experiments (three stallions and one ejaculate per stallion).

Figure 4 Effect of BSA in the presence of 2 mM EGTA on protein tyrosine phosphorylation in equine sperm. Sperm were incubated in MW+Bic (25 mM HCO_3^-) at 30×10^6 /ml at initial pH 7.25 for 4 h at 37 °C in air. The immunoblot shown is representative of three independent experiments (three stallions and one ejaculate per stallion).

Experiments 1, 2, 3 and 4 (Fig. 5). However, when sperm were incubated at 10×10^6 sperm/ml, those incubated in MW+++ demonstrated an increase in protein tyrosine phosphorylation compared with both that for the 30×10^6 sperm/ml treatments and that achieved for the 10×10^6 sperm/ml treatment in MW+Ca (Fig. 5). The pH of the samples was determined after 4 h incubation. In MW+++, the final pH values were 7.83 ± 0.07 and 7.43 ± 0.04 (mean $\pm$ S.E.M.) for samples incubated at 10×10^6 and 30×10^6 sperm/ml respectively; this difference was significant ($P < 0.05$). In MW+Ca, the final pH values were 7.12 ± 0.05 and 7.06 ± 0.02 for samples incubated at 10×10^6 and 30×10^6 sperm/ml respectively ($P < 0.05$).

Experiment 6: effect of medium pH on protein tyrosine phosphorylation

To determine whether the stimulatory effect of low sperm concentration found in Experiment 5 was related to the increase in pH observed during incubation, we studied the role of medium pH on protein tyrosine phosphorylation. We attempted to evaluate both initial high pH and a gradual rise in pH during incubation, as occurred in the low sperm concentration samples in Experiment 5. Processed sperm were diluted to 10×10^6 /ml (500 μ l) or 30×10^6 /ml (1 ml) in MW+Ca or in MW+++ that were adjusted to pH 7.25, 7.9, or 8.5 using 1 M NaOH. The samples were incubated at 37 °C in either air or in 5% CO_2 for 4 h. Incubation in CO_2 was performed to maintain the pH of bicarbonate-containing media near initial values.

Incubation in high-pH media induced protein tyrosine phosphorylation in sperm in both MW+Ca and MW+++ (Fig. 6). The absolute amount of protein tyrosine phosphorylation in response to different incubation conditions varied among the three stallions evaluated; however, an increase in protein tyrosine phosphorylation in relation to increasing pH, regardless of medium composition, was consistent. For ease of comparison, total band density in each treatment was calculated as a fold increase relative to that for MW+Ca at 7.25 in air for that stallion; sperm in this treatment remained at close to initial pH values after the 4-h incubation.

Results from Stallion SP (Fig. 6A) are presented as representative. For this stallion, band density increased 10- and 48-fold for MW+Ca at initial pH 7.9 and 8.5 respectively (lanes 4 and 7; all fold values are in comparison to MW+Ca at initial pH 7.25 (lane 1)). A similar pH-dependent increase in protein tyrosine phosphorylation was seen in sperm incubated in MW+++ in air, the fold increase being 20, 40, and 59 at initial pH 7.25, 7.9, and 8.5 respectively (lanes 3, 6 and 8); final pH values for these treatments were 8.0, 8.4, and 8.9 respectively. Incubation in MW+++ in 5% CO_2 atmosphere inhibited the rise in pH and inhibited protein tyrosine phosphorylation compared with that

Figure 5 Protein tyrosine phosphorylation in stallion sperm incubated at 10×10^6 or 30×10^6 /ml at initial pH 7.25 for 4 h at 37 °C in air in different media. The immunoblot shown is representative of three independent experiments (three stallions and one ejaculate per stallion); pH shown is that measured for the replicate pictured.

Figure 6 Effect of medium pH and composition on protein tyrosine phosphorylation in three different stallions (A, SP; B, AC; and C, RK). Sperm were incubated at 10×10^6 /ml for 4 h at 37 °C in air or in 5% CO₂ at the initial pH values given. Densitometric analysis (fold increase in arbitrary units compared to treatment 1) for all tyrosine-phosphorylated bands is reported under each lane.

achieved in MW+++ in air, the fold increase being 15 and 17 for initial pH 7.25 and 7.9 respectively (final pH values 7.6 and 7.7 respectively). Because initial pH 8.5 media could not be held at a stable pH in 5% CO₂, this treatment was not included. The increase in protein tyrosine phosphorylation appeared to be dependent on final medium pH, regardless of addition of BSA or bicarbonate to the medium. This may be seen in Fig. 6A by comparison of lanes 6 and 7, with density increases of 40- and 48-fold respectively for MW+++ and MW+Ca reaching pH 8.4 and 8.3 respectively. Similarly, equivalent increases in protein tyrosine phosphorylation were seen regardless of medium used in the four treatments having final pH values of 7.6–8.0 (lanes 2, 3, 4 and 5).

