Journal of Andrology, Vol. 31, No. 4, July/August 2010
Copyright © American Society of Andrology

Bicarbonate-Dependent Serine/Threonine Protein Dephosphorylation in Capacitating Boar Spermatozoa

FAHIMA A. ALNAGAR, PAUL BRENNAN,* AND IAN A. BREWIS*

From the Department of Infection, Immunity and Biochemistry, School of Medicine, Cardiff University, Cardiff, United Kingdom.

ABSTRACT: This study investigates the dynamics of serine/threonine (S/T) protein phosphorylation in sperm incubated under capacitating (C) conditions using the boar as a model system. For the first time, this approach has identified multiple dephosphorylation events that occur in a bicarbonate-dependent fashion. Different phospho-(S/T) kinase substrate antibodies were used, and dephosphorylation of 5 S/T phosphoproteins was observed in C sperm compared with noncapacitated (N) cells. Specifically, dephosphorylation of 96-, 90-, 64-, and 55-kd proteins was detected by immunoblotting using 2 phospho-Akt substrate antibodies and a phosphoprotein kinase A substrate antibody. In addition, dephosphorylation of a 105-kd protein was detected using a phospho-ATM/ATR substrate antibody. In contrast, no dephosphorylation was observed using a phosphoprotein kinase C substrate antibody, and increased tyrosine phosphorylation of 32- and 20-kd proteins was detected in C compared with N sperm. Immunolocalization experiments revealed subtle changes in the pattern expression as well as a reduction of phosphorylation in C sperm. Whereas sperm incubated in N medium containing dibutyryl cAMP (dbcAMP) and 3-isobutyl-1-methylxanthine (IBMX) did not show protein dephosphorylation, incubation in C medium with dbcAMP/IBMX showed dephosphorylation as well as increased phosphorylation of other proteins (p68, p51, and p29). Finally, calyculin A, a phosphatase inhibitor, prevented dephosphorylation of p96, p90, p64, and p55 but not p105. Based on these data, we propose 2 pathways of protein dephosphorylation that are active during capacitation and independent of cAMP. Together, this provides direct evidence for more complex S/T phosphorylation dynamics than has been previously described for sperm undergoing capacitation.

Key words: S/T, signaling, phosphorylation, mammalian, capacitation.

J Androl 2010;31:393–405

Spermatozoa must undergo a series of events in the female tract that enables them to fertilize an oocyte; collectively these events are known as capacitation. Discovered in the early 1950s (Austin, 1951; Chang, 1951), capacitation is believed to facilitate the development of hyperactivated motility, zona binding, and the acrosome reaction. Capacitation can also be induced in vitro by incubation of the sperm in specially defined media (Salicioni et al, 2007; Gadella et al, 2008).

Although there are variations in the media used for in vitro capacitation, most contain bicarbonate, calcium, and bovine serum albumin (BSA; Visconti and Kopf, 1998). Bicarbonate is particularly important for inducing capacitation in mammalian sperm in general and particularly in the boar (Okamura et al, 1985; Harrison, 1996; Flesch and Gadella, 2000). Bicarbonate activates an adenylyl cyclase known as SACY that is abundant in sperm and acts as a bicarbonate sensor to generate cAMP (Okamura et al, 1985; Chen et al, 2000). The subsequent activation of protein kinase A (PKA; Visconti et al, 1995; Flesch and Gadella, 2000) induces, via an unknown signaling mechanism, tyrosine phosphorylation of several proteins in boars (Kalab et al, 1998; Flesch et al, 1999; Harayama, 2003), mice (Visconti et al, 1995), bulls (Galantino-Homer et al, 1997), and humans (Leclerc et al, 1996). In addition, in vitro capacitation may be regulated by BSA probably because of its ability to serve as a sink for the removal of cholesterol from the sperm plasma membrane which causes membrane fluidity changes (Visconti et al, 1999a,b).

Protein phosphorylation is known to regulate sperm motility involving tail proteins, and this has been demonstrated in the boar (Holt and Harrison, 2002). It may also be important for zona pellucida recognition involving head proteins at fertilization (Salicioni et al, 2007; Boerke et al, 2008). Although sperm phosphoproteins have been elucidated in many species, their roles and regulation during capacitation are still not fully understood. The involvement of PKA has been demonstrated; inhibitors of PKA activity are able to inhibit

Fahima A. Alnagar is the recipient of a PhD scholarship from the Libyan Higher Education Ministry (Veterinary Medicine Faculty, Alfateh University, Tripoli).

Correspondence to: Dr Ian A. Brewis, Henry Wellcome Building, School of Medicine, Cardiff University, Heath Park, Cardiff CF14 4XN, United Kingdom (e-mail: brewisia@cardiff.ac.uk).

Received for publication May 21, 2009; accepted for publication November 18, 2009.

* These authors contributed equally to this article.

DOI: 10.2164/jandrol.109.008383

tyrosine phosphorylation as well as capacitation (Visconti et al, 1995; Leclerc et al, 1996). It has been hypothesized that PKA phosphorylates some enzymes/proteins as an intermediate step and that these phosphorylated PKA substrates are involved in capacitation-related protein tyrosine phosphorylation and its regulation (Visconti et al, 1995; Aitken et al, 1998; Visconti et al, 2002).

Many studies have investigated tyrosine phosphorylation in mammalian sperm (Salicioni et al, 2007), even though it is far less frequent in vertebrate somatic cells than serine/threonine (S/T) protein phosphorylation (1:1800:2000 for tyrosine:S:T phosphorylation events; Hunter, 1998). In contrast, there have been very few studies on S/T protein phosphorylation in sperm, and our understanding is therefore very limited. One major reason for this inequality is the general availability of antibodies that recognize phosphorylated tyrosine residues, whereas good-quality antibodies that specifically recognize phospho-(S/T) proteins/peptides have only become available in recent years. These new reagents are typically antibodies generated against the consensus sequences of individual kinases (eg, the PKB/Akt pathway) and represent useful reagents to survey the dynamics of S/T phosphorylation (Alessi et al, 1996).

The aim of this study was to investigate changes in phospho-(S/T) proteins in boar sperm incubated under capacitating (C) conditions using immunoblotting and immunofluorescence. We used 5 different phospho-(S/T) kinase substrate antibodies and detected dephosphorylation of 5 proteins. This is the first time it has been shown that multiple dephosphorylation events occur in a bicarbonate-dependent fashion in mammalian sperm. Moreover, studies with dibutyryl (dbcAMP)/3-isobutyl-1-methylxanthine (IBMX) and a phosphatase inhibitor suggest that dephosphorylation occurs through 2 pathways independent of cAMP. Together, these studies suggest that protein dephosphorylation may play a role in those events leading to sperm capacitation.

Materials and Methods

Sperm Preparation

Boar semen ejaculates were collected from Landrace boars and supplied in extender buffer (JSR Healthbred, Southburn, Driffield, Yorkshire, United Kingdom). Spermatozoa were isolated by centrifugation (750 $\times g$ for 20 minutes with brake off) through a 2-step discontinuous gradient of 35% and 70% isotonic Percoll (15 mL of each gradient overlaid with 15 mL of semen in extender buffer; Sigma-Aldrich Co Ltd, Poole, United Kingdom). Isotonic Percoll was prepared by first mixing Percoll with 10× M medium (1.37 M NaCl, 25 mM KCl, 200 mM HEPES, 100 mM glucose, pH 7.55) (12:1 ratio) and then checking the osmolality confirm it was 285 to 300 mOsm/kg. This mixture was diluted to the appropriate concentration with 1× M medium. After removal of the Percoll layers, the resultant loose pellet was resuspended in Tris-buffered sucrose solution (5 mM Tris, 0.25 M sucrose, pH 7.4; 10 mL) and subjected to further centrifugation (500 $\times g$ for 5 minutes). The pelleted cells were then resuspended in the appropriate buffer for incubation.

Sperm Incubation

Cells prepared as described were incubated under either noncapacitating (N) or C conditions at a concentration of 10 million/mL in 10 mL of media. For C conditions, Tyrode medium (100 mM NaCl, 21.7 mM lactate, 20 mM HEPES, 5 mM glucose, 3.1 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM pyruvate, 0.4 mM $MgSO_4$, 0.3 mM NaH_2PO_4, 15 mM $NaHCO_3$, 100 μg/mL kanamycin; 290–300 mOsm/kg, pH 7.4) containing 0.3% (wt/vol) BSA (Fraction V; Cat 05477, Sigma-Aldrich) was used with the cells in a tube with the screw cap loose at 38.5°C in a humidified incubator with 5% CO_2 in air (Parrish et al, 1988; Gadella and Harrison, 2000). N conditions were the same except that the Tyrode medium was prepared without $NaHCO_3$ (but with an additional 16 mM NaCl to maintain the same osmolality) and samples were incubated in airtight sealed tubes. Typically, samples were recovered for blotting and immunofluorescence experiments following a 1-hour incubation. In addition, the blotting data presented in Figures 1 through 3 also show samples recovered after 15 and 30 minutes. Finally, immunofluorescence data in Figure 1 following a 3-hour incubation are also presented.

