Molecular Human Reproduction Vol.10, No.5 pp. 355–363, 2004 DOI: 10.1093/molehr/gah046

Phosphorylation of the Arginine-X-X-(Serine/Threonine) motif in human sperm proteins during capacitation: modulation and protein kinase A dependency

C.O'Flaherty[1], E.de Lamirande and C.Gagnon

Urology Research Laboratory, Royal Victoria Hospital and Faculty of Medicine, McGill University, Montréal, Québec, Canada H3A 1A1

[1]To whom correspondence should be addressed at: Urology Research Laboratory, H6.44, Royal Victoria Hospital, 687 Pine Avenue West, Montréal, Québec, Canada H3A 1A1. E-mail: coflaher@yahoo.com

Sperm capacitation is a complex process that involves a protein kinase A (PKA)-dependent tyrosine phosphorylation of proteins. We studied the time-course, the modulation and the cellular localization of the phosphorylation of the Arginine-X-X-(Serine/Threonine) motif, characteristic of PKA substrates, in sperm proteins during capacitation. There was an increased phosphorylation of 80 (p80) and 105 (p105) kDa protein bands in human sperm treated with different capacitation inducers. Phosphorylation of p80 and p105 induced by fetal cord serum ultrafiltrate or the combination of 3-isobutyl-1-methylxanthine and dibutyryl cAMP was prevented by H89 and Rp-adenosine-3′,5′-cyclic monophosphorothionate, confirming the involvement of PKA in this effect. Inhibitors of protein kinase C, receptor type tyrosine kinase and mitogen-activated protein kinase kinase did not affect the Arginine-X-X-(Serine/Threonine) motif phosphorylation. Non-receptor type protein tyrosine kinase inhibitors, PP2 and herbimycin A, enzymatic antioxidants and a nitric oxide synthase inhibitor prevented the phosphorylation of p80 and p105 when sperm were incubated with fetal cord serum ultrafiltrate. The phosphorylated Arginine-X-X-Serine/Threonine motif was immunolocalized all along the flagellum and the fluorescent signal was higher in capacitating than in non-capacitating sperm. These results show for the first time the presence of a PKA-dependent phosphorylation of proteins in human sperm capacitation and its upstream modulation by reactive oxygen species and non-receptor type protein tyrosine kinase.

Key words: protein kinases/reactive oxygen species/spermatozoa/sperm capacitation/signal transduction

Introduction

Mammalian sperm must complete a series of morphological and metabolic changes collectively termed as 'capacitation' in order to acquire fertilizing capacity (Yanagimachi, 1994; de Lamirande *et al*., 1997). This is a poorly understood and complex process that involves different signal transduction elements, such as protein kinases A (PKA), C (PKC), protein tyrosine kinase (PTK), and the extracellular signal-regulated kinase (ERK) signalling pathway (de Lamirande *et al*., 1997; de Lamirande and Gagnon, 2002; Thundathil *et al*., 2002; Visconti *et al*., 2002).

The presence and activity of PKA have already been reported in bovine (Garbers *et al*., 1973), mouse (Visconti *et al*., 1997) and human (Lefièvre *et al*., 2002) sperm. The inhibition of sperm capacitation and the related protein tyrosine phosphorylation by H89 and Rp-adenosine-3′,5′-cyclic monophosphorothionate (Rp-cAMPS), both specific PKA inhibitors (Uguz *et al*., 1994; Leclerc *et al*., 1996; Galantino-Homer *et al*., 1997), suggested that PKA is involved in these processes. Conversely, treatment of sperm with phosphodiesterase inhibitors (ex: 3-isobutyl-1-methylxanthine (IBMX)] alone or in combination with a cell permeant analogue of cAMP (dibutyryl cAMP, dbcAMP) promoted sperm capacitation, confirming the importance of PKA in this process (Visconti *et al*., 1995; Leclerc *et al*., 1996). In human sperm treated with fetal cord serum ultrafiltrate (FCSu) as a capacitating agent, there was a 30% increase in PKA activity 30 min after the beginning of the incubation period (Lefièvre *et al*., 2002).

PKA is a ubiquitous tetrameric enzyme containing two regulatory (R) and two catalytic (C) subunits, and its activity is dependent on cAMP. PKA phosphorylates proteins on Serine (Ser) and Threonine (Thr) within the motif Arginine (Arg)-X-X-Ser/Thr (X represents any amino acid) (Bruce *et al*., 2002; Grøndborg *et al*., 2002). In human and mouse sperm, PKA was found in both the acrosomal cap and the flagellum (Pariset and Weinman, 1994; Visconti *et al*., 1997).

The cAMP/PKA system is needed for the tyrosine phosphorylation of 81 and 105 kDa proteins that occurs during capacitation induced by FCSu, bovine serum albumin (BSA), follicular fluid ultrafiltrate (FFu), progesterone, dbcAMP, and/or IBMX (Uhler *et al*., 1992; de Lamirande and Gagnon, 1995; Leclerc *et al*., 1996; Aitken *et al*., 1998; de Lamirande *et al*., 1998; Herrero *et al*., 2000). These proteins are from the fibrous sheath (Visconti *et al*., 1995; Aitken *et al*., 1998; Leclerc *et al*., 1998) and are antigenically related to A kinase anchoring proteins (AKAP) (Carrera *et al*., 1996).

There is accumulating evidence for the participation of reactive oxygen species (ROS) such as the superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and nitric oxide (NO•) in human sperm capacitation (de Lamirande *et al*., 1997; Aitken *et al*., 1998; Herrero and Gagnon, 2001), and the associated triggering of the protein tyrosine phosphorylation (Leclerc *et al*., 1997; Aitken *et al*., 1998). Levels of cAMP

are increased by NO• (Herrero *et al*., 2000), H_2O_2 (Aitken *et al*., 1998) and O_2^{-}• (Zhang and Zheng, 1996). However, superoxide dismutase (SOD, a O_2^{-}• scavenger) did not block the IBMX-induced capacitation, suggesting the participation of O_2^{-}• upstream of PKA during this process (de Lamirande *et al*., 1997).

Although there is consensus that PKA activity is associated with the increase of protein tyrosine phosphorylation (Visconti *et al*., 1995; Leclerc *et al*., 1996, 1997; Aitken *et al*., 1998), the events occurring between the early activation of PKA (Visconti *et al*., 1997; Lefièvre *et al*., 2002) and the late protein tyrosine phosphorylation remain unknown. It was hypothesized that PKA phosphorylates some enzymes/proteins as an intermediate step and that these phosphorylated PKA substrates are involved in the capacitation-related protein tyrosine phosphorylation. Therefore, our first objective was to determine whether the level of phosphorylated Arg-X-X-Ser/Thr motif, characteristic of PKA substrates, was modified in sperm proteins during capacitation. The second objective was to study the regulation of this phosphorylation by ROS and signal transduction kinases and determine its cellular localization.

Materials and methods

Materials

The following reagents were purchased from Sigma Chemical Company (USA): BSA (fatty acid-free), IBMX, dbcAMP (N^6,2′-*O*-dibutyryl cAMP), progesterone, H89 {N-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulphonamide}, L-NAME (N^G-nitro-L-arginine methyl ester), D-NAME (N^G-nitro-D-arginine methyl ester), phospho-Serine (phospho-Ser), phospho-Threonine (phospho-Thr) and phospho-Tyrosine (phospho-Tyr). DPI (diphenyliodonium chloride) was bought from Aldrich Chemical Company (USA). Spermine NONOate [*N*-(2-aminoethyl)-*N*-(2-hyrdoxy-3-methrosohydrazino)-1,2-ethylenedeamine] was bought from Cayman Chemical Company (USA). PD98059 (2′-amino-3′-methoxyflavone), chelerythrine, herbimycin A, PP2 {4-amino-5-(4-chloropenyl)-7-(*t*-butyl)pyrazolo[3,4-d] pyrimidine}, PP3 (4-amino-7-phenylpyrazol[3,4-d]-pyrimidine), tyrphostin A47, U126 [1,4-diamino-2,3-dicyano-1,4*bis*(2-aminophenylthio)butadiene], okadaic acid, bovine liver catalase (21 000 IU/mg), and Rp-cAMPS were purchased from Calbiochem (USA). SOD from bovine erythrocytes was bought from Roche Molecular Biochemicals (Canada). Percoll was purchased from Amersham Pharmacia Biotech (Canada). Nitrocellulose (0.22 μm pore size; Osmonics Inc., USA), the antibody raised against the phosphorylated Arg-X-X-Ser/Thr motif (anti-phospho PKA substrates antibody; Cell Signaling Technology, USA), donkey anti-rabbit IgG conjugated to horseradish peroxidase (Cedarlane Laboratories Ltd, Canada), an enhanced chemiluminiscence kit (Lumi-Light; Roche Molecular Biochemicals) and radiographic films (Fuji, Japan) were used for immunodetection of blotted proteins. The blocking agent of the anti-Arg-X-X-phospho (Ser/Thr) antibody was generously provided by Cell Signaling Technology, USA). Alexa Fluor 555 conjugate of streptavidin and Prolong Antifade kit were purchased from Molecular Probes (USA). All other chemicals were at least of reagent grade.