Viability and motility of sperm in Experiment 6 are presented in Fig. 7. Spermatozoal viability, as assessed by staining with propidium iodide, was not significantly different among MW+Ca samples at the different pH values ($P > 0.1$; Fig. 7A). Similarly, the viability of the samples incubated in MW+++ did not differ significantly among the different pH media ($P > 0.1$). Incubation in the presence of BSA and bicarbonate (MW+++), and in CO₂ vs air, appeared to be associated with lower viability; however, this difference was not significant ($P > 0.1$). There was no difference in the percentage of total motile sperm (total motility) among treatments ($P > 0.1$; Fig. 7B). No treatment was associated with hyperactivation-related motility parameters as used in our laboratory (ALH $> 12 \mu\text{m}$, VCL $> 300 \mu\text{m/s}$; Loux *et al.* 2010).

Experiment 7: effect of W-7, an inhibitor of calmodulin on protein tyrosine phosphorylation

Review of the final pH values of media in Experiment 1 demonstrated that medium pH did not account for the inhibiting effect of calcium on protein tyrosine phosphorylation. To evaluate a possible mechanism by which calcium inhibits protein tyrosine phosphorylation, we evaluated the effect of inhibition of the calcium-binding protein, calmodulin (CaM) as CaM regulates many calcium-dependent enzymes, notably, the protein phosphatase calcineurin (Crivici & Ikura 1995, Osawa *et al.* 1998). Sperm were incubated in MW+Bic (without calcium) with 2 mM EGTA or in MW+++ at initial pH 7.25 in air in the presence of different concentrations (10, 50, 100, 200, and 300 μM) of W-7, a CaM inhibitor. To study the role of pH in this inhibition, sperm were incubated at 10×10^6 (500 μl) or 30×10^6 /ml (1 ml), to induce differences in final pH independent of medium composition. For western blotting assays, 5 μg protein were loaded into each well; film exposure length was 5 min. Total band density in each treatment was calculated as a fold increase relative to that for MW+++ (Fig. 8; lane 2).

Sperm concentration affected final medium pH (Fig. 8) with the 10×10^6 treatments averaging 7.89 ± 0.01 (mean of all 10×10^6 treatments, all three replicates) and the 30×10^6 treatments averaging pH 7.51 ± 0.01 . As found in Experiment 3, there was an increase in protein tyrosine phosphorylation in MW+Bic with EGTA; this increase was greater for the 10×10^6 than for

Figure 7 Effect of medium pH and composition on viability and total motility. Sperm were incubated at 10×10^6 /ml for 4 h at 37 °C in air or in 5% CO₂ at different pH. Viability values are the mean $\pm$ S.E.M. of three ejaculates (three stallions and one ejaculate per stallion), and motility values are the mean $\pm$ S.E.M. of five ejaculates.

the 30×10^6 sperm/ml incubation. Incubation in MW+++ in the presence of increasing concentrations of W-7 demonstrated a dose-dependent increase in protein tyrosine phosphorylation for both sperm concentrations (Fig. 8); this increase was greater for the 10×10^6 treatment than for the 30×10^6 treatment (lanes 5 and 6 vs 11 and 12 respectively). For sperm incubated at 10×10^6 /ml, W-7 at 300 μ M yielded equivalent protein tyrosine phosphorylation to that found with incubation in 2 mM EGTA-containing medium. However, for sperm incubated at 30×10^6 /ml, treatment with 300 μ M W-7 did not restore protein tyrosine phosphorylation to levels achieved with EGTA (Fig. 8). Higher concentrations of W-7 appeared to be toxic, resulting in loss of phosphorylation (data not shown).