In experiments in which the influence of cAMP on S/T protein phosphorylation was evaluated, sperm were incubated for 1 hour under N or C conditions in the absence or presence of 1 mM dbcAMP (Sigma-Aldrich), which is a cAMP analog, and 100 μM IBMX (Sigma-Aldrich), which is a phosphodiesterase inhibitor. In experiments in which the role of phosphatases on S/T protein phosphorylation was investigated, cells were incubated for 1 hour under N or C conditions in the absence or presence of either 100 or 250 nM calyculin A (Sigma-Aldrich), which is a pharmacologic phosphatase inhibitor of protein phosphatase 2A (PP2A) and PP1 (IC_{50}, 0.5–1.0 nM and 2.0 nM, respectively; Ishihara et al, 1989).

Assessment of Sperm Motility, Viability, and Acrosomal Integrity

Sperm motility was assessed by subjective observation. The motility was observed in a 20-μL aliquot of sperm suspension on a slide under a bright-field microscope. Sperm viability was assessed using a LIVE/DEAD sperm viability kit (Invitrogen Ltd, Paisley, United Kingdom). SYBR14 dye (5 μL; 100 nM final concentration) was added to 1 $\times 10^6$ cells in 1 mL N or C medium following a 1- or 2-hour incubation and the mixture further incubated for 10 minutes at 38.5°C. Propidium iodide (5 μL; 12 μM final concentration) was then added, and the cells were incubated for a further 10 minutes at 38.5°C. Sperm were evaluated using a Leica fluorescence microscope, and 200 cells were counted for each treatment. Acrosomal status was

19394640, 2010, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.2164/jandrol.109.008383 by Libya Hinari NPL, Wiley Online Library on [08/09/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

19394640, 2010, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.2164/jandrol.109.008383 by Libya Hinari NPL, Wiley Online Library on [08/09/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

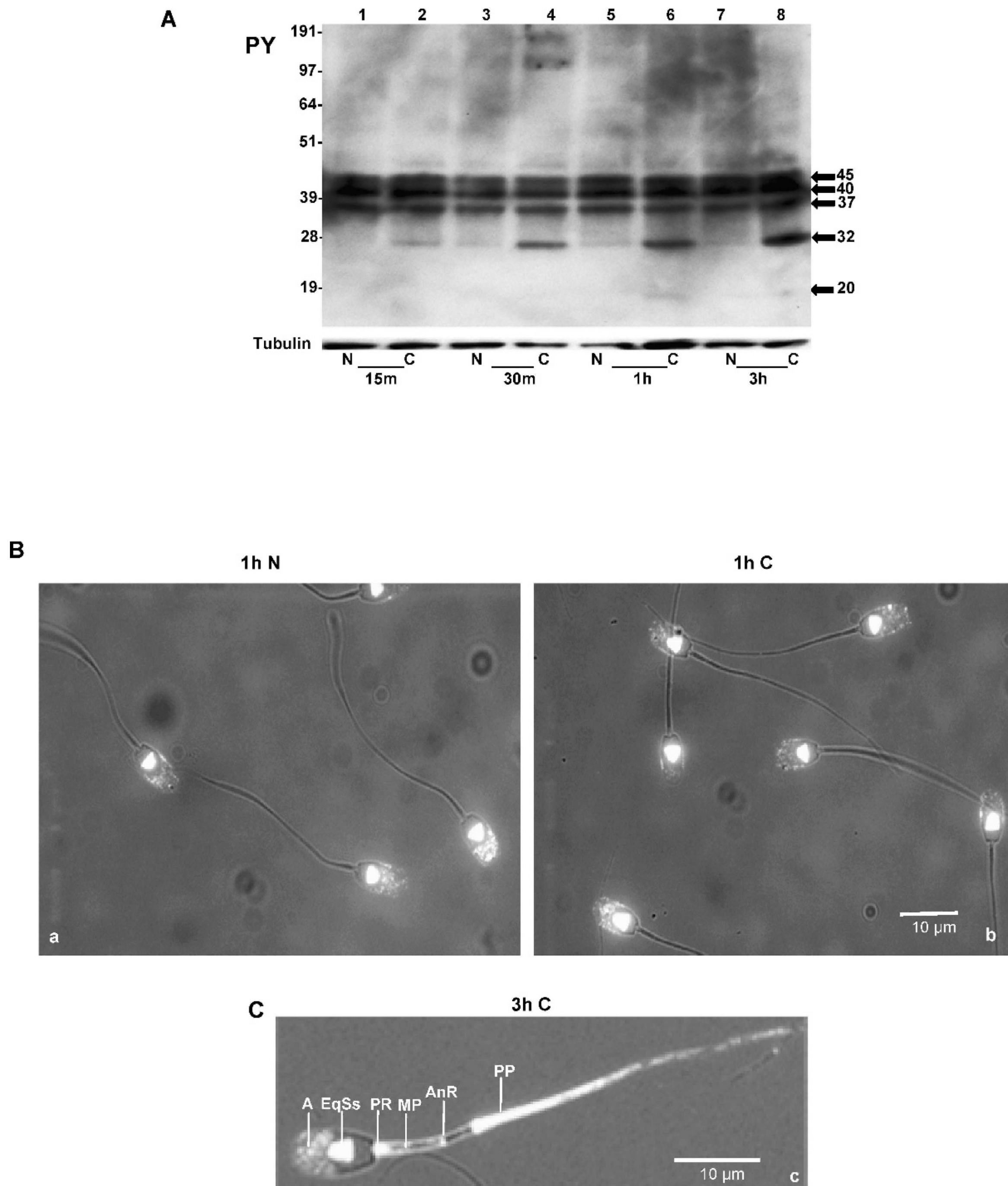
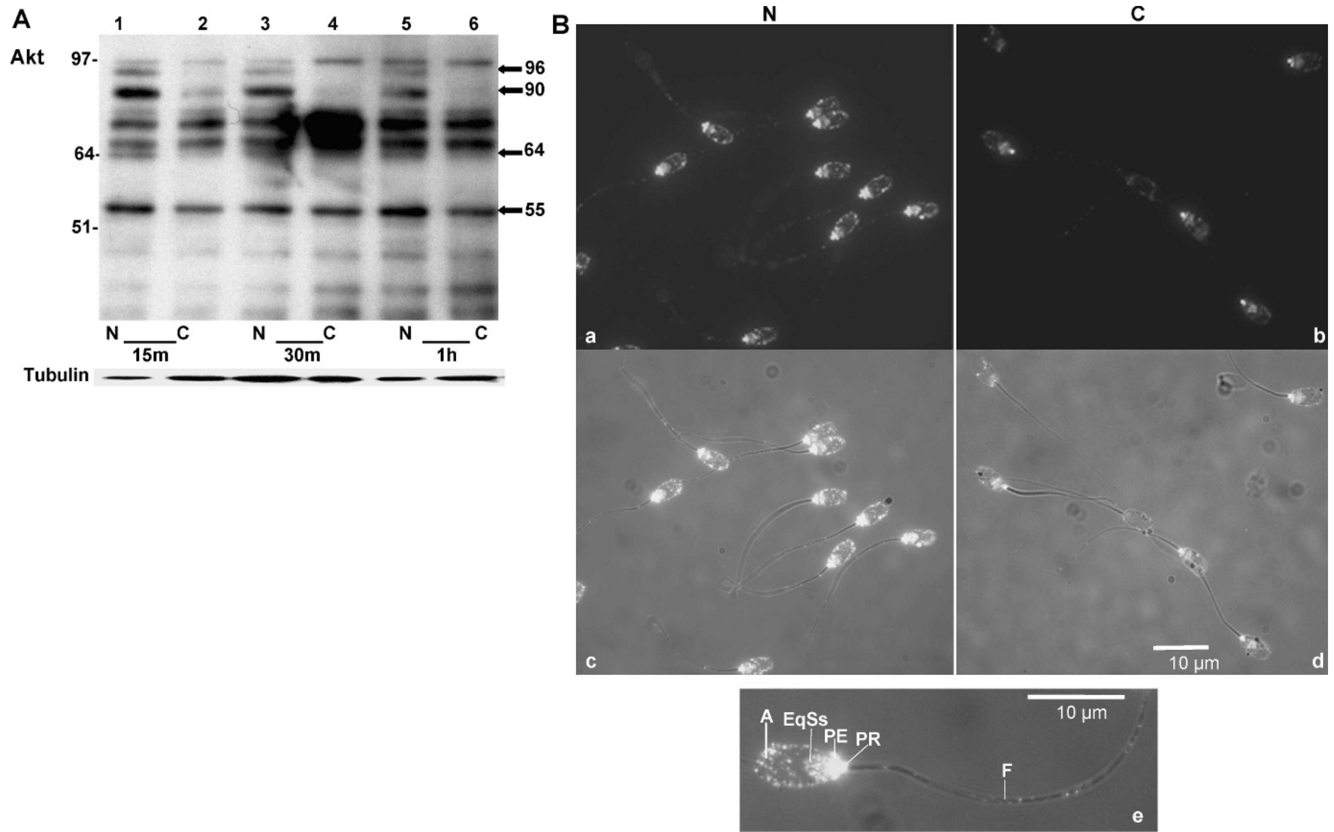
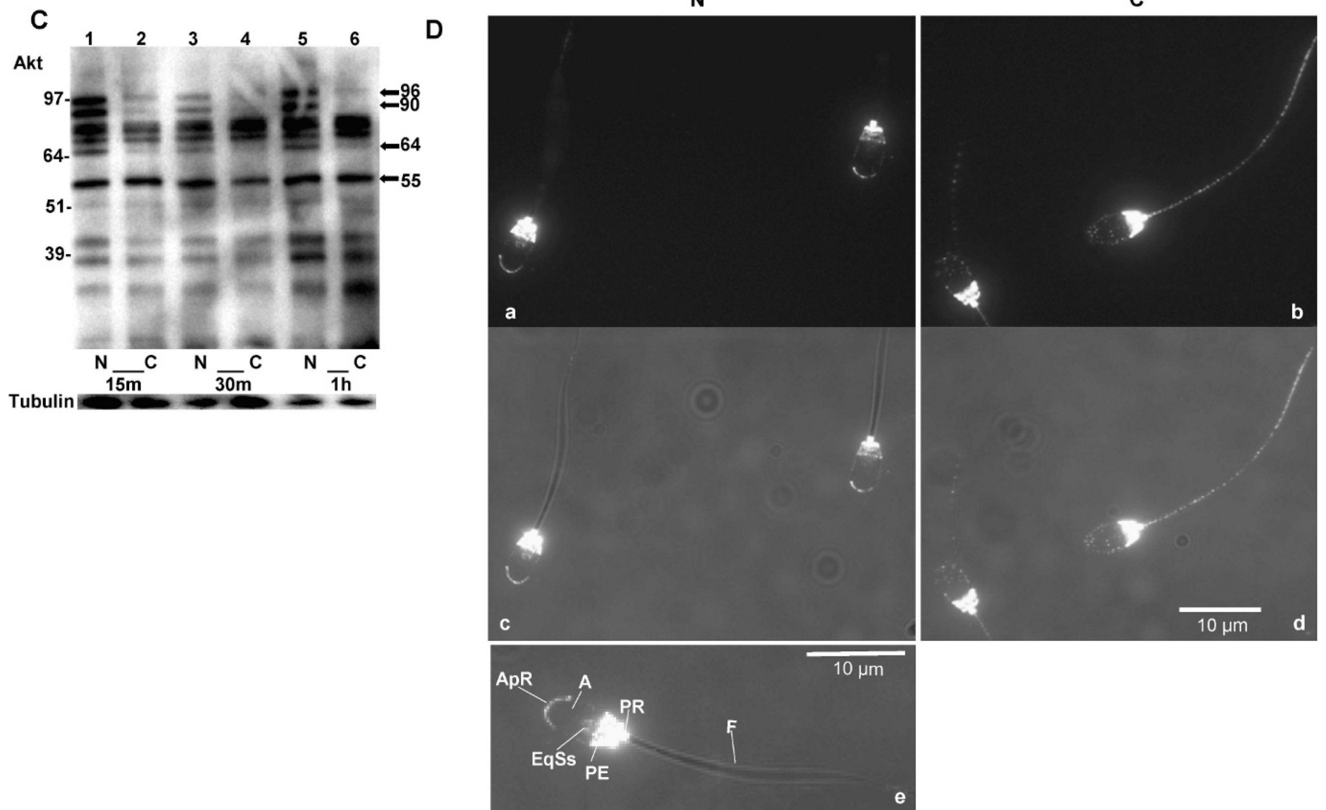


