Involvement of tyrosine kinase and cAMP-dependent kinase cross-talk in the regulation of human sperm motility

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Tyrosine phosphorylation and its upregulation by cAMP have been associated with capacitation and motility changes of spermatozoa. In the present study, washed spermatozoa were incubated for 6 h in protein-supplemented complete medium with or without kinase inhibitors to verify whether upstream activation of protein kinase A is indispensable for tyrosine phosphorylation and motility changes to occur in capacitating human spermatozoa. H89, a specific protein kinase A inhibitor, significantly inhibited the activity of sperm protein kinase A. However, this inhibition did not alter capacitation-related tyrosine kinase activation. Tyrosine phosphorylated proteins, motion parameters and the incidence of phosphotyrosine-immunoreactive spermatozoa were decreased only slightly. Conversely, genistein, a tyrosine kinase inhibitor which inhibited sperm tyrosine kinase but not protein kinase A, significantly reduced all the parameters studied. Spermatozoa incubated with cAMP and pentoxifylline showed a rapid enhancement of tyrosine phosphorylation

and some of the sperm motion parameters, particularly hyperactivation. Inclusion of H89 reduced cAMP stimulation of tyrosine kinase, and tyrosine phosphorylation and motion parameters were reduced almost to basal values. Treatment with genistein reduced tyrosine kinase activity, especially in the soluble fraction of sperm extracts. A decrease in tyrosine phosphorylation of soluble proteins, 105, 81, 55 and 48 kDa, correlated with a significant reduction in sperm motion parameters. Hyperactivation was reduced by tenfold. Tyrosine phosphorylated proteins in the insoluble fraction and the incidence of tyrosine phosphorylated-positive spermatozoa were not reduced markedly. Upstream protein kinase A activation may be a facilitatory rather than an indispensable step in the capacitation-induced tyrosine phosphorylation mediating motility changes in human spermatozoa. Triton- $\times$ 100 soluble tyrosine phosphorylated proteins, more than their insoluble counterparts, appear to be involved in the modulation of human sperm motion characteristics.

Introduction

Capacitation is a time-dependent process that involves numerous morphological and structural changes in the spermatozoon (Yanagimachi, 1994). These changes occur at two levels: the sperm plasma membrane and the intracellular compartment. At the intracellular level, there are changes in ions, increased glycolytic activity and oxygen consumption, and increased activity of the adenylyl cyclase–cAMP–protein kinase A system. However, the sperm plasma membrane appears to be the initial target of capacitation, as it loses its coating materials (decapacitation factors), experiences a net deglycosylation, changes its phospholipid composition, loses cholesterol, increasing membrane fluidity and protein mobility, displays new surface receptors, and ac-

tivates transductional mechanisms that facilitate the two most visible changes in capacitated spermatozoa: their ability to acrosome react and to develop hyperactivated motility (Yanagimachi, 1994).

Tyrosine phosphorylation in spermatozoa has been associated with capacitation (for a review, see Visconti and Kopf, 1998). However, its trigger, regulatory processes, kinases, phosphatases and target proteins, as well as the ultimate effectors of capacitation-related changes, have not been completely elucidated. Furthermore, there are some indications that these factors may not be the same in different species (Visconti *et al.*, 1995a, 1999; Carrera *et al.*, 1996; Luconi *et al.*, 1996; Vijayaraghavan *et al.*, 1997a). Owing to the head and tail compartmentalization of the sperm cell and the essentially different functions that these compartments display, zona pellucida binding and penetration, acrosome reaction at the head and motility at the tail, the above factors might not even be the same in these two compartments of the spermatozoon.