Fetal cord blood was collected at the birthing centre of the Royal Victoria Hospital (Montréal, QC, Canada) and follicular fluid was collected from pre-ovulatory follicles after gonadotrophin stimulation at the IVF centre of the Royal Victoria Hospital. In both cases, informed consent was obtained from the patients and the ethics board of the Royal Victoria Hospital approved the present study. Fetal cord blood and follicular fluid samples were centrifuged (1000 *g*, 30 min, 4°C), and supernatants were pooled and frozen at –20°C until used. The FCSu and FFu were prepared from three pools of 14–24 different samples using YM3 membranes with an exclusion limit of 3 kDa (Amicon, Canada) (de Lamirande and Gagnon, 1995). Silver stain indicated that no protein was present in 10% polyacrylamide gels loaded with FCSu or FFu alone. FCSu was used as capacitation inducer as it was shown to trigger capacitation and hyperactivated motility to similar levels and with similar kinetics as other agents such as BSA (de Lamirande *et al*.., 1997). Furthermore, as observed in other systems, FCSu-induced capacitation is associated with a cAMP-dependent tyrosine phosphorylation of fibrous sheath proteins (Leclerc *et al*., 1997), the generation of ROS (de Lamirande and Gagnon, 1995; Herrero *et al*., 2000), activation of PKA (Lefièvre *et al*., 2002), and modifications of the sulphydryl content of Triton-soluble proteins (de Lamirande and Gagnon, 2003).

Inhibitors and activators used in this study were dissolved in water or dimethylsulphoxide (DMSO). The concentration of DMSO in the incubation media never exceeded 1% (v/v), a condition that does not affect sperm capacitation. None of the chemicals tested caused a decrease in sperm motility over a 3.5 h incubation period at 37°C.

Sperm preparation

Semen samples from 12 healthy volunteers used in this study were normal according to World Health Organization (1992) criteria. Semen samples were washed on four-layer (95–65–40–20%) Percoll gradient buffered in HEPES-balanced saline (115 mmol/l NaCl, 4 mmol/l KCl, 0.5 mmol/l $MgCl_2$, 14 mmol/l fructose, 25 mmol/l HEPES, pH 8.0). Samples were centrifuged for 30 min at 2300 *g*, and sperm at the 65–95% Percoll interface and in the 95% Percoll layer were pooled and diluted to 250×10^6 cells/ml with the 95% Percoll solution. Only samples in which progressive motility was >70% were used. Sperm were further diluted to 50×10^6 cells/ml in Biggers–Whitten–Whittingham medium (BWW) (Biggers *et al*., 1971) devoid of bicarbonate and BSA and containing 1 mmol/l $CaCl_2$ and 25 mmol/l HEPES (pH 8.0).

SDS–PAGE and immunobloting

At the end of each experiment, treated samples were supplemented with electrophoresis buffer containing vanadate (100 μmol/l), β-glycerolphosphate (20 mmol/l), sodium fluoride (5 mmol/l), and okadaic acid (10 nmol/l), incubated at 100°C for 5 min and then centrifuged at 21 000 *g* for 5 min. Sperm proteins were electrophoresed on 10% polyacrylamide gels and electro-transferred to nitrocellulose membranes using 10 mmol/l CAPS (3-cyclohexylamino-1-propane sulphonic acid) buffer (pH 11) containing 10% methanol. The membranes were incubated with a solution of skim milk (5%, w/v) in Tris (20 mmol/l, pH 7.8)-buffered saline containing Tween 20 (0.1%, v/v) (TTBS) for 20 min. We used an antibody that detects a phosphorylated motif (Arg-X-X-Ser/Thr) characteristic of PKA substrates. It was diluted 1:1000 (v/v) in TTBS supplemented with 25 mg/ml BSA and 0.1% (w/v) sodium azide and then incubated with the membrane overnight at 4°C. After washing with TTBS, membranes were incubated with donkey anti-rabbit IgG conjugated with horseradish peroxidase [diluted 1:2500 (v/v) in TTBS] for 45 min at 20°C and washed again with TTBS. Positive immunoreactive bands were detected using the Lumi-Light chemiluminiscence kit. At the end of all the experiments, blots were rinsed in distilled water and silver-stained (Jacobson and Karsnas, 1990) to ascertain that the amount of protein loaded in each well was the same.

The anti-Arg-X-X-phospho-(Ser/Thr) antibody was preadsorbed with the blocking agent, as recommended by Cell Signaling Technology for 2 h at 20°C, to confirm the specificity of the antibody (Figure 1B). The anti-Arg-X-X-phospho-(Ser/Thr) antibody was also preadsorbed with a combination phospho-Ser, phospho-Thr and phospho-Tyr (each of them at a molar concentration 10 000-fold higher than that of the antibody) to confirm that the antibody did not non-specifically recognize any phosphorylated amino acid.

Immunolocalization of the Arg-X-X-phospho-(Ser/Thr) motif in capacitating sperm

Sperm suspensions were incubated without or with FCSu (10% v/v) at 37°C for 30 min and prepared for immunocytochemistry. Smears were prepared on Superfrost Plus slides (Fischer Scientific, Canada). After permeabilization by methanol and rehydration, smears were treated with 5% goat serum in PBS for 30 min, washed with PBS containing 0.1% Triton X-100 (PBS-T), and incubated with the anti-Arg-X-X-(phospho-Ser/Thr) antibody (dilution 1:100) for 2 h at 20°C. Smears were then washed with PBS-T and incubated with biotinylated goat anti-rabbit antibody (dilution 3:1000) for 1 h, and then with an Alexa Fluor 555 conjugate of streptavidin (1:500 w/v) in PBS-T. Smears were mounted with Prolong Antifade and observed under a Carl Zeiss (Germany) Axiophot microscope (exciter filter BP450–490) at ×1000 magnification. As controls, smears were incubated with the anti-Arg-X-X-(phospho-Ser/Thr) antibody preadsorbed with the blocking agent or with the biotinylated goat