Figure 1. Protein tyrosine phosphorylation in sperm incubated under capacitating (C) conditions is similar as that in previously reported studies. **(A)** Immunoblot showing proteins detected with anti-phosphotyrosine antibody for sperm incubated under noncapacitating (N) and C conditions for 15 minutes, 30 minutes, 1 hour, and 3 hours. **(B)** Immunolocalization of phosphoproteins detected with the same antibody as in Panel A in fixed sperm cells after a 1-hour incubation under N or C conditions. **(C)** Immunolocalization of phosphoproteins detected with the same antibody as in Panel A in fixed sperm cells after a 3-hour incubation under C conditions. The results shown are representative of at least 10 experiments performed with different sperm samples, and tubulin loading controls were used for all blots. A indicates acrosome; EqSs, equatorial subsegment; PR, posterior ring; MP, midpiece; ApR, apical ridge; PP, principal piece.

assessed by indirect immunofluorescence using a monoclonal antibody (designated as 18.6) as described previously (Brewis et al, 1996). Briefly, 18.6 recognizes a sperm epitope within the acrosomal content, and fixed cells displaying even fluorescence in the acrosomal region are scored as acrosome intact. Nuclear staining was also performed using 4,6 dimidine-2-phenylindole dihydrochloride (Invitrogen) added to the secondary antibodies (0.2 µL/100 mL).





19394640, 2010, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.2164/jandrol.109.008383 by Libya Hinari NPL, Wiley Online Library on [08/09/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis and Immunoblotting

Following incubation in either N or C medium, sperm aliquots (1×10^7 cells) were collected. These aliquots were centrifuged for 10 minutes at 5000 $\times$ *g*, washed in phosphate-buffered saline (PBS; 0.01 M phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4), and again centrifuged for 10 minutes at 5000 $\times$ *g*. The isolated sperm pellet was resuspended in sample buffer (NuPAGE LDS; Invitrogen) and heated at 70°C for 10 minutes. The sample was recentrifuged for 5 minutes, and the supernatant used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Sperm proteins equivalent to 1.5 to 2×10^6 cells were separated by SDS-PAGE (4%–12% Bis-Tris NuPAGE Novex gels; Invitrogen) and transferred onto polyvinylidene fluoride membranes for immunoblotting using a previously described alkaline phosphatase (AP) chemiluminescent detection protocol (Rowe and Jones, 2001). The phospho-(S/T) kinase substrate antibodies were supplied by Cell Signaling Technology (Beverly, Massachusetts): 2 phospho-(S/T) Akt substrate antibodies (polyclonal Cat. #9611; monoclonal Cat. #9614), a phospho-(S/T) PKA substrate monoclonal antibody (Cat. #9624), a phospho-(S) PKC substrate polyclonal antibody (Cat. #2261), and a phospho-(S/T) ATM/ATR substrate polyclonal antibody (Cat. #2851). The anti-phosphotyrosine monoclonal antibody (clone 4G10) was from Millipore UK Ltd (Watford, United Kingdom). All primary antibodies were used at a 1:1000 dilution for immunoblotting. The secondary antibodies used were goat anti-mouse IgG (H+L) AP conjugate or goat anti-rabbit IgG (H+L) AP conjugate (Bio-Rad Laboratories, Hemel Hempstead, United Kingdom) at a 1:10 000 dilution. An α-tubulin monoclonal antibody (1:1000 dilution; Cat. #T9026; Sigma-Aldrich) was used as a loading control for each blot (50-kd epitope).