Visconti *et al.* (1995b) demonstrated that cAMP can upregulate tyrosine phosphorylation at the same time that

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it facilitates the acquisition of sperm fertilizing ability. When administered exogenously, in some species, it can bypass the main requisites for sperm capacitation such as the presence of bicarbonate and cholesterol efflux (Visconti *et al.*, 1995b; Galantino-Homer *et al.*, 1997; Osheroff *et al.*, 1999; Visconti *et al.*, 1999). Although there appears to be a clear relationship between protein kinase A and tyrosine phosphorylation in human spermatozoa, whether upstream activation of protein kinase A is an indispensable condition for tyrosine kinase activation and tyrosine phosphorylation remains to be clearly established. Alternatively, activation of protein kinase A might play a facilitatory action on tyrosine kinase and tyrosine phosphorylation, contributing to its regulation together with other factors (for example, intracellular Ca^{2+} concentration, pH, redox status, ATP) that are known to change during capacitation. This facilitatory action on tyrosine phosphorylation would occur in addition to the role of protein kinase A as a modulator of capacitation events, effected by serine–threonine protein phosphorylation.

The present study has focused on the regulation of human sperm motion characteristics by protein tyrosine phosphorylation. In addition, the study has assessed cross-talk between cAMP-dependent kinase and tyrosine kinase. The findings were verified from different approaches by evaluating tyrosine kinase and protein kinase A activity, tyrosine phosphorylation of sperm proteins in both soluble and whole-cell extracts, and the incidence of tail tyrosine phosphorylation in capacitating spermatozoa incubated with and without cAMP and kinase inhibitors.

Materials and Methods

Reagents

Kinase inhibitors, H89 (*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulphonamide · 2HCl), H8 (*N*-[2-(methylamino)ethyl]-5-isoquinoline-sulphonamide · 2HCl), staurosporin and genistein (4,5,7-trihydroxyisoflavone) were obtained from Bio-Mol (Plymouth Meeting, PA). Ham's F10 medium was obtained from Gibco Biotechnology (Rockville, MD). Phosphotyrosine antibody (4G10) was purchased from Upstate Biotechnology (Lake Placid, NY), and fluorescein isothiocyanate anti-mouse IgG from Cappel (Aurora, OH) and anti-quenching buffer from Molecular Probes (Eugene, OR). *N*,2-*O*-dibutyryladenine 3',5'-cyclic monophosphate (dbcAMP), 3,7-dimethyl-1-(5-oxohexyl)xanthine (pentoxifylline), human serum albumin (HSA), BSA and all other reagents were obtained from Sigma Chemical Company (St Louis, MO).

Preparation of sperm sample

Human semen samples were obtained from consenting donors by masturbation after 3–5 days of abstinence

under a protocol approved by the Institutional Review Board. Only samples with normal semen analysis ($> 50 \times 10^6$ motile spermatozoa ml^{-1} , motility $> 50\%$) were used in the experiments. After liquefaction for 30 min, the seminal plasma was removed by washing in Ham's F10 + 0.35% HSA. Washed spermatozoa (10×10^6 cells ml^{-1}) were studied immediately ($t=0$) or after 6 h of incubation at 37°C in 5% CO_2 ($t=6$) with or without kinase inhibitors H89 (25 $\mu\text{mol l}^{-1}$), genistein (400 $\mu\text{mol l}^{-1}$), H8 (500 $\mu\text{mol l}^{-1}$) and staurosporin (1 $\mu\text{mol l}^{-1}$). The broad-spectrum kinase inhibitor, staurosporin, was used as a positive control for the assays. The negative controls consisted of medium with no inhibitors and up to 1% (v/v) dimethyl sulphoxide, similar to the final concentration of dimethyl sulphoxide in the experimental tubes. The concentrations of the inhibitors used in all the experiments were selected from those that did not show any sperm cytotoxic effects in membrane integrity and mitochondrial function assays (see below). Aliquots of different experimental variants were evaluated for protein kinase A and tyrosine kinase activity, protein tyrosine phosphorylation, incidence of phosphotyrosine-immunoreactive spermatozoa and sperm motion parameters.

In a second set of experiments aimed at enhancing the protein kinase A-mediated upregulation of tyrosine phosphorylation, swim-up spermatozoa were incubated with 1 mmol dibutyryl cAMP l^{-1} and 1 mmol pentoxifylline l^{-1} with or without tyrosine kinase (genistein, 400 $\mu\text{mol l}^{-1}$) or protein kinase A (H89, 25 $\mu\text{mol l}^{-1}$) inhibitors for 90 min at 37°C in 5% CO_2 . Again, aliquots of spermatozoa were characterized for their motility, motion characteristics, kinase activity and tyrosine phosphorylation.