Experiment 8: pH and calcium concentration of semen pre- and post-insemination

To evaluate whether the pH and calcium concentrations associated with protein tyrosine phosphorylation of stallion sperm *in vitro* in Experiment 6 were physiologically relevant, extracellular pH and calcium concentrations were determined for raw semen pre- and post-insemination into the uteri of estrous mares. The results for the four ejaculates evaluated are presented in Table 1. There was an increase of 0.26–0.61 pH units for each ejaculate after insemination; the post-insemination pH ranged from 7.70 to 8.06 and this was significantly different from the pH of the semen before insemination ($P < 0.01$). There was a minor but significant increase in calcium concentration after insemination, from 2.20 ± 0.40 to 2.70 ± 0.50 mM ($P < 0.05$).

Discussion

These results demonstrate for the first time that protein tyrosine phosphorylation in stallion sperm is modulated extensively by external pH and that, in simple capacitating medium, these sperm do not show a marked increase in protein tyrosine phosphorylation until the environmental pH approaches or exceeds 8. An increase in intracellular pH is held to be a basic requirement for capacitation (Parrish *et al.* 1989, Uguz *et al.* 1994, Vredenburg-Wilberg & Parrish 1995). Gradual rise in external pH (Galantino-Homer *et al.* 2004), but not elevated initial pH (Parrish *et al.* 1989) or 'pushbutton' intracellular alkalinization (Galantino-Homer *et al.* 2004), has previously been shown to increase capacitation (induced acrosome reaction and protein tyrosine phosphorylation) in bovine sperm. This pH-related increase in protein tyrosine phosphorylation is thought to be related to an increase in intracellular bicarbonate input, through $\text{Cl}^-/\text{HCO}_3^-$ (Wertheimer *et al.* 2008) or $\text{Na}^+/\text{HCO}_3^-$ cotransporters (Demarco *et al.* 2003), that activates SAC. The effect of pH in stallion sperm appears to be more intense than that seen in other species, as we found that protein tyrosine phosphorylation was effectively eliminated at lower pH. The pH requirement of equine sperm evidenced *in vitro* may be physiological, as the pH of uterine fluid after insemination, within the time that sperm destined for fertilization are still intrauterine (Brinsko *et al.* 1991) ranged from 7.7 to 8.0 (Table 1).

Incubation conditions had surprisingly profound effects on protein tyrosine phosphorylation in equine sperm. In MW+++ medium with an initial pH 7.25, phosphorylation was observed when incubation in air was performed at 10×10^6 sperm/ml but not at 30×10^6 sperm/ml (Fig. 5). This was related to acidification of the medium at the higher sperm concentration, presumably from an increase in sperm metabolic products. Acidification of the external milieu has previously been

Figure 8 Effect of different concentrations of an inhibitor of calmodulin, W-7, on protein tyrosine phosphorylation in equine sperm. Sperm were incubated in MW + + +, or in MW + Bic with 2 mM EGTA, at 10×10^6 /ml or 30×10^6 /ml for 4 h at 37 °C in air at initial pH 7.25. Densitometric analysis (fold increase in arbitrary units compared to treatment 2) for all tyrosine-phosphorylated bands is reported under each lane. The immunoblot shown is representative of three independent experiments (three stallions and one ejaculate per stallion).

described when bovine sperm are incubated in the presence of glucose, which, on glycolysis, leads to lactic acid production, a decrease in intracellular pH, and decrease in external medium pH associated with proton excretion (Parrish *et al.* 1989, Galantino-Homer *et al.* 2004). The decrease in intracellular pH is thought to be associated with a decrease in internal bicarbonate concentration and thus a reduction in the activity of sAC and the amount of cAMP generated, resulting in inhibition of protein tyrosine phosphorylation via reduction in PKA activity (Galantino-Homer *et al.* 2004).

In our study, the acidification of medium associated with the higher sperm concentration (30×10^6 /ml in 1 ml medium) prevented the rise in pH induced by gassing-off of bicarbonate under incubation in air. This was associated with the lack of protein tyrosine phosphorylation seen initially in Experiments 1, 2, 3 and 4. When in Experiment 5, sperm were prepared under the exact conditions described by McPartlin *et al.* (2008, 2009), i.e. 10×10^6 /ml in 500 μl, the lower sperm concentration, and perhaps the higher surface-area-to-medium ratio associated with the lesser volume, were associated with a higher final pH, and with greatly increased protein

tyrosine phosphorylation (Fig. 5). Subsequently, we found that incubation of sperm, at either concentration, in medium at higher initial pH was associated with greater protein tyrosine phosphorylation. Similarly, when medium contained 25 mM bicarbonate, incubation in air was associated with greater protein tyrosine phosphorylation than in the same medium incubated under 5% CO₂ (Fig. 6). Thus, in our studies, we examined three different mechanisms for varying pH: A) varying sperm concentration/volume, B) varying initial pH of the medium, and C) incubation of medium under 5% CO₂ vs under air. Protein tyrosine phosphorylation increased with increasing pH under each of these conditions, regardless of medium composition (addition of BSA or bicarbonate).