Indirect Immunofluorescence

Incubated sperm cells (1×10^5) were gently smeared onto a microscopic slide and allowed to air dry. Slides were fixed in 100% methanol for 5 minutes and allowed to air dry for 1 hour. Slides were then incubated for 1 hour with different antibodies at 38.5°C in a wet box. Please see "Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis and Immunoblotting," for details about the primary antibodies used and note that all were used at a 1:100 dilution except for the anti-phosphotyrosine antibody, which was used at a 1:200 dilution. Following incubation, the slides were rinsed with PBS and incubated for a further 1 hour with goat anti-mouse Alexa Fluor 488–conjugated antibody (1:300 dilution; Invitrogen) for the anti-phosphotyrosine antibody and with goat anti-rabbit Alexa Fluor 488–conjugated antibody (1:300 dilution; Invitrogen) for phospho-(S/T) kinase substrate antibodies. For each primary antibody used, a control slide was processed and analyzed for which the primary antibody incubation was excluded and the cells were just incubated with secondary antibody. Slides were washed with PBS and mounted with Slow Fade Light antifade solution (Dako UK Ltd, Ely, United Kingdom). Slides were assessed by epifluorescence ultraviolet microscopy at a 492-nm wavelength with ×40 oil objective lens magnification to determine localization of detected phosphoproteins.

Results

Protein Tyrosine Phosphorylation Is Increased in Sperm Incubated Under C Conditions

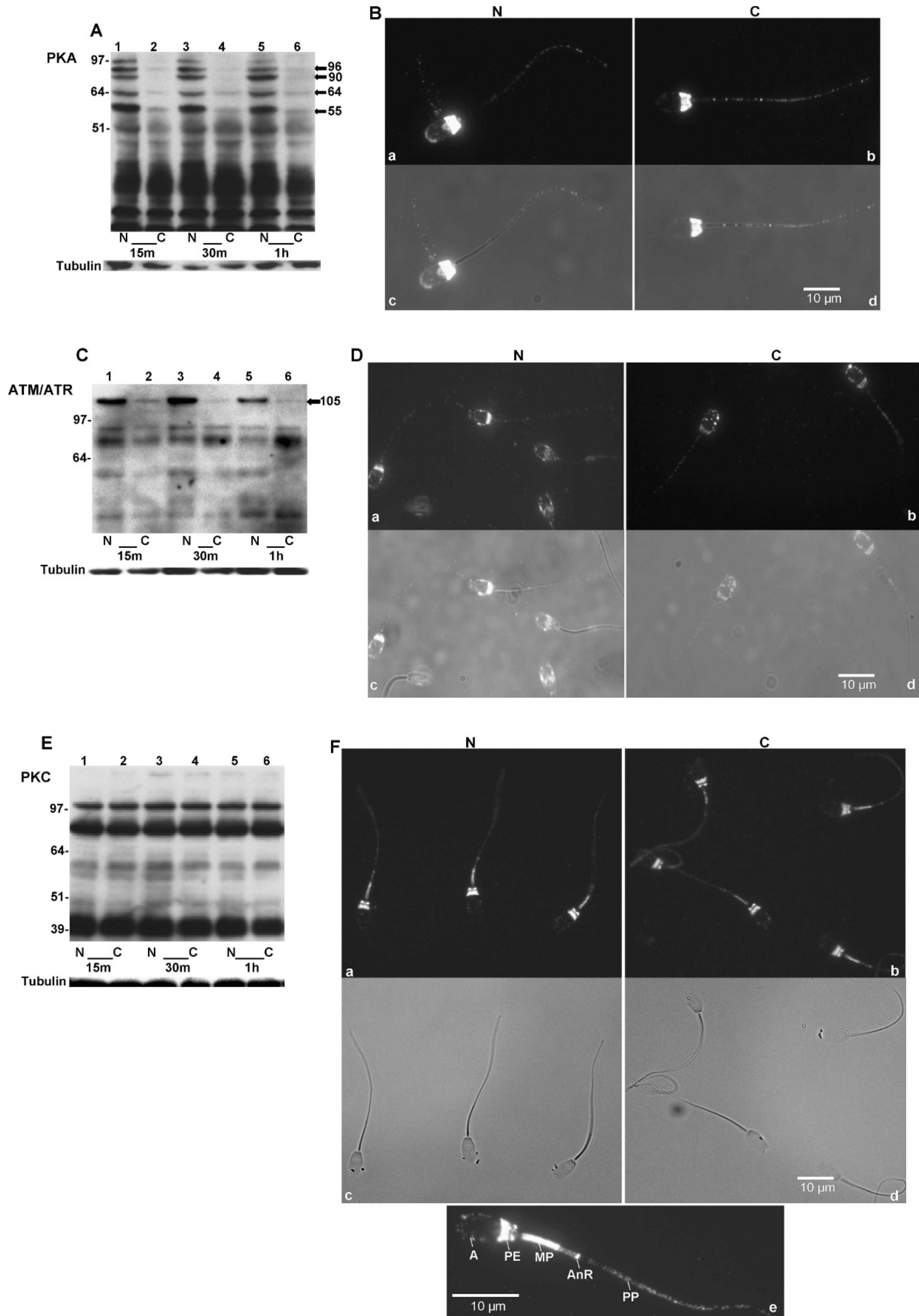
The first step of this study was to investigate tyrosine phosphorylation in sperm incubated under experimental C conditions. To this end, we generated protein extracts and prepared slides of fixed sperm cells and in parallel determined sperm viability and movement. Viability was assessed using a LIVE/DEAD sperm viability kit and classified as live (plasma membrane intact; green) or dead (plasma membrane damaged; red). The mean (±SEM) percentage of live sperm was 75% ± 1% (n = 4) after a 1-hour incubation under C conditions and in N cells was 81% ± 3% after a 1-hour incubation. Sperm cell motility was assessed by bright-field microscopy, and the majority of sperm incubated in C medium showed rapid progressive motility after a 5-minute incubation. The motility increased over time and reached a plateau after 2 hours of incubation, with a similar pattern after 3 hours. In contrast to C sperm, N sperm cells showed no movement through the microscopic field in all experiments but instead demonstrated a vibrational movement ("static vibrance") caused by sperm sticking to the microscope slide owing to a lower lateral head displacement than that of the C cells. This is consistent with observations by other researchers using the pig as a model system (Gadella, personal communication).

With confidence that we had viable sperm which was motile under C conditions, whole-cell lysates were

←

Figure 2. Loss of protein phosphorylation in sperm incubated under capacitating (C) conditions was detected with phospho-Akt substrate antibodies. Two phospho-Akt substrate antibodies, a polyclonal **(A and B)** and a monoclonal **(C and D)**, were used to analyze the changes in protein phosphorylation using immunoblotting **(A and C)** and immunolocalization **(B and D)** under noncapacitating (N) or C conditions. The immunolocalization results shown are following a 1-hour incubation. Ba, Bb, Da, and Db are fluorescence-only images, and paired images (fluorescence and light) are shown in Bc, Bd, Dc, and Dd, respectively. Be and De represent individual sperm cells from Bc and Dc, respectively. The results shown are representative of at least 5 experiments performed with different sperm samples, and tubulin loading controls were used for all blots. A, indicates acrosome; EqSs, equatorial subsegment; PE, postequatorial region; PR, posterior ring; MP, midpiece; F, flagellum.

19394640, 2010, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.2164/jandrol.109.008383 by Libya Hinari NPL, Wiley Online Library on [08/09/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License



19394640, 2010, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.2164/jandrol.109.008383 by Libya Hinari NPL, Wiley Online Library on [08/09/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

generated and proteins resolved by SDS-PAGE followed by Western blotting. Protein tyrosine phosphorylation was assessed by immunoblotting with an anti-phosphotyrosine monoclonal antibody (clone 4G10). Figure 1A shows protein tyrosine phosphorylation of 3 major bands (45, 40, and 37 kd) that were unchanged in sperm incubated under C conditions. The phosphorylation of a 32-kd band was increased in C compared with N sperm. In addition, a 20-kd band showed increased phosphorylation but to a much lesser extent than the 32-kd band. Note that all blots in this study were assessed for similar protein loading using an anti-tubulin loading control. Indirect immunofluorescence localization with the same antibody showed phosphorylated tyrosine in the acrosome and equatorial subsegment in N and C sperm incubated for 1 hour (Figure 1Ba and b), whereas C sperm incubated for 3 hours (Figure 1C) showed additional phosphorylation in the posterior ring, midpiece, and principal piece (not present in N sperm; data not shown but the same pattern as in Figure 1Ba). No immunofluorescence was observed in control slides (secondary antibody only; data not shown). These results for tyrosine phosphorylation were consistent with previous studies on boar sperm (Kalab et al, 1998; Flesch et al, 1999; Tardif et al, 2001; Harayama, 2003; Harayama et al, 2004; Harayama and Nakamura, 2008), and the 32-kd protein is likely to be the same as that observed after calcium-dependent phosphorylation during capacitation (Dubé et al, 2003). These data demonstrated that sperm tyrosine phosphorylation changed over time in a similar fashion as that previously reported and convinced us that our system was suitable for the study of boar sperm capacitation in vitro.