Assessment of sperm viability

Dose–response experiments were used to select the concentrations of the inhibitors that did not significantly compromise sperm viability. Viability was checked by two procedures: the hypo-osmotic swelling test (HOST) (Jeyendran *et al.*, 1984) and the mitochondrial function (MTT) (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2-H-tetrazolium) test (Levitz and Diamond, 1985). The HOST evaluates sperm plasma membrane integrity, whereas the MTT test is based on mitochondrial function. For the HOST, spermatozoa incubated with or without inhibitors were incubated further with hypo-osmotic medium (75 mmol fructose l^{-1} and 25 mmol sodium citrate l^{-1}) at 37°C and 5% CO_2 for 30 min and spermatozoa with coiled tails were counted as viable.

Another aliquot of spermatozoa was incubated with the MTT dye solution for 4 h. In live spermatozoa the dye was converted into a coloured formazan product, the absorbance of which was read at 570 nm. The concentration of inhibitors that did not display cytotoxic

effects on spermatozoa during a 6 h incubation were used for subsequent experiments. Sperm viability was also examined after each experiment to minimize the effect of sample variability.

Sperm motion parameters

Motility and motion parameters of spermatozoa were assessed in a computer-assisted motion analyser (Hamilton Thorn v.10.8) using a 20 μm deep chamber at 37°C. Settings were as follows: frames, 30; frame rate, 60 Hz; minimum contrast, 85; minimum cell size, 2 pixels; and minimum static contrast, 30. The following parameters were used to define hyperactivation: curvilinear velocity (VCL) $> 100 \mu\text{m s}^{-1}$, linearity of a curvilinear path (LIN) $< 65\%$, amplitude of lateral head displacement (ALH) $> 7.5 \mu\text{m}$ (Burkman, 1991).

The studied motion parameters can be defined as follows: VCL is the time-average velocity of a sperm head along its actual curvilinear path as perceived in two dimensions under the microscope; straight-line velocity (VSL) is the time-average velocity of a sperm head along the straight line between its first detected position and its last; average path velocity (VAP) is the time-average velocity of a sperm head along its average path; ALH is the magnitude of lateral displacement of a sperm head about its average path; and LIN is reported as a ratio VSL:VCL; straightness (STR) is the linearity of the average path (VSL/VAP); and beat-cross frequency (BCF) is the average rate at which the curvilinear path of a spermatozoon crosses its average path (World Health Organization, 1999).

Indirect immunofluorescence

Indirect immunofluorescence has been used to detect the incidence of tyrosine phosphorylated spermatozoa at different time points during a capacitating incubation. Spermatozoa ($10 \times 10^6 \text{ ml}^{-1}$) taken from the different experimental variants were washed in PBS+0.1% BSA and 10 μl of the samples were spotted on to eight-well slides. The slides were allowed to dry before proceeding. Spermatozoa were fixed and permeabilized in methanol for 30 min and then washed twice in PBS. Slides were incubated with phosphotyrosine antibody (4G10) for 1 h, washed twice with PBS and incubated with anti-mouse IgG-fluorescence isothiocyanate for 30 min at room temperature (25°C). After two washes in PBS, the slides were mounted in anti-quenching buffer (Slowfade light Antifade kit, Molecular Probes, Eugene, OR) and examined under a Nikon E800 epifluorescence microscope connected to a digital imaging system (Spot II, v.3.0 software, Sterling Heights, MI). Not fewer than 200 cells were counted.