These data appear to explain much of the discrepancy between the findings of McPartlin *et al.* (2008) and those of Baumber *et al.* (2003), Pommer *et al.* (2003), and Thomas *et al.* (2006). While all these studies used media containing calcium, BSA, and bicarbonate, in the former study, sperm were incubated at 10×10^6 /ml in air, whereas in the latter studies, sperm were incubated in a 5% CO₂ atmosphere; Pommer *et al.* (2003) and

Table 1 pH and Ca²⁺ concentration values of equine raw semen before insemination and of uterine fluid 15–30 min after insemination.

Ejaculate	Stallion	pH pre-insemination	pH post-insemination	[Ca ²⁺] pre-insemination (mM)	[Ca ²⁺] post-insemination (mM)
1	SP	7.40	7.70	0.97	1.2
2	SP	7.71	8.06	2.9	3.6
3	AC	7.50	7.76	2.5	3.2
4	CL	7.25	7.88	2.5	2.8
Mean ± S.E.M.		7.47 ± 0.1*	7.85 ± 0.08 [†]	2.2 ± 0.4 [‡]	2.7 ± 0.5 [§]

*[†]P < 0.01; [‡]P < 0.05.

Thomas *et al.* (2006) also used a higher sperm concentration ($20 \times 10^6/\text{ml}$).

In our studies, remarkably, as noted earlier, the response of sperm to medium pH was independent of the addition of BSA and bicarbonate to the medium (Fig. 6). Protein tyrosine phosphorylation in the absence of BSA or other cholesterol acceptor is notable, as this has been considered to be an absolute requirement for capacitation (Go & Wolf 1985, Visconti *et al.* 1999). We found that protein tyrosine phosphorylation occurred readily in medium without added bicarbonate if the pH was high or if Ca^{2+} was removed from the medium. However, this does not indicate that external bicarbonate is not required for protein tyrosine phosphorylation in stallion spermatozoa, as HCO_3^- forms in medium due to CO_2 in surrounding air. McPartlin *et al.* (2008) found that in $\text{MW} + \text{Ca} + \text{BSA}$, incubation of stallion sperm under 100% N_2 appeared to decrease the amount of protein tyrosine phosphorylation compared with that obtained on incubation in air and that the sperm appeared to be responsive to as little as 1 mM bicarbonate (McPartlin *et al.* 2008). For this reason, incubation of sperm in media without bicarbonate and Ca^{2+} , or of differing pH, in an environment without CO_2 would be necessary to assess the absolute requirement for bicarbonate in equine sperm capacitation.

The effect of addition of bicarbonate is generally considered in relation to its direct effect on sAC. However, bicarbonate addition should also be considered in light of its effect on medium pH. In our study, bicarbonate-containing media increased in pH during incubation, especially under air (Fig. 6), and external pH had a major effect on protein tyrosine phosphorylation. Protein tyrosine phosphorylation induced by bicarbonate was clearly inhibited in Ca^{2+} -containing media at our initial sperm concentration of 30×10^6 sperm/ml (Experiments 1 and 3). This result appears to be in contrast with the findings of McPartlin *et al.* (2008) in which, in calcium-containing medium, both BSA and bicarbonate increased protein tyrosine phosphorylation. However, the surprisingly strong effect of sperm concentration, and thus final medium pH, on the apparent effects of these components, as shown in Experiment 5 (Fig. 5), explains this discrepancy: the final pH after incubation at $30 \times 10^6/\text{ml}$ was ~ 7.5 , whereas the final pH after incubation at $10 \times 10^6/\text{ml}$, as performed by McPartlin *et al.* (2008), was ~ 7.9 .