Loss of S/T Protein Phosphorylation in Sperm Incubated Under C Conditions

Having established the conditions for in vitro sperm capacitation, we investigated the changes in S/T protein phosphorylation using a range of phospho-(S/T) kinase substrate antibodies. The first antibody we tested was a phospho-(S/T) Akt substrate antibody. This commercial polyclonal antibody was produced by immunizing rabbits with phospho-Akt substrate peptides, and the resulting antibodies have been shown to have a specificity of R/K-X-R/K-X-X-T*/S* (X represents any amino acid). The results in Figure 2A show distinct bands, particularly in proteins above 50 kd. Although some bands did not change, we observed that 4 proteins of 96, 90, 64, and 55 kd were reduced in C sperm when compared with N sperm throughout a 1-hour incubation (Figure 2A). It should be noted that phosphatase inhibitors were not routinely added during sperm washing and extraction prior to SDS-PAGE, but control experiments using a broad-range phosphatase inhibitor cocktail added to both the wash buffer and extraction buffer immediately prior to SDS-PAGE did not affect the results presented (data not shown). Immunolocalization with this antibody detected phospho-(S/T) protein in the flagellum (low levels), acrosome, equatorial subsegment, postequatorial region, and posterior ring in N sperm incubated for 1 hour (Figure 2Ba). Less phosphorylation was present in C sperm incubated for 1 hour (Figure 2Bb). No immunofluorescence was observed in control slides (secondary antibody only), and the same control was employed for all the immunofluorescence studies using phospho-(S/T) kinase substrate antibodies. In all cases, no fluorescence was observed (data not shown).

Acrosomal integrity was assessed in all experiments by observing the immunofluorescence micrographs, and we confirmed that the acrosomes were intact in the majority (>90%) of cells. We also assessed both cell viability and acrosomal integrity (with a well-established immunofluorescence assay using the 18.6 monoclonal antibody) before and after washing immediately prior to SDS-PAGE. At least 90% of cells were acrosome intact, and no differences were observed between washed and nonwashed cells and between N and C sperm. In addition, no differences in cell viability were observed (data not shown).

The 4 phosphoproteins of 96, 90, 64, and 55 kd were detected in N sperm but were dephosphorylated in C sperm by a different phospho-(S/T) Akt substrate antibody, which is a monoclonal antibody that recognizes the motif R-X-R-X-X-T*/S* (Figure 2C). Immunolocalization with this antibody showed that phospho-

←

Figure 3. Protein phosphorylation dynamics in sperm incubated under capacitating (C) conditions using 3 other phosphokinase substrate antibodies. Protein phosphorylation was analyzed using a phospho-serine/threonine (S/T) PKA substrate antibody **(A and B)**, phospho-(S/T) ATM/ATR substrate antibody **(C and D)**, and phospho-(S) PKC substrate antibody motif **(E and F)**. Immunoblotting **(A, C, and E)** and immunolocalization **(B, D, and F)** data under noncapacitating (N) or C conditions are presented. The immunolocalization results shown are following a 1-hour incubation. Ba, Bb, Da, and Db are fluorescence-only images and paired images (fluorescence and light) are shown in Bc, Bd, Dc, and Dd, respectively. Fa and Fb are fluorescence-only images, and paired light microscopy images are shown in Fc and Fd, respectively. Note that Fe represents an individual sperm cell from Fa. The results shown are representative of at least 5 experiments performed with different sperm samples, and tubulin loading controls were used for all blots. A indicates acrosome; PE, postequatorial region; MP, midpiece, AnR, annular ring; PP, principal piece.

19394640, 2010, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.2164/jandrol.109.008383 by Libya Hinari NPL, Wiley Online Library on [08/09/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

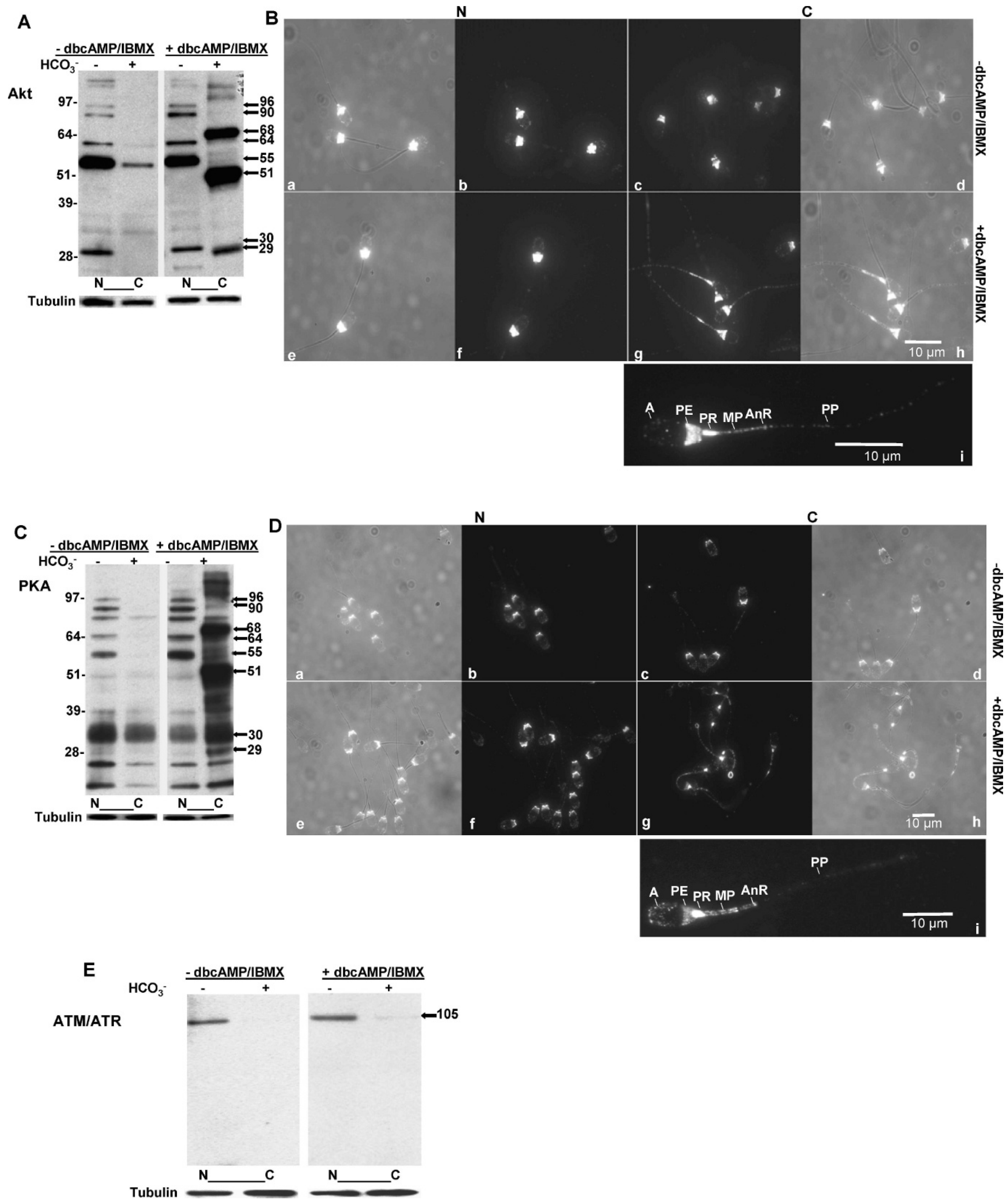


Figure 4. The effect of dibutyryl cAMP (dbcAMP)/3-isobutyl-1-methylxanthine (IBMX) on protein serine/threonine (S/T) phosphorylation in sperm incubated under capacitating (C) conditions. Cells were treated with dbcAMP/IBMX under both noncapacitating (N) and C conditions. Subsequently, they were analyzed by immunoblotting **(A, C, and E)** and immunolocalization **(B and D)**, as described. Protein phosphorylation was analyzed using a phospho-(S/T) Akt substrate polyclonal antibody **(A and B)**, a phospho-(S/T) PKA substrate antibody **(C and D)**, and a

19394640, 2010, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.2164/jandrol.109.008383 by Libya Hinari NPL, Wiley Online Library on [08/09/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

(S/T) proteins were localized in the apical ridge, acrosome, equatorial subsegment (low levels), postequatorial region, posterior ring, and flagellum (low levels) in N sperm incubated for 1 hour (Figure 2Da). No phosphorylation was observed in C sperm incubated for 1 hour in the apical ridge and equatorial subsegment (Figure 2Db).