Solubilization of spermatozoa

Spermatozoa from all experimental variants were washed twice with PBS and centrifuged at 400 g for

10 min. Spermatozoa ($10 \times 10^6 \text{ cells ml}^{-1}$) were solubilized in 10 μl SDS solubilization buffer (0.0187 mol Tris-HCl l^{-1} , pH 6.8, containing 2% (w/v) SDS, 10% (v/v) glycerol, 1 mmol vanadate l^{-1} and 1 mmol phenylmethylsulphonyl fluoride (PMSF)) for 30 min at 25°C. The sperm extract was then mixed with 10 μl SDS-PAGE buffer (0.0625 mol Tris-HCl l^{-1} , pH 6.8, 2% SDS, 10% glycerol and 2% (v/v) 2-mercaptoethanol) and heated at 100°C for 5 min. Samples were loaded on to 7.5% (w/v) polyacrylamide gels and subjected to electrophoresis. An equivalent of 1×10^6 spermatozoa was applied to each lane. Aliquots of spermatozoa were treated with Triton buffer (50 mmol Tris-HCl l^{-1} (pH 7.5), 10 mmol benzamide l^{-1} , 5 mmol EDTA l^{-1} , 10 mmol EGTA l^{-1} , 1 mmol vanadate l^{-1} , 1 mmol PMSF l^{-1} and 0.05% Triton-X100) and centrifuged at 15 800 g for 20 min to study soluble proteins. The supernatant was taken as the solubilized fraction and was used for protein kinase A assay and to study tyrosine phosphorylation of soluble proteins by western blot analysis.

Western blot analysis

Samples were run on SDS-PAGE at a constant voltage of 125 V. Proteins were transferred on to Immobilon P (Millipore, Bedford, MA) at a constant voltage (100 V) for 2 h and blocked with superblocker (Pierce, Rockford, IL) overnight at room temperature. Immobilon sheets were incubated with a 1:10 000 dilution of monoclonal phosphotyrosine antibody (4G10) for 2 h in TBST (0.02 mol Tris-HCl l^{-1} , 0.15 mol NaCl l^{-1} , 0.05% (v/v) Tween-20, pH 7.6) containing 5% BSA. After washing twice with TBST, Immobilon sheets were incubated with anti-mouse secondary antibody (IgG-horseradish peroxidase), washed and then developed with Amplified-Opti4CN kit from Bio-Rad (Hercules, CA). Images were taken with Spot II Digital Imaging System and molecular weights were calculated using Kodak Imaging System (v. 2.1) (New Haven, CT).

Kinase activity

Washed or swim-up spermatozoa ($10 \times 10^6 \text{ ml}^{-1}$) were solubilized in 100 μl homogenizing buffer (20.0 mmol Hepes l^{-1} , pH 7.4, 2.0 mmol EDTA l^{-1} , 1.0 mmol dithiothreitol l^{-1} , 0.1 mmol PMSG l^{-1} , 0.1 mmol vanadate l^{-1} , 1.0 mmol $\text{MgCl}_2 \text{l}^{-1}$, 100.0 mmol NaCl l^{-1} and 0.05% Triton-X100) for 15 min at 4°C. A Triton-X100 soluble fraction was obtained by sonicating spermatozoa ($10 \times 10^6 \text{ ml}^{-1}$) in homogenizing buffer and centrifuging at 15 800 g for 30 min at 4°C. The supernatant was used as the Triton-soluble fraction of sperm extracts. Kinase activity was determined in whole-cell and Triton-soluble extracts at room temperature.

ELISA-based tyrosine kinase and protein kinase A kits were purchased from Cal-Biochem (San Diego, CA). Standard curves were run along with test samples in

Fig. 1. Changes in protein tyrosine kinase (PTK) activity, incidence of phosphotyrosine-immunoreactive spermatozoa and motion parameters after a 6 h capacitating incubation in Ham's F10 + 0.35% human serum albumin at 37°C in 5% CO₂. Data have been normalized against baseline preincubation values (equal to 100%, indicated by dashed line). Superscripts indicate significance of data compared with baseline value (100%) by Wilcoxon's signed-rank test (b: $P < 0.001$; c: $P < 0.05$). ALH: amplitude of lateral head displacement, HA: hyperactivation, LIN: linearity, MOT: motility, PROG: progressive velocity, PY-Sp: phosphotyrosine-immunoreactive sperm tails, VCL: curvilinear velocity ($n = 24$).

each experiment and their activity expressed as U mg⁻¹ protein. The protein content was determined using the Bio-Rad kit (Hercules, CA).