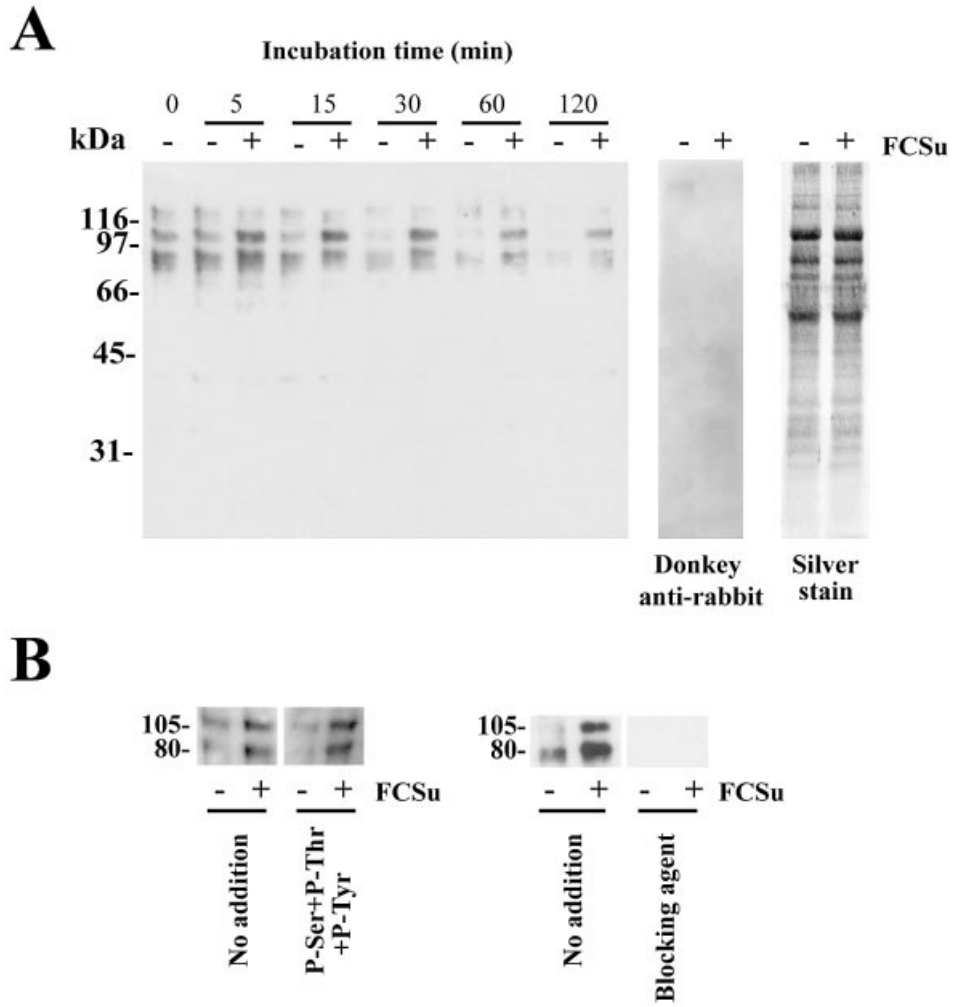

Figure 1. (**A**) Time-course for the phosphorylation of the Arg-X-X-Ser/Thr motif in human sperm during capacitation induced by fetal cord serum ultrafiltrate (FCSu). Percoll-washed sperm resuspended in Biggers–Whitten–Whittingham medium were incubated in the absence (–) or presence (+) of FCSu (10%, v/v). Proteins from 0.5×10^6 cells were loaded in each well, electrophoresed (10% polyacrylamide gels), electrotransferred, and immunoblotted using the anti-Arg-X-X-(phospho-Ser/Thr) antibody. The position of the molecular mass markers is indicated on the left. Blot of sperm proteins incubated with the donkey anti-rabbit antibody is presented as a control. A silver stain blot is presented on the right. (**B**) Specificity of the antibody. Incubation with the preadsorbed anti-Arg-X-X-phospho-(Ser/Thr) antibody with a mixture of phospho-Ser (P-Ser), phospho-Thr (P-Thr) and phospho-Tyr (P-Tyr) (each at molar concentration 10 000-fold higher than that of the antibody), or with the blocking agent (prepared as manufacturer recommended). Results are from one experiment representative of three others ($n = 4$) performed with semen samples from different donors.

anti-rabbit antibody alone or with the Alexa Fluor 555 conjugate of streptavidin alone.

Phosphorylation of the Arg-X-X-Ser/Thr motif during capacitation: time-course and involvement of PKA

Sperm samples in BWW were incubated without (control) or with the capacitation inducer FCSu (10%, v/v) and aliquots were taken at 0, 5, 15, 30, 60 and 120 min. Because the phosphorylation reached a plateau after 15–30 min incubation and because the maximal PKA activity of FCSu-treated sperm occurs at 30 min (Lefièvre *et al*., 2002), the following experiments were performed with a 30 min incubation time. Sperm were also incubated with progesterone (10 µmol/l), FFu (10% v/v), IBMX (0.5 mmol/l), the combination IBMX (0.1 mmol/l) + dbcAMP (1 mmol/l) or BSA (3 mg/ml). All incubations were performed at 37°C. Only for BSA-treated sperm were cells submitted to a one-step wash over a 20% Percoll layer (2000 *g*, 5 min) to remove most the BSA present in the incubation medium; sperm were collected by aspiration of the loose pellet through the Percoll layer as previously described (de Lamirande *et al*., 1998).

To confirm that PKA is involved in the phosphorylation of proteins recognized by the antibody used, sperm were incubated with FCSu (10% v/v) or IBMX (0.1 mmol/l) + dbcAMP (1 mmol/l) in the presence of H89 (10 µmol/l), Rp-cAMPS (200 µmol/l) or the combination of H89 + Rp-cAMPS.

A Triton X-100 extraction was also performed. Control and capacitating sperm were concentrated to 200×10^6 cells/ml. After the addition of vanadate (100 µmol/l), β-glycerolphosphate (20 mmol/l), sodium fluoride (5 mmol/l), and okadaic acid (10 nmol/l), sperm were incubated with Triton X-100 (0.2%, v/v) for 10 min on ice, and then centrifuged (12 000 *g*). The Triton-soluble and -insoluble (resuspended to the original volume with HEPES-balanced saline containing vanadate, β-glycerolphosphate, sodium fluoride, okadaic acid and Triton X-100 at the same concentrations used above) fractions were used for immunoblotting as described above.

Phosphorylation of the Arg-X-X-Ser/Thr motif during capacitation: regulation by ROS and kinases

The role of ROS on the phosphorylation of the Arg-X-X-Ser/Thr motif in human sperm was first studied by the addition of 50 µmol/l H_2O_2 or 100 µmol/l spermine-NONOate (sp-NONOate) to sperm. The effect of catalase (0.1 mg/ml), SOD (0.1 mg/ml), DPI (100 µmol/l), L-NAME (1 mmol/l) or D-NAME (1 mmol/l) on the phosphorylation of the Arg-X-X-(Ser/Thr) motif induced by FCSu or IBMX + dbcAMP was also studied. In another set of experiments, the

effect of SOD + catalase + L-NAME on sperm treated with IBMX + dbcAMP was studied.

To determine the role of kinases in the phosphorylation of the Arg-X-X-Ser/Thr motif, sperm were supplemented or not with FCSu (10%, v/v), in the absence or presence of chelerythrine (10 µmol/l), tyrphostin A47 (10 µmol/l), PD98059 (100 µmol/l) or U126 (0.3 µmol/l), herbimycin A (10 µmol/l), PP2 (10 nmol/l) or PP3 (10 nmol/l). All the inhibitors used in this study were added to sperm 30 min before the capacitation inducers.

Results

Phosphorylation of the Arg-X-X-Ser/Thr motif increases in sperm incubated under capacitating conditions

The antibody raised against the Arg-X-X-(phospho-Ser/Thr) motif recognized few sperm proteins bands of 40, 80, 105, 116 and 140 kDa (Figure 1A). When sperm were incubated under capacitating conditions (FCSu), there was an increased phosphorylation of the 80 (p80) and 105 (p105) kDa protein bands as compared with that noted in sperm in BWW alone. This FCSu-related increase was observed as early as 5 min after the beginning of the incubation, plateaued at 15 and 30 min and then decreased (Figure 1A). In sperm incubated with BWW alone there was a basal phosphorylation which was always lower than that observed in capacitating sperm, and even decreased with time. Because of this early phosphorylation, and because the maximal PKA activity of FCSu-treated sperm occurs at 30 min (Lefièvre *et al*., 2002), the following experiments were performed with a 30 min incubation time. Only the two protein bands p80 and p105 will be presented since the changes in the Arg-X-X-(phospho-Ser/Thr) motif related with capacitation were observed only in these proteins. We also observed a time-dependent diminution of the phosphorylation of the Arg-X-X-(Ser/Thr) motif in proteins of 40 and 140 kDa, but there was no difference between sperm incubated in the presence of FCSu or BWW alone and the phosphorylation decreased with time. Blots of sperm proteins incubated with the second antibody alone did not present any bands (Figure 1A).