We hypothesized that the inhibitory effect of BSA seen at low pH could be related to the presence of calcium bound to BSA, since, as determined by atomic absorption, media containing 2.6% BSA without added calcium was shown to contain 0.1 mM Ca^{2+} (Marin-Briggiler *et al.* 2003, 2005). As seen in Experiment 3 of our study, concentrations of Ca^{2+} as low as 0.02 mM inhibited protein tyrosine phosphorylation in stallion sperm at low pH. However, addition of 2 mM EGTA to BSA-containing medium did not completely recover

protein tyrosine phosphorylation (Fig. 4). This result indicates that while some inhibition appears to be due to associated calcium, BSA also inhibits protein tyrosine phosphorylation in a non-calcium-related manner. The effect of BSA appears to be dependent on the pH of the medium, as BSA did not inhibit protein tyrosine phosphorylation at high pH (Fig. 6).

An inhibitory effect of Ca^{2+} on protein tyrosine phosphorylation has previously been described in human sperm (Luconi *et al.* 1996, Marin-Briggiler *et al.* 2003, 2005), although to a lesser extent than that seen in this study. Carrera *et al.* (1996) attributed the inhibitory effect of Ca^{2+} to stimulation of the calcium-CaM-dependent protein phosphatase 2B (calcineurin). These authors demonstrated that the inhibitory effect of calcium ionophore on protein tyrosine phosphorylation of human sperm was reversed not only by incubation with calmidazolium, an inhibitor of CaM, or with deltamethrin, an inhibitor of calcineurin but also by incubation with okadaic acid, a phosphatase inhibitor with weak anti-calcineurin activity. These authors hypothesized that modulation of correct phosphorylation/dephosphorylation via calcium-CaM-calcineurin (or other phosphatases) may be important in establishing the appropriate kinetics of protein tyrosine phosphorylation during capacitation. Leclerc *et al.* (1998) reported reversal of calcium-induced inhibition of protein tyrosine phosphorylation in human sperm using the specific CaM inhibitor, W-7, and suggested another possible mechanism, i.e. the CaM-regulated sperm cyclic nucleotide PDE. High activity of this enzyme in the presence of Ca^{2+} would maintain cAMP at low levels and thus inhibit cAMP-stimulated protein tyrosine phosphorylation. In bovine sperm, PDE activity is effectively constitutive (Galantino-Homer *et al.* 2004), so that such regulation is unlikely; however, the situation in stallion sperm is unknown. Other authors hypothesize that the inhibitory effect of external Ca^{2+} is due to the energy required to export Ca^{2+} from the cell; this increase in ATP requirement would lower the ATP available to protein tyrosine kinases (Baker *et al.* 2004). However, this explanation cannot be reconciled with our results, as our findings demonstrate that protein tyrosine phosphorylation occurs in the presence of Ca^{2+} in stallion sperm under appropriate pH conditions (Experiment 6).

The inhibitory effect of Ca^{2+} was pH dependent, as medium with calcium inhibited protein tyrosine phosphorylation at low pH but not at high pH (Fig. 5). Similarly, W-7, which blocks the action of CaM by binding to its ligand-binding site (Osawa *et al.* 1998), completely reversed the Ca^{2+} -related inhibition of protein tyrosine phosphorylation at pH 7.89 (lower sperm concentration) but did not completely reverse calcium-related inhibition at pH 7.5 (higher sperm concentration; Fig. 8). These pH-dependent differences in calcium-CaM action could be related to the reported

decrease in calcineurin phosphatase activity at alkaline pH (Pallen & Wang 1983); thus, the ability of W-7 to completely restore protein tyrosine phosphorylation in high-pH medium (30×10^6 /ml) could be due to the lesser activity of the CaM-regulated phosphatase at high pH. In addition, increasing pH may be associated with a decrease in total CaM content. A decrease in CaM levels in sperm has been reported during capacitation in the bull and monkey (Chan & Dukelow 1985, Leclerc *et al.* 1992). As capacitation is associated with an increase in intracellular pH in most species (Visconti *et al.* 2011), there may be a pH-related mechanism for regulating total amounts of CaM present within sperm. In addition, there may be additional pH-dependent controls on protein tyrosine phosphorylation that are not CaM regulated. This is supported by the finding that even in the absence of calcium (MW+Bic+2 mM EGTA treatment), protein tyrosine phosphorylation was higher in the 10×10^6 sperm/ml (pH 7.91) incubation than for the 30×10^6 sperm/ml (pH 7.55; Fig. 8).