One Unit of protein kinase A activity is defined as the amount of enzyme required to catalyse the transfer of 1 pmol phosphate into the substrate (RFARKGSLRQKNV) in 1 min at 30°C. One Unit of tyrosine kinase activity is defined as the amount of enzyme required to catalyse the transfer of 1 pmol phosphate to the peptide substrate

(EAIYAAPFAKKK) present at 100 μmol l⁻¹ in 1 min at 30°C in 25 μl reaction mixture.

Statistical analysis

The Wilcoxon's signed-rank test was used to analyse control normalized data. Mean percentages were compared with a hypothetical control mean of 100% (the control was spermatozoa incubated without inhibitor). In the cAMP plus pentoxifylline experiments, data were analysed using Welch's test or paired t test depending on dispersion. All statistical analyses were performed with Prism software (Graphpad, San Diego, CA).

Results

Tyrosine phosphorylation and motility changes during a capacitating incubation

As previously reported, there was a significant increase in tyrosine kinase activity in human spermatozoa incubated for 6 h under capacitating conditions (Fig. 1). In addition, in parallel, there was an increase in the number and intensity of tyrosine phosphorylated sperm proteins and in the incidence of phosphotyrosine-immunoreactive spermatozoa at the end of such capacitating incubation (Figs 1, 2). The percentage of phosphotyrosine-immunoreactive spermatozoa increased from 13% at $t = 0$ to 33% at $t = 6$. Although sperm motility and progressive motility remained unchanged, some of the motion characteristics, such as VCL, ALH and the percentage of hyperactivated (HA) spermatozoa, increased in magnitude (Fig. 1).

Fig. 2. Matching pair of photomicrographs. Immunolocalization of tyrosine phosphorylated proteins in human spermatozoa at the end of a capacitating incubation. Spermatozoa were fixed and permeabilized with methanol and immunolabelled with a phosphotyrosine antibody (4G10) and fluorescein isothiocyanate-conjugated anti-mouse IgG. (a) Fluorescent and (b) phase contrast photomicrographs. Scale bar represents 10 μm.

Fig. 3. Effect of protein tyrosine kinase (PTK, ■) and protein kinase A (PKA, ▨) inhibitors (H89, H8, genistein (GEN), staurosporin (STA)) on PTK and PKA activity in whole sperm extracts measured by a colorimetric immunoreaction (ELISA) detecting tyrosine- and serine-phosphorylated target peptides. Data are expressed as mean $\pm$ SE ($n=24$ for PTK; $n=10$ for PKA) and represent the percentage of control (control is PTK or PKA activity of spermatozoa after 6 h of incubation at 37°C in 5% CO₂ without the inhibitors; dashed line). Wilcoxon's signed-rank test was used to analyse data (versus control = 100%). Superscripts indicate significance according to the Wilcoxon's test (a: $P < 0.0001$; b: $P < 0.001$).

Role of tyrosine kinase and protein kinase A in regulation of motility of human spermatozoa

The involvement of protein kinase A in the capacitation-related regulation of motility parameters and tyrosine phosphorylation was investigated by incubating washed spermatozoa with 25 μ M H89 I⁻¹, 500 μ M H8 I⁻¹, 400 μ M genistein I⁻¹ or 1 μ M staurosporin I⁻¹ in Ham's F10 + 0.35% HSA at 37°C in 5% CO₂ for 6 h. The concentrations of the inhibitors used in these experiments were not toxic to human spermatozoa, as assessed by membrane integrity (HOST) and mitochondrial function (MTT) assays.

H89, a specific inhibitor of protein kinase A, significantly inhibited the activity of sperm protein kinase A (Fig. 3). However, this inhibition did not affect total tyrosine kinase activity, and only diminished the incidence of phosphotyrosine-immunoreactive spermatozoa by 20% (Fig. 4). Motion parameters were slightly decreased and hyperactivation was not affected (Table 1). Genistein, a tyrosine kinase inhibitor, did not alter protein kinase A activity (Fig. 3). However, as expected, it did inhibit sperm tyrosine kinase activity, decreasing the incidence of phosphotyrosine-positive spermatozoa and the magnitude of motion parameters, including hyperactivation (Fig. 4, Table 1). H8, a PKA inhibitor of wider spectrum, inhibited both sperm protein kinase A and tyrosine kinase, decreasing the incidence of tyrosine phosphorylated-immunoreactive spermatozoa (Figs 3,4). Motility and