The two protein bands p80 and p105 were not detected when the anti-Arg-X-X-(phospho-Ser/Thr) antibody was preadsorbed with the blocking agent, confirming the specificity of the antibody. (Figure 1B). Furthermore, the anti-Arg-X-X-(phospho-Ser/Thr) antibody preadsorbed with a mixture of phospho-Ser, phospho-Thr and phospho-Tyr was as efficient as the untreated antibody in binding to p80 and p105 (Figure 1B), confirming that the antibody did not recognize unspecifically all phospho amino acid. To confirm that the same amount of protein was loaded in all the wells, blots were always silver-stained at the end of the experiments (Figure 1A).

The proteins p80 and p105 carrying the Arg-X-X-(phospho-Ser/Thr) motif were found in the sperm Triton-insoluble fraction (Figure 2). The protein band at 40 kDa recognized by the antibody in whole sperm samples and not modified by capacitation conditions (Figure 1A) was found in the Triton-soluble fraction (Figure 2).

Other inducers used to study *in vitro* capacitation, FFu, progesterone (Uhler *et al*., 1992; de Lamirande and Gagnon, 1995; de Lamirande *et al*., 1998; Leclerc *et al*., 1998), IBMX, IBMX + dbcAMP (Visconti *et al*., 1995; Leclerc *et al*., 1996) and BSA (Aitken *et al*., 1998; Herrero *et al*., 2000) were also tested. The effects of different biological fluids and pharmacological compounds used in this study on human sperm capacitation are summarized in Table I. There was an increase in the phosphorylation of the Arg-X-X-Ser/Thr motif in all the cases (Figure 3). These data indicated that the effect observed was related to the capacitation process and was not specific for FCSu. Moreover, the levels of phosphorylation were low

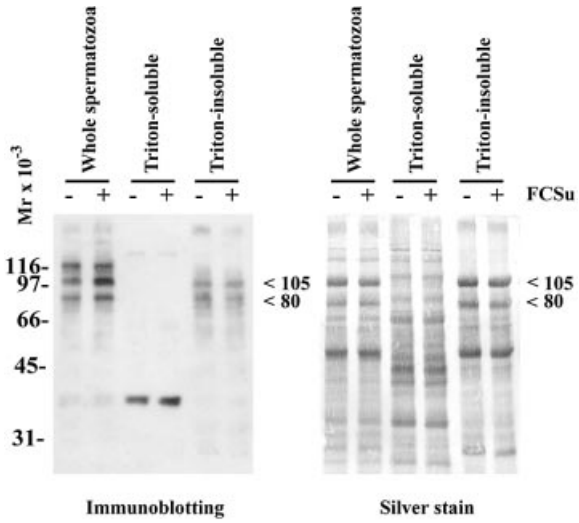

Figure 2. Presence of the phosphorylated Arg-X-X-Ser/Thr motif in Triton-soluble and -insoluble sperm extracts. Percoll-washed sperm were incubated for 30 min in the absence (–) or presence (+) of fetal cord serum ultrafiltrate (FCSu). Extracts were prepared as described in Materials and methods. Proteins from whole sperm (0.5×10^6 cells/well), Triton-soluble (equivalent to 2.5×10^6 cells/well) and Triton-insoluble (0.5×10^6 cells/well) extracts, were immunoblotted as described previously. A silver-stained blot is shown to present the relative amount of protein in each well. Results are from one experiment representative of one other (n = 2) performed with semen samples from different donors.

Table I. Effects of different biological fluids and compounds on human sperm capacitation

	References
Activators	
Fetal cord serum ultrafiltrate	de Lamirande and Gagnon, 1993
Follicular fluid ultrafiltrate	de Lamirande *et al*., 1995
Bovine serum albumin	Aitken *et al*., 1998; Herrero *et al*., 2000
Progesterone	Uhler *et al*., 1992
IBMX (phosphodiesterase inhibitor)	Leclerc *et al*., 1996
dbcAMP (permeable cAMP analogue)	Leclerc *et al*., 1996
Superoxide anion ($O_2^{-\bullet}$)	de Lamirande and Gagnon, 1995
Hydrogen peroxide (H_2O_2)	Griveau *et al*., 1994; Leclerc *et al*., 1997
Sp-NONOate (a NO• generator)	Zini *et al*., 1995; Thundathil *et al*., 1993
Inhibitors	
Thyrphostin A47 (inhibitor of receptor-type tyrosine kinase)	Leclerc *et al*., 1997
PD98059 (inhibitor of MEK activation by Raf)	de Lamirande and Gagnon, 2002
U126 (inhibitor of phosphorylated MEK action)	de Lamirande and Gagnon, 2002
Chelerythrine (inhibitor of PKC)	Thundathil *et al*., 2002
H89 (inhibitor of PKA)	Leclerc *et al*., 1996
Rp-cAMPS (inactive analogue of cAMP)	Leclerc *et al*., 1996; Aitken *et al*., 1998
Superoxide dismutase ($O_2^{-\bullet}$ scavenger)	de Lamirande and Gagnon, 1993
Catalase (H_2O_2 scavenger)	Girevau *et al*., 1994
L-NAME (inhibitor of nitric oxide synthase)	Herrero *et al*., 2000
DPI (inhibitor of flavin containing enzymes such as NADPH oxidases)	de Lamirande *et al*., 1997
PP2 (inhibitor of non-receptor type tyrosine kinase)	Thundathil *et al*., 2002
Herbimycin A (inhibitor of non-receptor type tyrosine kinase inhibitor)	Leclerc *et al*., 1997

in sperm incubated in BWW alone and the effect of FCSu was reproducible as the data presented in Figure 3 are from three different donors.

PKA is involved in the phosphorylation of the Arg-X-X-Ser/Thr motif

The Arg-X-X-(Ser/Thr) motif is characteristic of PKA substrates. The pharmacological increase of intracellular cAMP by treatment with IBMX or IBMX + dbcAMP induces sperm capacitation (Leclerc *et al*., 1996; Aitken *et al*., 1998) and increases in Arg-X-X-(Ser/Thr) phosphorylation (Figure 4A), suggesting a role for PKA. Conversely, the phosphorylation of the Arg-X-X-Ser/Thr motif in p80 and p105 induced by FCSu was partially prevented by H89, (a specific PKA inhibitor) or Rp-cAMPS (a cell-permeant inactive analogue of cAMP), but the inhibition was complete when both compounds were present (Figure 4B). Additional indication of the involvement of PKA in the phosphorylation of the Arg-X-X-(Ser/Thr) motif was obtained when pharmacological conditions, IBMX + dbcAMP, were used to promote sperm capacitation. The prevention of the phosphorylation of these bands was partial with Rp-cAMPS and complete with H89 or the combination of H89 + Rp-cAMPS (Figure 4A).

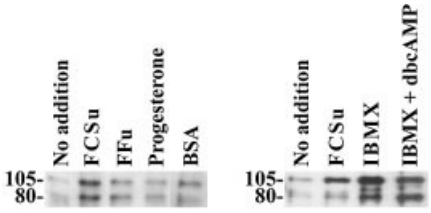

Figure 3. Different capacitation inducers trigger an increase in the phosphorylation of the Arg-X-X-Ser/Thr motif. Sperm were incubated with fetal cord serum ultrafiltrate (FCSu) (10%, v/v), follicular fluid ultrafiltrate (FFu) (10%, v/v), progesterone (10 µmol/l), bovine serum albumin (BSA) (3 mg/ml), 3-isobutyl-1-methylxanthine (IBMX) (0.5 mmol/l), or IBMX (0.1 mmol/l) + dbcAMP (1 mmol/l) in Biggers–Whitten–Whittingham medium for 30 min. Proteins from 0.5×10^6 cells were loaded in each well, and immunoblotted using the Arg-X-X-(phospho-Ser/Thr) motif antibody. Results presented are from the same gel and are from one experiment representative of four (FFu, IBMX), two (progesterone), six (IBMX + dbcAMP) others performed with semen samples from different donors.