Based on our *in vivo* findings, i.e. that sperm undergo a significant increase in environmental pH after insemination, and on the profound effect of external pH on protein tyrosine phosphorylation as shown in our *in vitro* experiments, we hypothesize that the relatively low pH of stallion semen, in association with the calcium content, prevents protein tyrosine phosphorylation of equine sperm via a calcium–CaM-mediated mechanism until they are within the uterine lumen, where increased pH allows a decrease in CaM-mediated phosphatase activity, decrease in CaM content, and/or increased activity of protein kinases. The activation of sAC by bicarbonate and the inhibition of the Ca^{2+} –CaM pathway by high pH could act in parallel, resulting in an increase in protein tyrosine phosphorylation compatible with capacitation.

In our study, preparation of sperm at 10×10^6 /ml in 500 μl , using MW + + + at initial pH of 7.9 or 8.5, with incubation in air, showed the greatest increase in protein tyrosine phosphorylation. This increase could be physiological since, as shown in Table 1, the pH in the mare uterus 15–30 min after insemination ranges from 7.70 to 8.06, and sperm viability and total motility were not affected by these incubation conditions (Fig. 7). However, more work needs to be done to clarify whether the increases in protein tyrosine phosphorylation observed in the media in our study, especially in media without BSA, are associated with other capacitation-related events such as membrane cholesterol loss or an increased capability to undergo the acrosome reaction. Incubation in MW + + + at 10×10^6 /ml in 500 μl in air at an initial pH of 7.25 was compatible with successful IVF when paired with a treatment stimulating hyperactivated motility (McPartlin *et al.* 2009). As reported by McPartlin *et al.* (2009), we did not observe a significant increase in the percentage of sperm showing hyperactivated motility parameters in any of our treatments, and

hence, the physiology of induction of hyperactivated motility in equine sperm requires further research.

To the best of our knowledge, this is the first time that the key roles of external pH, calcium, and CaM in the regulation of protein tyrosine phosphorylation of equine sperm have been reported. Because of the profound importance of external pH, future studies in this area should consider the effect on pH of all methodological aspects used, and these should be detailed in resulting reports. More work is needed to fully understand the mechanisms by which external pH regulates the overall capacitation process in stallion sperm; however, the delineation of the environmental requirements for protein tyrosine phosphorylation, as presented here, should aid greatly in progress toward development of a repeatable method for IVF in this species.

Materials and Methods

Materials

Triton X-100, BSAs, and EGTA were purchased from Sigma–Aldrich, Inc. A0281: globulin and fatty acid free, purified by a combination of heat-shock and ethanol fractionation. A7030: globulin and fatty acid free, extracted using heat-shock fractionation. A8806: fatty acid free and low endotoxin, prepared by a modified Cohn cold ethanol fractionation method. A6003: fatty acid free, prepared by a modified Cohn cold ethanol fractionation method. A2153: prepared using the Cohn cold ethanol fractionation method. A3803: fatty acid free, prepared using heat-shock fractionation and further purified to reduce fatty acid content. Acrylamide, ammonium persulfate, 2-mercaptoethanol, Tween 20, and DC Protein Assay were purchased from Bio-Rad (Hercules, CA, USA). Protease inhibitor cocktail tablets (complete EDTA-free) were obtained from Roche (Basel, Switzerland). Antiphosphotyrosine MAB (clone 4G10) and PVDF membrane were from Millipore (Billerica, MA, USA). Anti-mouse IgG HRP-conjugated secondary antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). ECL detection reagents and Hyperfilm ECL were obtained from Amersham. W-7 was purchased from Enzo Life Sciences (Farmingdale, NY, USA).

Media

The basal medium used was modified MW, which consisted of 100 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl_2 , 5.5 mM glucose (anhydrous), 22 mM HEPES, 2.4 mM sodium L-lactate, and 1.0 mM pyruvic acid. Medium with Ca^{2+} was prepared by replacing the sodium L-lactate with 2.4 mM calcium L-lactate hydrate (MW+Ca). For some media, 7 mg/ml BSA and/or 25 mM NaHCO_3 were added (MW+BSA, MW+Bic). Media containing Ca^{2+} , BSA, and bicarbonate were designated MW + + +. Bicarbonate was added 1 h before the experiment and the medium was maintained at 37 °C until the beginning of the experiment, at which time the pH was checked again and adjusted if necessary. All media were adjusted with NaCl to an osmolality of 290–300 mOsm/kg and to pH 7.25 unless otherwise indicated.