Fig. 4. Effect of protein tyrosine kinase and protein kinase A inhibitors (H89, H8, genistein (GEN), staurosporin (STA)) on the incidence of phosphotyrosine-immunoreactive sperm tails after a 6 h incubation in Ham's F10 and human serum albumin at 37°C in 5% CO₂. Spermatozoa were fixed and permeabilized with methanol and labelled with phosphotyrosine monoclonal antibody (4G10) and fluorescein isothiocyanate-conjugated anti-mouse IgG. Data (mean $\pm$ SE, $n=24$) are expressed as a percentage of control (after 6 h of incubation at 37°C in 5% CO₂ without inhibitors; dashed line). Wilcoxon's signed-rank test was used to analyse the normalized data (versus control = 100%). Superscripts indicate significance obtained with the Wilcoxon's test (a: $P < 0.0001$).

progressive motility were decreased, whereas velocity was affected differentially according to the parameters considered (Table 1). The VAP and the VSL were reduced, whereas VCL was not affected. With increased ALH and decreased LIN, the percentage of hyperactivation spermatozoa was augmented. Similar changes, albeit more pronounced, were observed when staurosporin, which also inhibited protein kinase A and tyrosine kinase, was included in the medium.

Western blot analysis of whole sperm extracts revealed a slight decrease in the phosphotyrosine-immunoreactive bands of spermatozoa treated with H89 (Fig. 5). However, the decrease was more pronounced when spermatozoa were treated with H8. Genistein and staurosporin, as reported previously, significantly inhibited phosphotyrosine-immunoreactive bands. Two bands of approximately 162 and 83 kDa were reduced in H89-treated spermatozoa and absent in H8-, genistein- and staurosporin-treated cells. In a similar way to staurosporin, although in most cases to a lesser extent, H8 significantly reduced all bands that were seen in the western blots of Triton-X100 solubilized spermatozoa. Conversely, treatment with H89 induced only a minor decrease in the intensity of the bands (Fig. 6).

cAMP-dependent enhancement of tyrosine-phosphorylated proteins and motion parameters

In an attempt to characterize further the possible interaction between protein kinase A and tyrosine kinase

Table 1. Effect of kinase inhibitors on changes in sperm motility that occur during a 6 h capacitating incubation

Inhibitors	Inhibitor ($\mu\text{mol l}^{-1}$)	Percentage motile (%)	Progressive motility (%)	VAP (%)	VSL (%)	VCL (%)	ALH (%)	BCF (%)	STR (%)	LIN (%)	HA (%)
H89	25	86.3 ± 3.5^a	75.7 ± 4.8^a	87.4 ± 2.3^a	84.4 ± 2.6^a	92.1 ± 3.2^c	96.6 ± 2.9	94.9 ± 3.5	94.3 ± 1.7^b	92.5 ± 2.8	106.9 ± 13.2
H8	500	69.6 ± 5.0^a	45.2 ± 4.7^a	74.5 ± 4.0^a	68.5 ± 3.4^a	92.1 ± 4.1	113.2 ± 4.2^b	92.1 ± 3.7^c	87.2 ± 1.2^a	75.2 ± 2.3^a	160.2 ± 29.1
GEN	400	63.6 ± 4.9^a	55.4 ± 4.9^a	67.2 ± 2.5^a	63.9 ± 2.9^a	73.9 ± 3.0^a	78.4 ± 4.8^a	93.5 ± 4.1	94.8 ± 1.5^b	88.8 ± 3.3^a	38.9 ± 9.9^a
STA	1	46.5 ± 5.4^a	24.1 ± 3.6^a	85.6 ± 3.8^b	65.0 ± 3.1^a	105.6 ± 4.5	110.0 ± 8.8	103.8 ± 6.7	79.8 ± 2.9^a	65.0 ± 3.1^a	286.9 ± 66.1^c

Washed spermatozoa were incubated with or without protein kinase A (H8, H89), protein tyrosine kinase (genistein, GEN) and wide spectrum kinase (staurosporin, STA) inhibitors in Ham's F10 and human serum albumin at 37°C for 6 h. Motility and motion parameters were analysed using a Hamilton Thorne motion analyser (v 10.8).