ROS modulate the phosphorylation of the Arg-X-X-Ser/Thr motif in capacitating human sperm

The role of ROS, such as $O_2^{-\bullet}$, H_2O_2 and NO• in human sperm capacitation is now well established (de Lamirande *et al*., 1997; Aitken *et al*., 1998; Herrero *et al*., 2001). As ROS were shown to affect intracellular cAMP levels (Zhang and Zheng, 1996; Aitken *et al*., 1998; Herrero *et al*., 2000) and therefore PKA activity, the possible role of ROS in the modulation of the phosphorylation of the Arg-X-X-Ser/Thr motif detected in previous experiments was evaluated. SOD and catalase (scavengers of $O_2^{-\bullet}$ and H_2O_2, respectively) and L-NAME (a NO• synthase inhibitor) totally (on p80) and partially (on p105) prevented the increase in Arg-X-X-Ser/Thr phosphorylation of p80 and p105 bands in sperm treated with FCSu (Figure 5A); D-NAME (the inactive analogue of L-NAME) did not prevent the phosphorylation. The combination of SOD, catalase and L-NAME completely prevented the phosphorylation in both p80 and p105 (Figure 5A). On the other hand, when sperm were incubated with IBMX + dbcAMP in the presence of SOD, catalase or L-NAME, alone or in combination, no inhibition of the phosphorylation of the Arg-X-X-Ser/Thr motif on p80 and p105 was observed (Figure 5B). Exogenous addition of ROS using sp-NONOate (NO• donor) and H_2O_2 caused an increase in the intensity of the p80 and p105 bands (Figure 5C). The combination of xanthine and xanthine oxidase as a source of $O_2^{-\bullet}$ could not be used since it also requires addition of catalase to remove the H_2O_2 generated, an enzyme that prevents the effect of FCSu (Figure 5A). However, DPI—an inhibitor of flavin-containing enzymes such as NADPH oxidases (Hancock and Jones, 1987; O'Donnell *et al*., 1993) that inhibits the $O_2^{-\bullet}$ production in capacitating human sperm (de Lamirande *et al*., 1997)—caused a decrease in the phosphorylation of the Arg-X-X-Ser/Thr motif both in capacitating and non capacitating sperm, suggesting an involvement of $O_2^{-\bullet}$ in the modulation of the phosphorylation of this motif during capacitation with FCSu (Figure 5A).

PTK but not PKC or mitogen-activated protein kinase kinase (MEK) is involved in the modulation of the phosphorylation of the Arg-X-X-Ser/Thr motif in capacitating sperm

Herbimycin A and PP2 (inhibitors of non-receptor type PTK), but not tyrphostin A47 (inhibitor of receptor type PTK), chelerythrine (inhibitor of PKC) or PD98059 (100 µmol/l) and U126 (0.3 µmol/l) (inhibitors of MEK) totally prevented the phosphorylation of the

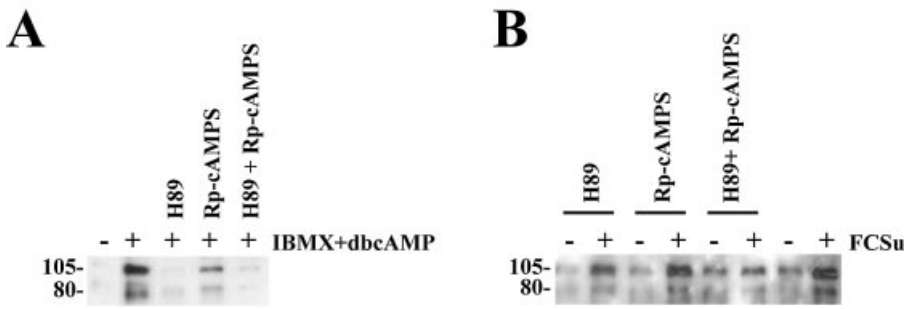

Figure 4. Participation of protein kinas A (PKA) on the phosphorylation of the Arg-X-X-Ser/Thr motif in sperm treated with 3-isobutyl-1-methylxanthine (IBMX) + dbcAMP or fetal cord serum ultrafiltrate (FCSu). Sperm were incubated (**A**) without (–) or with (+) IBMX (0.1 mmol/l) + dbcAMP (1 mmol/l) or (**B**) without (–) or with (+) fetal cord serum ultrafiltrate (FCSu) (10%, v/v), in the absence or presence of H89 (10 µmol/l), Rp-cAMPS (200 µmol/l) or the combination of H89 and Rp-cAMP. Proteins from 0.5×10^6 sperm were loaded in each well, and immunoblotted as described before. Results of one experiment representative of six (H89) or four (Rp-cAMPS and H89 + Rp-cAMPS) in the set of experiments performed with FCSu; of six (IBMX + dbcAMP), two (IBMX + dbcAMP without or with H89 or Rp-cAMPS) performed with sperm from different semen donors. Lines have been pasted but belong to the same blot and have the same film exposure.

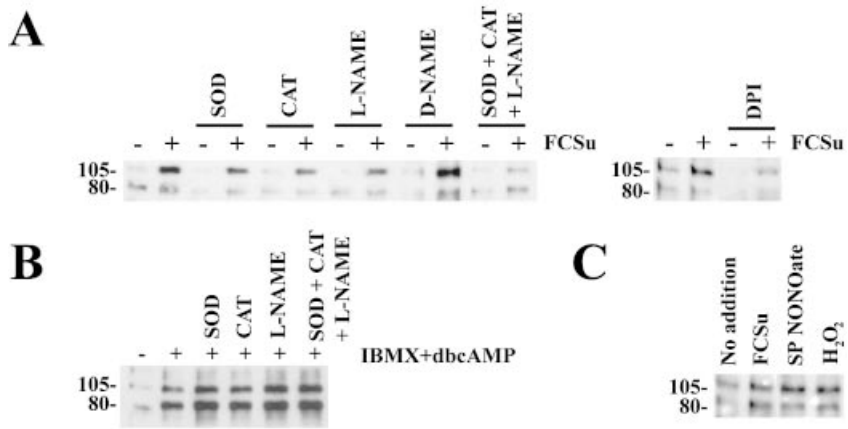

Figure 5. Reactive oxygen species are involved in the phosphorylation of the Arg-X-X-Ser/Thr motif during fetal cord serum ultrafiltrate (FCSu)-induced capacitation. Percoll-washed sperm were treated with superoxide dismutase (SOD) (0.1 mg/ml), catalase (CAT, 0.1 mg/ml), diphenyliodonium chloride (DPI) (0.1 mmol/l), L-NAME (N^G-nitro-L-arginine methyl ester) (1 mmol/l), D-NAME (1 mmol/l) or the combination of SOD, CAT and L-NAME, and incubated in the absence (–) or the presence (+) of FCSu (10% v/v) (**A**) or IBMX (0.1 mmol/l) + dbcAMP (1 mmol/l). (**C**) Percoll-washed sperm were treated with FCSu, H_2O_2 (50 µmol/l) or Sp-NONOate (100 µmol/l). Proteins from 0.5×10^6 sperm were immunoblotted as described previously. Results of one experiment representative of four (SOD, DPI), seven (catalase), nine (L-NAME) and three (D-NAME, Sp-NONOate, H_2O_2, and **B**) others performed with sperm from different semen donors. Lines have been pasted but belong to the same blot and have the same film exposure.