We extended our study using a phospho-(S/T) PKA substrate antibody raised against the motif R-R-X-S*/T*. This antibody detected a wider range of proteins compared with the phospho-Akt substrate antibody, particularly in the lower region of the gel. For example, it detected a 35-kd band that was unchanged during capacitation. Interestingly, this antibody also detected 4 proteins in N sperm of the same size as those detected by the phospho-Akt substrate antibody: 96, 90, 64, and 55 kd (Figure 3A). None of these proteins were detected in C sperm. Immunolocalization with this antibody detected phosphoproteins in the flagellum (low levels), apical ridge, equatorial subsegment, and postequatorial region in N sperm incubated for 1 hour (Figure 3Ba). A similar pattern was observed in C sperm, but less phosphorylation was present in the equatorial subsegment (Figure 3Bb).

A phospho-(S/T) ATM/ATR substrate antibody, raised against the motif (Hyd-S*/T*-Q), was investigated. In contrast with the previous antibodies, this antibody detected only 3 bands. Interestingly, one of these bands, a 105-kd phosphoprotein was absent in C sperm compared with N sperm (Figure 3C). Phosphoproteins were localized in the apical ridge, acrosome, postequatorial region, and flagellum (low levels) in N and C sperm incubated for 1 hour (Figure 3Da and b). Finally, we tested a phospho-(S) PKC substrate antibody raised against the motif (R/K-X-S*-Hyd-R/K) (Figure 3E and Fa and b) but observed no changes in phosphorylation by immunoblotting and immunofluorescence.

Addition of dbcAMP/IBMX Is Not Sufficient to Cause S/T Protein Dephosphorylation

To determine the effect of cAMP on S/T protein phosphorylation during capacitation, washed sperm were incubated with dbcAMP/IBMX under N and C conditions for 1 hour. Cell extracts were generated, and proteins were separated by SDS-PAGE followed by Western blotting. S/T protein phosphorylation was first detected with the Akt substrate antibody. No loss of phosphorylation was detected in N sperm in the presence of dbcAMP/IBMX (Figure 4A). As observed previously, the phosphoproteins of 96, 90, 64, and 55 kd were absent in C compared with N sperm. Interestingly, additional phosphoproteins (eg, bands were observed at 68, 51, and 29 kd) were observed in C sperm in the presence of dbcAMP/IBMX compared with C sperm incubated in the absence of dbcAMP/IBMX or N sperm. Phospho-(S/T) proteins detected by this antibody were localized in the acrosome (low levels), equatorial subsegment, and postequatorial region in N and C sperm incubated in the absence of dbcAMP/IBMX (Figure 4Ba and b). This antibody was localized in similar cellular regions in N cells in the presence of dbcAMP/IBMX (Figure 4Bc). The phosphorylation was enhanced in the postequatorial region, posterior ring, and midpiece following a 1-hour incubation with dbcAMP/IBMX in C sperm (Figure 4Bd).

S/T protein phosphorylation was also detected using the phospho-PKA substrate antibody (Figure 4C and D). As observed with the phospho-Akt substrate antibody, treatment of N sperm with dbcAMP/IBMX did not cause the loss of protein phosphorylation, which was observed in C sperm. Again, enhanced protein phosphorylation was observed in C sperm in the presence of dbcAMP/IBMX compared with C sperm incubated without dbcAMP/IBMX or N sperm (Figure 4C). Immunofluorescence analysis with phospho-PKA substrate antibody detected phosphoproteins localized in the postequatorial region in N sperm incubated in the presence or absence of dbcAMP/IBMX (Figure 4Da and c). Phosphorylation was enhanced in the posterior ring, midpiece, and principal piece but reduced in the postequatorial region in C sperm in the presence but not in the absence of dbcAMP/IBMX (Figure 4Db and d).

Incubation of sperm with dbcAMP/IBMX had no effect on the 105-kd protein detected by phospho-ATM/ATR substrate antibody in N cells but not in C cells (Figure 4E). Finally, for all of the experiments investigating the effects of cAMP on S/T phosphorylation, controls were performed to determine protein tyrosine phosphorylation by immunoblotting. In all cases, increased tyrosine phosphorylation of many phosphoproteins was observed in C sperm following addition of dbcAMP/IBMX (data not shown).

←

phospho-(S) ATM/ATR substrate antibody **(E)**. Bb, Bc, Bf, Bg, Db, Dc, Df, and Dg are fluorescence-only images, and paired images (fluorescence and light) are shown in Ba, Bd, Be, Bh, Da, Dd, De, and Dh, respectively. Bi and Di represent individual sperm cells from Bg and Dg, respectively. The results shown are representative of at least 3 experiments performed with different sperm samples, and tubulin loading controls were used for all blots. A indicates acrosome; PE, postequatorial region; PR, posterior ring; MP, midpiece; AnR, annular ring; PP, principal piece.

19394640, 2010, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.2164/jandrol.109.008383 by Libya Hinari NPL, Wiley Online Library on [08/09/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Calyculin A Inhibits Most Protein Dephosphorylation Observed in Sperm Incubated Under C Conditions

The final step in this study was to investigate the effect of calyculin A, which is a PP2A and PP1 phosphatase inhibitor, to assess whether protein dephosphorylation was caused by these classes of phosphatase. Sperm were incubated in the presence or absence of calyculin A at concentrations of 100 and 250 nM under N or C conditions. No obvious differences in sperm motility in C sperm were observed. Protein extracts were generated and processed as before. Calyculin A prevented the loss of detection of 4 bands detected with the phospho-PKA substrate antibody in C sperm: p96, p90, p64, and p55 (Figure 5B). It also prevented the loss of similar bands detected with the Akt substrate polyclonal antibodies (Figure 5C) and Akt substrate monoclonal antibody (data not shown). However, calyculin A did not prevent the loss of the 105-kd protein detected by phospho-ATM/ATR substrate antibody in N cells but not in C cells (Figure 5A). This suggests 2 distinct mechanisms of protein dephosphorylation in boar sperm incubated under C conditions.

Discussion

This project was initiated to investigate S/T phosphorylation in sperm incubated under C conditions. We chose phosphokinase substrate antibodies that have been used to investigate other signaling systems but have not been widely used to study mammalian sperm proteins. Using these reagents, we identified the loss of phosphorylation of 5 different molecular-weight proteins, 4 through a calyculin A–sensitive phosphatase and 1 through a calyculin A–independent pathway. These dephosphorylation events could not be caused by dbcAMP/IBMX treatment alone. Together, these data indicate 2 pathways of phosphatase activity that are activated in sperm incubated under C conditions downstream of the bicarbonate sensor in a cAMP-independent manner.

We deliberately chose to study S/T phosphorylation using 4 antibodies that have not been used previously in sperm, including substrate antibodies for Akt (2 antibodies), ATM/ATR, and PKC. With the Akt substrate antibodies, we observed dephosphorylation of 5 proteins in sperm incubated under capacitating conditions, whereas increased phosphorylation of other proteins was observed following treatment with dbcAMP/IBMX. These changes contrasted with the PKC substrate antibody, which detected similar proteins under all experimental conditions. We detected dephosphorylation with a PKA substrate antibody that we chose as a comparison because it had been used in other studies (Harrison, 2004; O'Flaherty et al, 2004; Harayama and Miyake, 2006; Harayama and Nakamura, 2008; Kaneto et al, 2008). One previous study reported decreased phosphorylation of a single 100 kd phospho-(S/T) protein during capacitation using a different anti-phosphoserine antibody in the hamster (Jha and Shivaji, 2002). However, the majority of studies have focused on increases in phosphorylation. Thus, this study is the first to document multiple dephosphorylation events that occur in a bicarbonate-dependent fashion. Together, this provides direct evidence for more complex S/T phosphorylation dynamics than is generally described for sperm undergoing capacitation.