Values expressed as means $\pm$ SEM ($n = 24$) represent percentages of control (6 h of incubation without inhibitors).

Superscripts indicate significant differences when compared with control (no inhibitors) by the Wilcoxon signed-rank test (a: $P < 0.0001$; b: $P < 0.005$; c: $P < 0.05$).

ALH: amplitude of lateral head displacement; BCF: beat-cross frequency; HA: hyperactivated; LIN: curvilinear path; STR: straightness; VAP: average path velocity; VCL: curvilinear velocity; VSL: straight line velocity.

Table 2. Effect of protein kinase A and protein tyrosine kinase inhibitors on cAMP and pentoxifylline (PTX)-stimulated sperm motion parameters

	Motility	Progressive motility	VAP (%)	VSL (%)	VCL (%)	ALH (%)	BCF (%)	STR (%)	LIN (%)	HA (%)
cAMP/PTX	90.7 ± 3.7^c	82.7 ± 5.8^b	113.8 ± 3.7^a	108.9 ± 4.9	129.6 ± 3.9^a	136.9 ± 4.3^a	99.7 ± 3.7	95.5 ± 1.5^b	87.0 ± 3.5^b	508.5 ± 86.4^a
H89 + cAMP/PTX	70.0 ± 3.7^a	63.2 ± 4.6^c	93.5 ± 2.6^a	91.2 ± 2.9^b	104.6 ± 2.6^a	118.9 ± 3.8^a	102.1 ± 3.7	96.0 ± 0.9	89.8 ± 2.8	241.5 ± 26.6^c
GEN + cAMP/PTX	52.1 ± 4.1^a	31.4 ± 3.4^a	63.6 ± 1.7^a	58.1 ± 2.2^a	79.8 ± 2.5^a	94.9 ± 5.2^a	95.3 ± 3.9	88.3 ± 1.1^a	75.2 ± 2.2^c	56.7 ± 9.4^a

Swim-up spermatozoa were incubated with or without kinase inhibitors (25 $\mu\text{mol l}^{-1}$ H89 or 400 $\mu\text{mol l}^{-1}$ genistein (GEN)) for 30 min and subsequently stimulated with cAMP and PTX (1 mmol l^{-1} each) for 1 h. Motility and motion parameters were analysed with a Hamilton-Thorne motion analyser (v 10.8) and normalized against control (medium alone, no cAMP and PTX).

CAMP and PTX data were compared with control (100%) with the Wilcoxon's signed-rank test.

The two variants containing kinase inhibitors were compared with cAMP/PTX using Welch's t test.

Superscripts indicate significance (a: $P < 0.0001$; b: $P < 0.001$; c: $P < 0.05$).

ALH: amplitude of lateral head displacement; BCF: beat-cross frequency; HA: hyperactivated; LIN: curvilinear path; STR: straightness; VAP: average path velocity; VCL: curvilinear velocity; VSL: straight line velocity.

Fig. 5. Effect of protein tyrosine kinase and protein kinase A inhibitors on sperm protein tyrosine phosphorylation at baseline ($t=0$) and at 6 h of incubation ($t=6$) in Ham's F10 and human serum albumin at 37°C in 5% CO₂. Proteins were extracted from washed spermatozoa in SDS-buffer, loaded on to a 7.5% (w/v) polyacrylamide gel (1×10^6 l⁻¹ cells per lane), separated by electrophoresis, transferred to Immobilon P sheets and immunolabelled with phosphotyrosine antibody (4G10). Lanes were loaded with proteins extracted from untreated control spermatozoa ($t=0$ and $t=6$) or spermatozoa incubated with the inhibitors (H89, H8, genistein (GEN), staurosporin (STA)) for 6 h. The lane with genistein was run on a different gel using the same sperm sample. Images were taken with a SPOT II digital camera. Molecular masses of bands were determined using Kodak digital imaging system (v 2.1) ($n=12$). Arrowheads indicate the approximate molecular mass of the main visible protein bands. MMs: molecular markers.

in human spermatozoa, swim-up spermatozoa were incubated in Ham's F10 + 0.35% HSA at 37°C in 5% CO₂ for 90 min, under protein kinase A stimulation via a combination of 1 mmol dibutyryl cAMP l⁻¹ and 1 mmol pentoxifylline l⁻¹, a phosphodiesterase inhibitor.