Collection and processing of semen

Semen was collected from three fertile stallions using an artificial vagina (Missouri Model AV, Nasco, Fort Atkinson, WI, USA). A nylon in-line filter (Animal Reproduction Systems, Chino, CA, USA) was used to eliminate the gel fraction. The sperm-rich fraction was diluted 2:1 (v:v) in the designated MW medium with or without added calcium, according to treatment, and held for 30 min at 37 °C. The sperm suspension was centrifuged at 100 *g* for 1 min at room temperature (22–24 °C; RT) to remove particulate matter, then the supernatant was transferred to a 15 ml conical tube, and centrifuged again at 600 *g* for 5 min at RT. The spermatozoal pellet was resuspended in 1.5 ml of the designated medium that had been warmed to 37 °C. The sperm concentration was then determined using a Nucleocounter SP-100 (Chemometec, Allerød, Denmark) and an aliquot of the suspension was diluted to achieve a final concentration of 10 million (500 µl final volume) or 30 million sperm (1 ml final volume). Aliquots of the final sperm suspension were placed in 5 ml round bottom plastic tubes from BD Falcon (San Jose, CA, USA). The tubes were incubated at 37 °C in either air or 5% CO₂ for 4 h. Each experiment was performed using one ejaculate from each of the three stallions.

Western blotting

After 4 h, the sperm were centrifuged at 5000 *g* for 3 min at RT and washed at 4 °C in PBS supplemented with the protein tyrosine phosphatase inhibitor sodium orthovanadate (0.2 mM). After centrifugation, the supernatant was removed, and the pellet was resuspended in lysis buffer consisting of 62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% Bromophenol Blue (Laemmli 1970), and a protease inhibitor cocktail (complete EDTA-free; Roche). Samples were then centrifuged at 10 000 *g* for 10 min at 4 °C and the protein concentration of the supernatant was determined using a Bio-Rad DC Protein Assay according to the manufacturer's instructions. After protein determination, 2-mercaptoethanol (2.5%) was added to the lysates before heating for 5 min at 95 °C. Aliquots of lysate containing the designated amount of protein (5 or 15 µg) were loaded in 10% polyacrylamide gels and separated according to their molecular weight by SDS-PAGE electrophoresis. The proteins were then transferred to Immobilon P PVDF membranes (Millipore). Membranes were blocked for 1 h at room temperature using 3% BSA in a Tris buffer saline-Tween 20 solution (TBS-T) containing 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, and 0.1% (v/v) Tween 20. Immunodetection of tyrosine-phosphorylated proteins was performed using the antiphosphotyrosine MAB diluted 1:5000 in blocking buffer for 60 min at room temperature. After incubation with the primary antibody, membranes were washed for 10 min with TBS-T. Then, the membranes were incubated for 45 min with a secondary goat anti-mouse HRP-coupled IgG antibody, diluted 1:5000 in blocking buffer. After incubation with the secondary antibody, membranes were washed for 30 min, incubated for 5 min with ECL Kit (Amersham Corp., Arlington Heights, IL, USA), and exposed to Hyperfilm ECL film; exposure lengths varied as detailed above. Autoradiographs were scanned and the intensity of the protein

bands was measured using Gel Pro Analyzer Software (Media Cybernetics, Bethesda, MD, USA).

For densitometry analysis, the entire lane was used instead of a determined band, as there did not appear to be a constitutive band present in all treatments, and it was unclear which bands denoted functional protein tyrosine phosphorylation. The lane for the treatment anticipated to be the least stimulatory in each trial was used as a baseline for fold increase to control for differences among stallions and for differences in gel processing. Densitometry detected increased density in the control lane in all stallions after background was removed.

Viability assay

Sperm viability was determined using the Nucleocounter according to the manufacturer's instructions, as previously validated (Foster *et al.* 2011). For determination of total sperm concentration, 10 µl sperm solution was diluted with 1 ml detergent reagent S100 and was loaded into a cassette containing propidium iodide. The cassette loaded with the sample was analyzed and the total number of cells in the sample was obtained. Sperm viability was assessed by diluting 20 µl sperm sample with PBS (200 µl) at pH 7.1, placing this in the propidium iodide-containing cassette and analyzing with the Nucleocounter set for this dilution, thus determining the number of nonviable cells. The percentage of viable sperm was determined by subtracting the nonviable cells from the total number of cells and dividing by the total number of cells.