There was an overlap with the bands detected with the Akt substrate antibodies and the PKA substrate antibodies, particularly under N conditions. Despite the overlap, when the Akt substrate antibodies and the PKA substrate antibody were compared using immunofluorescence, the pictures look subtly different, probably owing to the detection of other proteins by the antibodies. Although different motifs of S/T PKA substrate antibody were used in this study than previously used by others on boar sperm, the immunolocalization of these phosphoproteins was similar (Harayama, 2003; Adachi et al, 2008). Interestingly, phosphorylation seemed to be lost in the head region with the Akt substrate monoclonal antibody (Figure 2D), which may suggest a role in zona binding. There is a similarity between the motifs recognized by the Akt and PKA substrate antibodies: the R-R-X-S*/T* motif of the PKA substrate would be included within the R-X-R-X-X-S*/T* of the Akt substrate antibody. Given this, we would predict that p96, p90, and p55 contain a motif that is made up of both sequences, perhaps R-X-R-R-X-S*/T*. This is valuable information that we will aim to use to identify these proteins as the next step in our studies.

These experiments show that S/T dephosphorylation, across all 5 proteins studied, occurs within 15 minutes of incubation of sperm in C conditions. This rapid response indicates a direct role for the bicarbonate sensor in stimulating protein dephosphorylation. The rapid dephosphorylation is paralleled by previous reports that merocyanine-reported boar sperm membrane fluidization occurs within minutes after bicarbonate addition (Gadella and Harrison, 2000). However, treatment of sperm with dbcAMP/IBMX in N conditions did not result in dephosphorylation, demonstrating that elevation of cAMP alone was not sufficient to cause dephosphorylation. This further supports the observation that the bicarbonate sensor is a critical regulator of the changes that occur inside sperm cells undergoing capacitation.

19394640, 2010, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.2164/jandrol.109.008383 by Libya Hinari NPL, Wiley Online Library on [08/09/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

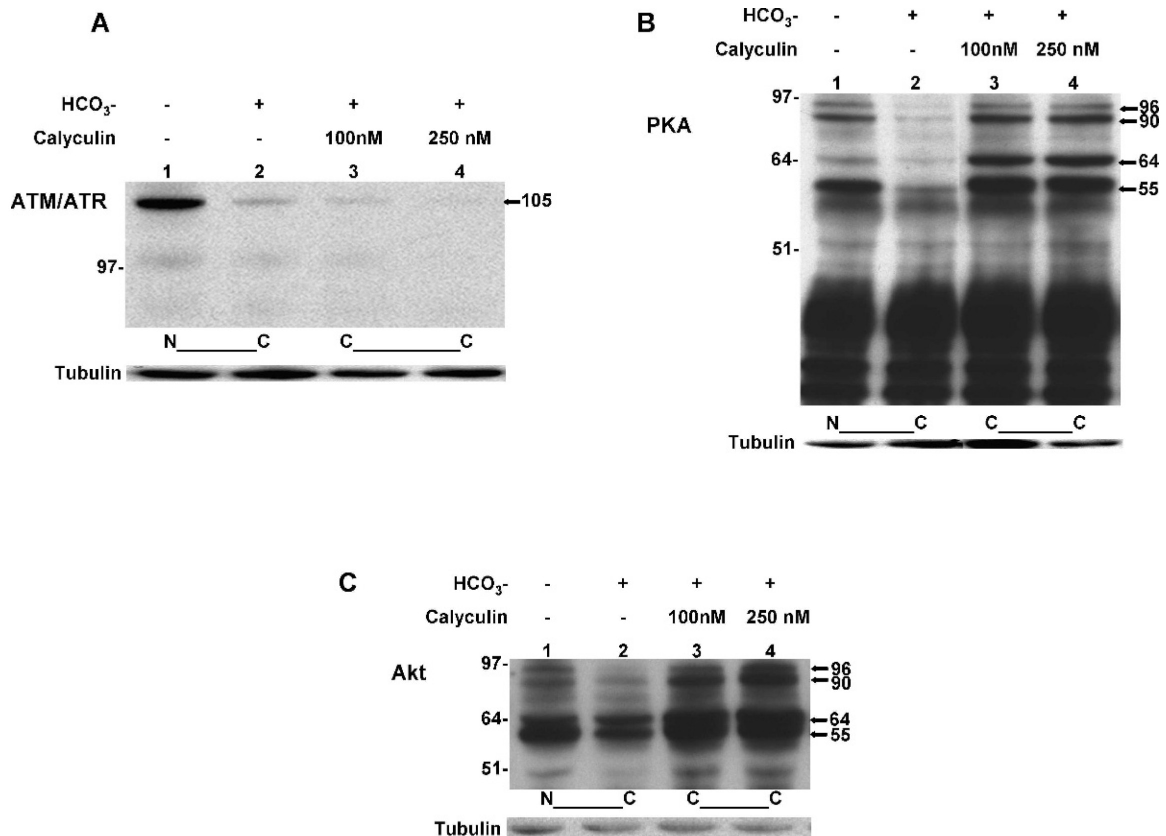


Figure 5. The effect of calyculin A on serine/threonine (S/T) dephosphorylation in sperm incubated under capacitating (C) conditions. Cells were treated with 2 concentrations of calyculin A, as indicated, under both noncapacitating (N) and C conditions. Subsequently, protein phosphorylation was analyzed by immunoblotting using a phospho-(S/T) ATM/ATR substrate antibody **(A)**, a phospho-(S/T) PKA substrate antibody **(B)**, and a phospho-(S/T) Akt substrate polyclonal antibody **(C)**. The results shown are representative of at least 3 experiments performed with different sperm samples, and tubulin loading controls were used for all blots.

One of the dephosphorylation events that was detected by the phospho-ATM/ATR substrate antibody was independent of the phosphatase inhibitor calyculin A, whereas dephosphorylation of the other proteins was sensitive to this compound. This suggests that 2 different pathways lead to protein S/T dephosphorylation: one involving either PP1 and/or PP2A and one that is independent. We investigated other inhibitors, including okadaic acid, but we could not demonstrate any dramatic changes in phosphorylation patterns. Calyculin A has previously been used as a phosphatase inhibitor in boar sperm, and it greatly enhanced the phosphorylation of S/T PKA proteins (Harrison, 2004) and caused increased sperm motility (Holt and Harrison, 2002). A recent study by Adachi et al (2008) using immunolocalization but not Western blotting reported S/T dephosphorylation in the postacrosomal region during capacitation. These proteins showed increased phosphorylation when sperm were incubated with calyculin A. It is possible that one of the bands observed in our study may also be the protein(s) observed by Adachi et al (2008), suggesting a possible functional role for these phosphorylated proteins before and after ejaculation in boar sperm.

Since dephosphorylation has been demonstrated as affecting multiple proteins in sperm incubated under C conditions, the next step is to identify these proteins. Unfortunately, the size of these proteins does not suggest any likely candidates based on the literature. Furthermore, although these are kinase substrate antibodies, it is possible that these proteins do not lie downstream of the kinases that denote the name of the antibody. This was shown to be true for the phospho-Akt substrate antibody, which also recognizes phosphorylation of S6 which is regulated by S6 kinase not Akt (Kane et al, 2002). Thus, we are currently undertaking a proteomic approach to characterize proteins of the appropriate molecular weight in sperm in which we will search for the R-X-R-R-X-S*/T* motif. In combination with immunoprecipitation, we believe this approach has the potential to identify these proteins from boar sperm.

In conclusion, S/T protein dephosphorylation in sperm incubated under C conditions has been reported

19394640, 2010, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.2164/jandrol.109.008383 by Libya Hinari NPL, Wiley Online Library on [08/09/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

for the first time in boar sperm. It affects multiple proteins, and this observation has not previously been reported in mammalian sperm. Interestingly, our data show 2 pathways of protein dephosphorylation, with one regulated by PP1 and/or PP2A. Identification of these proteins may shed further light on the possible function of such dephosphorylation events during sperm capacitation or on the role of the protein S/T phosphorylation in N conditions prior to deposition in the female tract.

Acknowledgment

We thank Professor Harry Moore (University of Sheffield) for the 18.6 monoclonal antibody.

References

Adachi J, Tate S, Miyake M, Harayama H. Effects of protein phosphatase inhibitor calyculin A on the postacrosomal protein serine/threonine phosphorylation state and acrosome reaction in boar spermatozoa incubated with a cAMP analog. *J Reprod Fertil*. 2008;54(3):171–176.

Aitken RJ, Harkiss D, Knox W, Paterson M, Irvine DS. A novel signal transduction cascade in capacitating human spermatozoa characterised by a redox-regulated, cAMP-mediated induction of tyrosine phosphorylation. *J Cell Sci*. 1998;111(Pt 5):645–656.

Alessi DR, Caudwell FB, Andjelkovic M, Hemmings BA, Cohen P. Molecular basis for the substrate specificity of protein kinase B; comparison with MAPKAP kinase-1 and p70 S6 kinase. *FEBS Lett*. 1996;399(3):333–338.