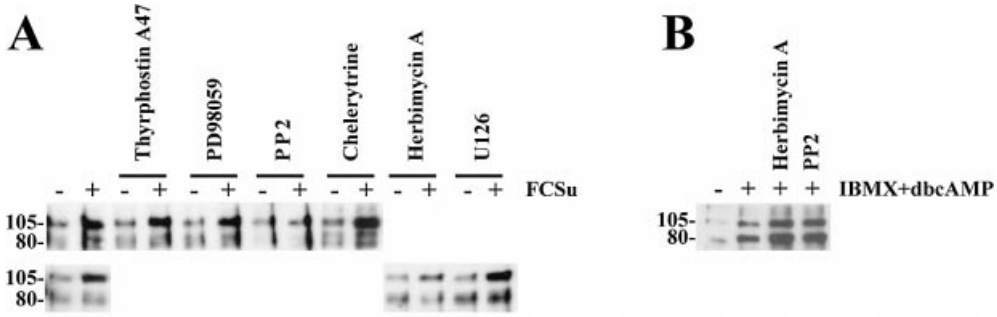

Figure 6. Non-receptor type protein tyrosine kinase (PTK), but not protein kinase C (PKC) or mitogen-activated protein kinase kinase (MEK) regulate the phosphorylation of the Arg-X-X-Ser/Thr motif during fetal cord serum ultrafiltrate (FCSu)-induced capacitation. Percoll-washed sperm were treated with tyrphostin A47 (10 µmol/l), PD98059 (100 µmol/l), U126 (0.3 µmol/l), chelerythrine (10 µmol/l), herbimycin A (10 µmol/l) or PP2 (10 nmol/l) in the absence (–) or presence (+) of FCSu (10%, v/v) (**A**) or IBMX (0.1 mmol/l) + dbcAMP (1 mmol/l) (B). Proteins from 0.5×10^6 sperm were loaded in each well, and immunoblotted using the Arg-X-X-(phospho-Ser/Thr) motif antibody. Results of one experiment representative of four (tyrphostin A47, PD98059, herbimycin A and U126), 5 (PP2), and 6 (chelerythrine) others in the set of experiments performed with FCSu, and three others in the set of experiments performed with IBMX and dbcAMP (**B**) using sperm from different semen donors.

Arg-X-X-Ser/Thr motif in FCSu-treated sperm (Figure 6A). Moreover, tyrphostin A47 and chelerythrine produced a slight increase in the phosphorylation pattern. PP3 (the inactive analogue of PP2) did not inhibit the phosphorylation of this motif (data not shown). On the other hand, the phosphorylation of the Arg-X-X-(Ser/Thr) motif in sperm treated with IBMX + dbcAMP was not prevented but rather increased by hermymicin A or PP2 (Figure 6B).

Immunolocalization of the Arg-X-X-(phospho-Ser/Thr) motif in human sperm

Immunocytochemistry with the anti-Arg-X-X-(phospho-Ser/Thr) antibody indicated that sperm proteins containing this motif were all along the flagellum and brighter in the mitochondrial helix region (Figure 7A). The proportion of sperm labelled with the antibody was the same in capacitating as in non-capacitating sperm but the fluorescence was more intense in capacitating than in non-capacitating sperm (Figure 7A). As observed with immunoblots, preadsorption of the antibody with its blocking peptide reduced the signal to that observed with the second antibody alone (Figure 7B).

Discussion

The data presented above indicated that the phosphorylation of the Arg-X-X-(Ser/Thr) motif is increased in p80 and p105 during capacitation induced by different physiological (FCSu, FFu, progesterone, and BSA) and pharmacological (IBMX, dbcAMP alone or in combination) agents and depends, directly or indirectly, on PKA activity. This phosphorylation of p80 and p105 appeared to be modulated by ROS and a non-receptor type PTK, both acting upstream of PKA.

The involvement of PKA during capacitation has been demonstrated by activating this kinase with dbcAMP (direct effect) and IBMX (indirect effect through inhibition of phosphodiesterases), or by using PKA inhibitors such as H89 and Rp-cAMPS (Leclerc *et al*., 1996; Visconti *et al*., 1995; Aitken *et al*., 1998). Furthermore, PKA activity peaks in human sperm 30 min after the beginning of FCSu-induced capacitation (Lefièvre *et al*., 2002) and it is needed for the next steps (such as tyrosine phosphorylation increasing after 1–2 h of capacitation) of capacitation to proceed (de Lamirande *et al*., 1997). Although the participation of PKA is well established in sperm

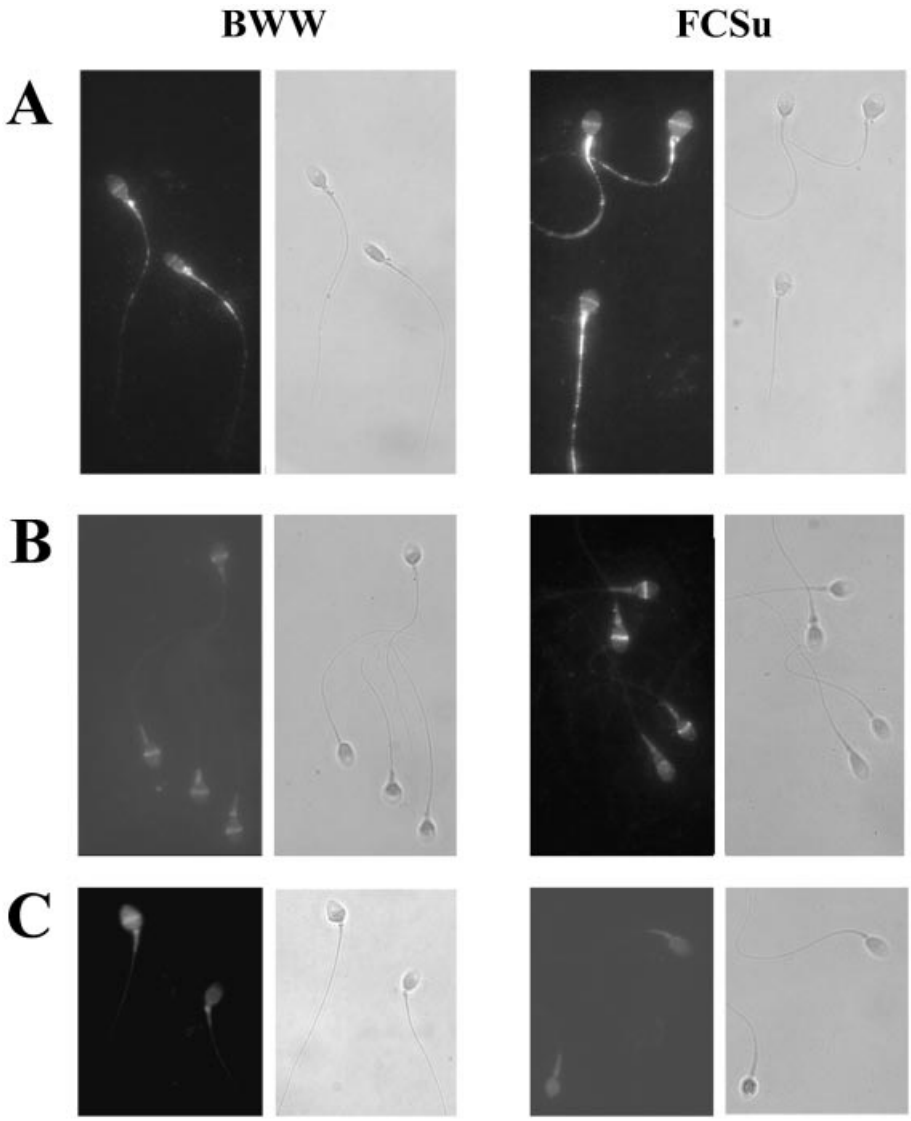

Figure 7. Immunolocalization of the Arg-X-X-(phospho-Ser/Thr) motif in human sperm. Sperm incubated with Biggers–Whitten–Whittingham medium alone or with fetal cord serum ultrafiltrate (FCSu) for 30 min, and then immunocytochemistry was done using (**A**) anti-Arg-X-X-(phospho-Ser/Thr) antibody alone or (**B**) preadsorbed with the blocking agent. (**C**) sperm treated with FCSu were incubated with the biotinylated goat anti-rabbit antibody or with the Alexa Fluor 555 conjugate of streptavidin alone as controls. Fluorescence and phase contrast microphotographs of the same microscopic field are presented. Same time of exposure was used for all fluorescent or phase contrast microphotographs. Results of one experiment representative of three others (n = 4) performed on sperm from different donors.

capacitation, there are only few reports on the Ser/Thr phosphorylation of sperm proteins during capacitation. Human sperm treated with radioactive phosphate showed an increased phosphorylation of proteins of 78, 71, 56 and 57.5 kDa during capacitation (Furuya *et al*., 1993). Immunoblotting experiments using anti-phospho-Ser and anti-phospho-Thr antibodies indicated an increase in Ser/Thr phosphorylation in 18, 35 43–55, 94, 110 and 190 kDa Triton-soluble proteins during capacitation (Naz, 1999); however, the effect of kinase inhibitors was not tested, so that no conclusion on the participation of a specific kinase could be drawn.