Motility assessment

Sperm motility was analyzed by CAsMA. An aliquot (6 µl) of each sample semen was placed in a warmed (37 °C) counting chamber with a fixed height of 20 µm (Leja Standard Count 2 Chamber slides; Leja Products, B.V., Nieuw-Vennep, The Netherlands). The slide was then placed on a stage set at 37 °C and inserted into the computerized sperm motion analyzer (IVOS version 12.2L; Hamilton Thorne Biosciences, Beverly, MA, USA) for evaluation. A minimum of ten microscopic fields were evaluated. Parameters assessed were percent total motility, percent progressive motility, curvilinear velocity (VCL), smoothed path velocity (VAP), straight-line velocity (VSL), straightness (STR), amplitude of lateral head movement (ALH), and linearity (LIN).

Semen pH and Ca²⁺ concentration pre- and post-insemination

Semen was collected from three mature Quarter Horse stallions of normal fertility. The pH and calcium concentrations were determined in the raw semen sample and then in fluid recovered from the uterus 15–30 min after insemination of raw semen into estrous mares. All experimental procedures were performed according to the 'United States Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training' and were approved by the Laboratory Animal Care Committee at Texas A&M University.

Immediately after semen collection, 0.5 ml undiluted semen was placed in a borosilicate glass tube, and the pH was

measured using a Fisher Scientific Accumet XL60 pH meter coupled to a gel-filled pencil-thin mercury-free pH combination electrode (Thermo Fisher Scientific, Wilmington, DE, USA). A separate 0.5 ml aliquot of undiluted semen was centrifuged at 600 *g* for 5 min, and the supernatant filtered through a 0.2 µm filter. Calcium concentration was determined by the Texas Veterinary Medical Diagnostic Laboratory using a calcium- α -cresolphthalein complex automatic colorimetric assay.

Undiluted semen (50 ml) was used for insemination. Four Quarter-type mares ranging from 10 to 15 years of age were inseminated when a follicle ≥ 35 mm in diameter was visualized by transrectal ultrasonography, accompanied by signs of uterine edema and an open cervix. One mare received an injection of hCG, 2000 IU i.v., the day before insemination. For insemination, the perineum was washed with povidone iodine and dried and then semen was instilled into the body of the uterus transcervically.

To retrieve the fluid from the uterus after insemination, the perineum was washed in the same manner, 15 min after insemination. A flexible artificial insemination pipette was introduced through the cervix into the uterine body. The operator then localized the fluid in the uterus via ultrasonography per rectum. The tip of the pipette was manipulated per rectum to collect fluid. A 60 ml syringe was attached to the free end of the pipette and a second operator aspirated with a continuous low pressure during manipulation of the pipette. A sample of 0.2–3 ml of uterine fluid was retrieved within 15–30 min after insemination. The pH and Ca^{2+} concentration of the fluid were measured on aliquots of this fluid, as described earlier; however, no centrifugation and filtration was performed on uterine fluid from ejaculate 3 due to the low volume recovered.

Statistical analysis

The data were first examined using the Kolmogorov–Smirnov's test to determine their distribution. In view of the Gaussian distribution of the data, differences in viability and motility parameters among treatments were determined by a one-way ANOVA. Differences in pH of the media and differences in pH and Ca^{2+} concentrations before and after insemination were evaluated using a paired, one-tail *T* test. Statistical significance was set at $P < 0.05$. Analyses were performed using Minitab version 15.0 for Windows. Statistical analysis was not performed in the densitometry data obtained by western blotting due to high stallion-to-stallion variability.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

Supported by the Link Equine Research Endowment Fund, Texas A&M University and the Legends Premier Stallion Auction Fund, Texas A&M University. L González-Fernández was supported by a postdoctoral contract (PO10003) from

Consejería de Empleo, Empresa e Innovación, Junta de Extremadura, Spain and by Fondo Social Europeo (FSE). B Macías-García was funded by a grant from the Spanish Ministry of Education FPI program (BES-2008-001880; EEBB-I-12-04378).

Acknowledgements

The authors wish to thank the generous collaboration of the laboratory of the Section of Theriogenology, Texas A&M University College of Veterinary Medicine, and particularly Mrs Sheila Teague for her help in collection of semen from stallions. They also thank Ms Sheila Spacek for highly efficient technical assistance.

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Received 28 February 2012

First decision 27 March 2012

Revised manuscript received 28 June 2012

Accepted 26 July 2012