Austin CR. Observations on the penetration of the sperm in the mammalian egg. *Aust J Sci Res B*. 1951;4(4):581–596.

Boerke A, Tsai PS, Garcia-Gil N, Brewis IA, Gadella BM. Capacitation-dependent reorganization of microdomains in the apical sperm head plasma membrane: functional relationship with zona binding and the zona-induced acrosome reaction. *Theriogenology*. 2008;70(8):1188–1196.

Brewis IA, Clayton R, Barratt CL, Hornby DP, Moore HD. Recombinant human zona pellucida glycoprotein 3 induces calcium influx and acrosome reaction in human spermatozoa. *Mol Hum Reprod*. 1996;2(8):583–589.

Chang MC. Fertilizing capacity of spermatozoa deposited into the fallopian tubes. *Nature*. 1951;168(4277):697–698.

Chen Y, Cann MJ, Litvin TN, Iourgenko V, Sinclair ML, Levin LR, Buck J. Soluble adenylyl cyclase as an evolutionarily conserved bicarbonate sensor. *Science*. 2000;289(5479):625–628.

Dubé C, Tardif S, LeClerc P, Bailey JL. The importance of calcium in the appearance of p32, a boar sperm tyrosine phosphoprotein, during in vitro capacitation. *J Androl*. 2003;24(5):727–733.

Flesch FM, Colenbrander B, van Golde LM, Gadella BM. Capacitation induces tyrosine phosphorylation of proteins in the boar sperm plasma membrane. *Biochem Biophys Res Commun*. 1999; 262(3):787–792.

Flesch FM, Gadella BM. Dynamics of the mammalian sperm plasma membrane in the process of fertilization. *Biochim Biophys Acta*. 2000;1469(3):197–235.

Gadella BM, Harrison RA. The capacitating agent bicarbonate induces protein kinase A-dependent changes in phospholipid transbilayer behavior in the sperm plasma membrane. *Development*. 2000;127(11):2407–2420.

Gadella BM, Tsai PS, Boerke A, Brewis IA. Sperm head membrane reorganisation during capacitation. *Int J Dev Biol*. 2008;52(5–6):473–480.

Galantino-Homer HL, Visconti PE, Kopf GS. Regulation of protein tyrosine phosphorylation during bovine sperm capacitation by a cyclic adenosine 3′5′-monophosphate-dependent pathway. *Biol Reprod*. 1997;56(3):707–719.

Harayama H. Viability and protein phosphorylation patterns of boar spermatozoa agglutinated by treatment with a cell-permeable cyclic adenosine 3′,5′-monophosphate analog. *J Androl*. 2003;24(6): 831–842.

Harayama H, Miyake M. A cyclic adenosine 3′,5′-monophosphate-dependent protein kinase C activation is involved in the hyperactivation of boar spermatozoa. *Mol Reprod Dev*. 2006;73(9):1169–1178.

Harayama H, Muroga M, Miyake M. A cyclic adenosine 3′,5′-monophosphate-induced tyrosine phosphorylation of Syk protein tyrosine kinase in the flagella of boar spermatozoa. *Mol Reprod Dev*. 2004;69(4):436–447.

Harayama H, Nakamura K. Changes of PKA and PDK1 in the principal piece of boar spermatozoa treated with a cell-permeable cAMP analog to induce flagellar hyperactivation. *Mol Reprod Dev*. 2008;75(9):1396–1407.

Harrison RA. Capacitation mechanisms, and the role of capacitation as seen in eutherian mammals. *Reprod Fertil Dev*. 1996;8(4): 581–594.

Harrison RA. Rapid PKA-catalysed phosphorylation of boar sperm proteins induced by the capacitating agent bicarbonate. *Mol Reprod Dev*. 2004;67(3):337–352.

Holt WV, Harrison RA. Bicarbonate stimulation of boar 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. *J Androl*. 2002;23(4):557–565.

Hunter T. The Croonian Lecture 1997. The phosphorylation of proteins on tyrosine: its role in cell growth and disease. *Philos Trans R Soc Lond B Biol Sci*. 1998;353(1368):583–605.

Ishihara H, Martin BL, Brautigan DL, Karaki H, Ozaki H, Kato Y, Fusetani N, Watabe S, Hashimoto K, Uemura D, Hartshorne DJ. Calyculin A and okadaic acid: inhibitors of protein phosphatase activity. *Biochem Biophys Res Commun*. 1989;159(3):871–877.

Jha KN, Shivaji S. Protein serine and threonine phosphorylation, hyperactivation and acrosome reaction in in vitro capacitated hamster spermatozoa. *Mol Reprod Dev*. 2002;63(1):119–130.

Kalab P, Peknicová J, Geussová G, Moos J. Regulation of protein tyrosine phosphorylation in boar sperm through a cAMP-dependent pathway. *Mol Reprod Dev*. 1998;51(3):304–314.

Kane S, Sano H, Liu SC, Asara JM, Lane WS, Garner CC, Lienhard GE. A method to identify serine kinase substrates. Akt phosphorylates a novel adipocyte protein with a Rab GTPase-activating protein (GAP) domain. *J Biol Chem*. 2002;277(255):22115–22118.

Kaneto M, Krisfalusi M, Eddy EM, O'Brien DA, Miki K. Bicarbonate-induced phosphorylation of p270 protein in mouse sperm by cAMP-dependent protein kinase. *Mol Reprod Dev*. 2008;75(6):1045–1053.

Leclerc P, de Lamirande E, Gagnon C. Cyclic adenosine 3′,5′mono-phosphate-dependent regulation of protein tyrosine phosphorylation in relation to human sperm capacitation and motility. *Biol Reprod*. 1996;55(3):684–692.

O'Flaherty C, de Lamirande E, Gagnon C. Phosphorylation of the arginine-X-X-(serine/threonine) motif in human sperm proteins during capacitation: modulation and protein kinase A dependency. *Mol Hum Reprod*. 2004;10(5):355–363.

Okamura N, Tajima Y, Soejima A, Masuda H, Sugita Y. Sodium bicarbonate in seminal plasma stimulates the motility of mamma-

19394640, 2010, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.2164/jandrol.109.008383 by Libya Hinari NPL, Wiley Online Library on [08/09/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

lian spermatozoa through direct activation of adenylate cyclase. *J Biol Chem*. 1985;260(17):9699–9705.

Parrish JJ, Susko-Parrish J, Winer MA, First NL. Capacitation of bovine sperm by heparin. *Biol Reprod*. 1988;38(5):1171–1180.

Rowe M, Jones M. Detection of EBV latent proteins by western blotting. *Methods Mol Biol*. 2001;174:229–242.

Salicioni AM, Platt MD, Wertheimer EV, Arcelay E, Allaire A, Sosnik J, Visconti PE. Signalling pathways involved in sperm capacitation. *Soc Reprod Fertil Suppl*. 2007;65:245–259.

Tardif S, Dubé C, Chevalier S, Bailey JL. Capacitation is associated with tyrosine phosphorylation and tyrosine kinase-like activity of pig sperm proteins. *Biol Reprod*. 2001;65(3):784–792.

Visconti PE, Bailey JL, Moore GD, Pan D, Olds-Clarke P, Kopf GS. Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. *Development*. 1995;121(4):1129–1137.

Visconti PE, Galantino-Homer H, Ning X, Moore GD, Valenzuela JP, Jorgez CJ, Alvarez JG, Kopf GS. Cholesterol efflux-mediated signal transduction in mammalian sperm. β-cyclodextrins initiate transmembrane signaling leading to an increase in protein tyrosine phosphorylation and capacitation. *J Biol Chem*. 1999a;274(5):3235–3242.

Visconti PE, Kopf GS. Regulation of protein phosphorylation during sperm capacitation. *Biol Reprod*. 1998;59(1):1–6.

Visconti PE, Ning X, Fornes MW, Alvarez JG, Stein P, Connors SA, Kopf GS. Cholesterol efflux-mediated signal transduction in mammalian sperm: cholesterol release signals an increase in protein tyrosine phosphorylation during mouse sperm capacitation. *Dev Biol*. 1999b;214(2):429–443.

Visconti PE, Westbrook VA, Chertihin O, Demarco I, Sleight S, Diekman AB. Novel signaling pathways involved in sperm acquisition of fertilizing capacity. *J Reprod Immunol*. 2002;53(1–2):133–150.

19394640, 2010, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.2164/jandrol.109.008383 by Libya Hinari NPL, Wiley Online Library on [08/09/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License