The antibody against the phosphorylated Arg-X-X-(Ser/Thr) motif was shown to be a powerful tool to study PKA substrates. In a mass spectrometry-based proteomic approach for identification of Ser/Thr-phosphorylated proteins in HeLa and 293T cells, a novel PKA substrate named 'Frigg' with a phospho-Ser/Thr with an Arg in the -3 position was recognized using this antibody. There was an increase in the phosphorylation of the Arg-X-X-(Ser/Thr) motif in 'Frigg' when this protein was incubated with the catalytic subunit of PKA (Grøndborg *et al*., 2002). Moreover, forskolin increased the phosphorylation of the Arg-X-X-(Ser/Thr) motif, as evaluated with the anti-Arg-X-X-(phospho-Ser/Thr) antibody, in the inositol 1,4,5-triphosphate receptors of parotid acinar cells; H89 and Rp-cAMPS inhibited this effect (Bruce *et al*., 2002), confirming the involvement of PKA and the use of the antibody to determine PKA substrates in cells. Here we used the antibody against the Arg-X-X-(phospho-Ser/Thr) motif, in combination with agents that stimulate (directly with dbcAMP, indirectly with IBMX) or inhibit (H89 and Rp-cAMPS) PKA, to study the PKA-dependent Ser/Thr phosphorylation in capacitating human sperm. It is not possible at this time to demonstrate that p80 and p105 are directly phosphorylated by PKA. However, these proteins appeared to include the motif Arg-X-X-(phospho-Ser/Thr) characteristic of PKA substrates and recognized by the antibody. Furthermore, the early phosphorylation of the Arg-X-X-Ser/Thr motif observed in this study is coincident with the maximal PKA activity

measured in FCSu-induced capacitation (Lefièvre *et al*., 2002). Also, the fact that H89 and Rp-cAMPS prevented the increase in phosphorylation of p80 and p105 in FCSu- and IBMX + dbcAMP-treated sperm (Figure 4) strongly suggests that this phenomenon is PKA dependent.

The inducers FCSu, FFu, progesterone, BSA, IBMX and dbcAMP previously shown to trigger capacitation (Uhler *et al*., 1992; de Lamirande and Gagnon, 1995; Visconti *et al*., 1995; Leclerc *et al*., 1996; Aitken *et al*., 1998; de Lamirande *et al*., 1998; Herrero *et al*., 2000) and associated phosphorylation events (Leclerc *et al*., 1996; de Lamirande and Gagnon, 2002; Thundathil *et al*., 2002) promoted the Arg-X-X-(Ser/Thr) phosphorylation of p80 and p105 (Figure 3), confirming that the effect observed was dependent on the capacitation process rather than on a specific agent.

Although a significant increase in phosphorylation was always observed on p80 and p105 (Figure 1), there was sometimes a mild increase in the phosphorylation of a 116 kDa protein (Figure 2) that was variable between donors and between samples from the same donor.

The concept that ROS, at low levels, have a positive role in human sperm capacitation has grown in recent years (de Lamirande *et al*., 1997; Aitken *et al*., 1998; Herrero *et al*., 2000). ROS are involved in different capacitation-related events such as the increase in cAMP levels and activation of the cAMP/PKA-dependent pathway (Zhang and Zheng, 1996; Leclerc *et al*., 1997; Aitken *et al*., 1998; Herrero *et al*., 2000), the tyrosine phosphorylation of fibrous sheath proteins (Carrera *et al*., 1996; Leclerc *et al*., 1997) and the double phosphorylation of the Thr-Glutamine-Tyr motif in sperm proteins of low (de Lamirande and Gagnon, 2002) and high (Thundathil *et al*., 2003) molecular masses. The phosphorylation of the Arg-X-X-(Ser/Thr) motif in p80 and p105 was prevented by a combination of ROS scavengers (SOD and catalase) and the NOS inhibitor L-NAME, as well as by an inhibitor of sperm $O_2^{-\bullet}$ generation (DPI) during incubation with FCSu (Figure 5A), therefore suggesting a role for ROS in this process. Interestingly, the three ROS, $O_2^{-\bullet}$, H_2O_2 and NO• that induce sperm capacitation and the related protein tyrosine phosphorylation through the cAMP/PKA-dependent pathway (Leclerc *et al*., 1996; Aitken *et al*., 1998; Herrero *et al*., 2000) also appear to be involved in the phosphorylation of the Arg-X-X-(Ser/Thr) motif. The participation of ROS appears to be upstream of PKA activation since SOD, catalase and L-NAME (Figure 5B) did not prevent the phosphorylation of the Arg-X-X-(Ser/Thr) motif of p80 and p105 in sperm incubated with IBMX + dbcAMP, a condition that bypasses the first steps of sperm capacitation allowing the direct activation of PKA (Visconti *et al*., 1995; Leclerc *et al*., 1996).

Herbimycin A and PP2, but not tyrphostin A47, chelerythrine, PD98059 and U126, prevented the phosphorylation of the Arg-X-X-(Ser/Thr) motif, suggesting the participation of non-receptor type PTK (but not MEK or PKC) in the regulation of the PKA-dependent phosphorylation of p80 and p105. Moreover, the lack of inhibition by herbimycin A and PP2 on the protein Arg-X-X-(Ser/Thr) phosphorylation of sperm incubated with IBMX + dbcAMP strongly suggests that a tyrosine kinase acts upstream of PKA activation. This would also imply that at least two different PTK are involved in capacitation; one would act at the beginning of capacitation and be related to PKA activation and the other would be related to the protein tyrosine phosphorylation of fibrous sheath proteins. It is presently not known how the early activation of a PTK could trigger PKA during capacitation. However, a PTK was shown to induce an increase of intracellular cAMP in porcine heart vascular smooth muscle cells (El-Mowafy and White, 1998) and in HT4.7 neural cells (Stringfield and Morimoto, 1997).

Protein tyrosine phosphorylation is associated with capacitation and increases progressively from 1 h after the beginning of incubation under capacitating conditions (Visconti *et al*., 1995; Leclerc *et al*., 1996; Aitken *et al*., 1998). The early PKA-related phosphorylation of the Arg-X-X-(Ser/Thr) motif observed in this study would also be in agreement with the cAMP/PKA dependency of protein tyrosine phosphorylation (Visconti *et al*., 1995; Leclerc *et al*., 1996; Aitken *et al*., 1998).

The molecular masses (Figure 1) and the Triton-insoluble nature (Figure 2) of the proteins recognized by the anti-Arg-X-X-Ser/Thr antibody could suggest that these proteins belong to the fibrous sheath of sperm. However, immunocytochemistry experiments indicated that proteins carrying the Arg-X-X-(phospho-Ser/Thr) motif are located all along the flagellum (Figure 7A) and not strictly on the principal piece as it was observed for proteins carrying phosphorylated Tyr (Leclerc *et al*., 1997) or double phosphorylated Thr-Glu-Tyr (Thundathil *et al*., 2003). The fluorescence was more intense in capacitating than in non-capacitating sperm (Figure 7A) but the proportion of sperm labelled with the antibody was the same in the two sperm populations. This observation is important because it indicates another difference between proteins carrying the Arg-X-X-(phospho-Ser/Thr) motif and those carrying phosphorylated Tyr or double-phosphorylated Thr-Glu-Tyr; in the latter cases, capacitation was associated with an increase in the proportion of sperm with bright fluorescence rather than an increase in the level of fluorescence on the whole population of sperm. The nature of the proteins carrying the Arg-X-X-(phospho-Ser/Thr) remains to be elucidated.

In conclusion, this study shows, for the first time, that the phosphorylation of the Arg-X-X-(Ser/Thr) motif, characteristic of PKA substrates, is increased during human sperm capacitation and that this phosphorylation is dependent on PKA and modulated by ROS and non-receptor type protein tyrosine kinase both acting upstream of PKA. These (Ser/Thr)-phosphorylated proteins could be the link between the early increase of PKA activity and the late protein tyrosine phosphorylation during human sperm capacitation.

Acknowledgements

We thank Drs Alice Benjamin and Lucie Morin for collecting fetal cord blood. We also thank the staff of the IVF centre at the Royal Victoria Hospital, McGill University for providing follicular fluid. We wish to acknowledge the participation of volunteers in this study. C.G. was supported by a grant of the Canadian Institutes for Health Research (CIHR), Canada.

References

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

Biggers JD, Whitten WK and Whittingham DG (1971) The culture of mouse embryos in vitro. In Daniel JC (ed) Methods in Mammalian Embryology. WH Freeman, San Fransisco, USA, pp 86–116.

Bruce JIE, Shuttleworth TJ, Giovannucci DR and Yule DI (2002) Phosphorylation of inositol 1,4,5-triphosphate receptors in parotid acinar cells. J Biol Chem 277,1340–1348.

Carrera A, Moss J, Ning XP, Gerton GL, Tesarik J, Kopf GS and Moss SB (1996) Regulation of protein tyrosine phosphorylation in human sperm by a calcium/calmodulin dependent mechanism: identification of A kinase anchor proteins as major substrates for tyrosine phosphorylation. Dev Biol 180,284–296.

de Lamirande E and Gagnon C (1993) A positive role for the superoxide anion in triggering hyperactivation and capacitation of human spermatozoa. Int J Androl 16,21–25.

de Lamirande E and Gagnon C (1995) Capacitation-associated production of superoxide anion by human spermatozoa. Free Radic Biol Med 18,487–496.

de Lamirande E and Gagnon C (2002) The extracellular signal-regulated

kinase (ERK) pathway is involved in human sperm function and modulated by the superoxide anion. Mol Hum Reprod 8,124–135.

de Lamirande E and Gagnon C (2003) Redox control of changes in protein sulfhydryl levels during human sperm capacitation. Free Radic Biol Med 35,1271–1285.

de Lamirande E, Leclerc P and Gagnon C (1997) Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. Mol Hum Reprod 3,175–194.

de Lamirande E, Harakat A and Gagnon C (1998) Human sperm capacitation induced by biological fluids and progesterone, but not by NADH or NADPH, is associated with the production of superoxide anion. J Androl 19,215–225.

El-Mowafy AM and White RE (1998) Evidence for a tyrosine kinase-dependent activation of the adenylyl cyclase/PKA cascade downstream from the G-protein-linked endothelin ETA receptor in vascular smooth muscle. Biochem Biophys Res Commun 251,494–500.

Furuya S, Endo Y, Osumi K, Oba M and Suzuki S (1993) Effects of modulators of protein kinase C on human sperm capacitation. Fertil Steril 59,1285–1290.

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

Garbers DL, First NL and Lardy HA (1973) Properties of adenosine 3′,5′-monophosphate-dependent protein kinases isolated from bovine epididymal spermatozoa. J Biol Chem 248,875–879.

Griveau JF, Renard P and LeLannou D (1994) An in vitro promoting role for hydrogen peroxide in human sperm capacitation. Int J Androl 17,300–307.

Grøndborg M, Kristiansen TZ, Stensballe A, Andersen JS, Ohara O, MannM, Jensen ON and Pandey A (2002) A mass spectrometry-based proteomic approach for identification of Serine/Threonine-phosphorylated proteins by enrichment with phospho-specific antibodies. Mol Cell Proteom 1,517–527.

Hancock JT and Jones OT (1987) The inhibition by diphenyleneiodonium and its analogues of superoxide generation by macrophages. Biochem J 242,103–107.

Herrero MB and Gagnon C (2001) Nitric oxide: a novel mediator of sperm function. J Androl 22,349–356.

Herrero MB, Chatterjee S, Lefièvre L, de Lamirande E and Gagnon C (2000) Nitric oxide interacts with the cAMP pathway to modulate capacitation of human spermatozoa. Free Radic Biol Med 29,522–536.

Jacobson G and Karsnas P (1990) Important parameters in semi-dry electrophoresis transfer. Electrophoresis 11,46–52.

Johnson LR, Foster JA, Haig-Ladewing L, VanScoy H, Rubin CS, Moss SB and Gerton GL (1997) Assembly of AKAP82, a protein kinase A anchor protein, into the fibrous sheath of mouse sperm. Dev Biol 192,340–350.

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

Leclerc P, de Lamirande E and Gagnon C (1997) Regulation of protein tyrosine phosphorylation and human sperm capacitation by reactive oxygen species derivatives. Free Radic Biol Med 22,643–656.

Leclerc P, de Lamirande E and Gagnon C (1998) Interaction between Ca^{2+}, cyclic 3′,5′ adenosine monophosphate, the superoxide anion, and tyrosine phosphorylation pathways in the regulation of human sperm capacitation. J Androl 19,434–443.

Lefièvre L, Jha KN, de Lamirande E, Visconti PE and Gagnon C (2002) Activation of protein kinase A during human sperm capacitation and acrosome reaction. J Androl 23,709–716.

Moss SB and Gerton GL (2001) A-kinase anchor proteins in endocrine systems and reproduction. Trends Endocrinol Metab 12,434–440.

Naz RK (1999) Involvement of protein serine and threonine phosphorylation in human sperm capacitation. Biol Reprod 60,1402–1409.

O'Donnell BV, Tew DG, Jones OT and England PJ (1993) Studies on the inhibitory mechanism of iodonium compounds with special reference to neutrophil NADPH oxidase. Biochem J 290,41–49.

Pariset C and Weinman S (1994) Differential localization of two isoforms of the regulatory subunit RIIα of cAMP-dependent protein kinase in human sperm: biochemical and cytochemical study. Mol Reprod Dev 39,415–422.

Stringfield TM and Morimoto BH (1997) Modulation of cyclic AMP levels in a clonal neural cell line by inhibitors of tyrosine phosphorylation. Biochem Pharmacol 53,1271–1278.

Thundathil J, de Lamirande E and Gagnon C (2002) Different signal transduction pathways are involved during human sperm capacitation induced by biological and pharmacological agents. Mol Hum Reprod 8,811–816.

Thundathil J, de Lamirande E and Gagnon C (2003) Nitric oxide regulates the phosphorylation of the threonine-glutamine-tyrosine motif in proteins of human spermatozoa during capacitation. Biol Reprod 68,1291–1298.

Uguz C, Vredenburgh WL and Parrish JJ (1994) Heparin-induced capacitation but not intracellular alkalinization of bovine sperm is inhibited by Rp-adenosine-3′,5′-cyclic monophosphorothioate. Biol Reprod 51,1031–1039.

Uhler ML, Leung A, Chan SYW and Wang C (1992) Direct effect of progesterone and antiprogesterone on human sperm hyperactivated motility and acrosome reaction. Fertil Steril 58,1191–1198.

Visconti PE, Moore GD, Bailey JL, Leclerc P, Connors SA, PanD, Olds-Clarke P and Kopf GS (1995) Capacitation in mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. Development 121,1139–1150.

Visconti PE, Johnson LR, Oyasaki M, Fornes M, Moss SB, Gerton GL and Kopf GS (1997) Regulation, localization, and anchoring of protein kinase A subunits during mouse sperm capacitation. Dev Biol 192,351–363.

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

World Health Organization (1992) Laboratory Manual for the Examination of Human Semen and Semen–Cervical Mucus Interaction. 3rd edn, Cambridge University Press, Cambridge, UK, pp 1–107.

Yanagimachi R (1994) Mammalian fertilization. In Knobil E and Neill JD (eds) The Physiology of Reproduction, Vol 2. Raven Press, New York, pp 189–317.

Zhang H and Zheng R (1996) Promotion of human sperm capacitation by superoxide anion. Free Radic Res 24,261–268.

Submitted on December 18, 2003; resubmitted on January 16, 2004; accepted on January 25, 2004