Although barely modifying total motility or progressive motility, cAMP plus pentoxifylline significantly increased VCL, ALH and especially, hyperactivation (Table 2, Fig. 7). Furthermore, cAMP plus pentoxifylline induced a parallel increase in protein kinase A activity in the soluble fraction of sperm extracts. Tyrosine kinase activity was increased too, particularly in the whole extract (Fig. 8). The incidence of tyrosine phosphorylated-positive spermatozoa was augmented too (Fig. 9). When spermatozoa were incubated with cAMP plus pentoxifylline, almost all the bands in the western blots of whole sperm extracts were increased in intensity (Fig. 10). However, the soluble fraction showed a more selective pattern. Only bands with molecular masses of about 105, 81, 55 and 48 kDa were consistently augmented (Fig. 11).

Inclusion of H89, an inhibitor of the catalytic subunit of protein kinase A, returned soluble protein kinase A activity to basal values (that is, without cAMP plus

Fig. 6. Effect of protein tyrosine kinase and protein kinase A inhibitors on tyrosine phosphorylation of soluble (Triton-X100) sperm proteins at baseline ($t=0$) and after a 6 h incubation ($t=6$) in Ham's F10 and human serum albumin at 37°C in 5% CO₂. Proteins were extracted from washed spermatozoa in SDS-buffer, solubilized with 0.05% Triton-X100, loaded on to a 7.5% (w/v) polyacrylamide gel (1×10^6 l⁻¹ cells per lane), separated by electrophoresis, transferred to Immobilon P sheets and immunolabelled with phosphotyrosine antibody (4G10). Lanes were loaded with proteins extracted from untreated control spermatozoa ($t=0$ and $t=6$) or spermatozoa incubated with the inhibitors for 6 h (H89, H8, genistein (GEN), staurosporin (STA)). The lane with genistein was run on a different gel using the same sperm sample. Note that the order of the lanes is not the same as that of Fig. 5. Images were taken with a SPOT II digital camera. Molecular masses of bands were determined using a Kodak digital imaging system (v 2.1) ($n=5$). Arrowheads indicate the approximate molecular masses of the main visible protein bands. MMs: molecular markers.

pentoxifylline) and significantly reduced total tyrosine kinase activity in whole sperm extracts (Fig. 8). Soluble tyrosine kinase activity was reduced to a lesser extent. Most bands in western blots of whole-sperm extracts decreased in intensity (Fig. 10), and the incidence of tyrosine phosphorylated-immunoreactive spermatozoa was significantly reduced (Fig. 9). In the soluble fraction, the cAMP-dependent increase of the 55 and 48 kDa bands was consistently reversed (Fig. 10), as were most of the enhanced motion parameters such as velocity, ALH and hyperactivation (Table 2, Fig. 7).

The inclusion of genistein resulted in inhibition of both tyrosine kinase and protein kinase A activity in the soluble fraction. Tyrosine kinase activity in the whole extract was less affected (Fig. 8). The incidence of tyrosine phosphorylated-immunoreactive spermatozoa as well as the intensity of phosphotyrosine-immunoreactive bands in western blots of whole-sperm extracts were decreased only partially (Figs 9, 10). Conversely, there was a very clear decrease in the 105, 81, 55 and 48 kDa bands of the soluble fraction (Fig. 11). Occasionally, there was a further reduction in low molecular mass

Fig. 7. Effect of protein tyrosine kinase (PTK) and protein kinase A (PKA) inhibitors on sperm hyperactivated motility stimulated by cAMP and pentoxifylline (PTX). Swim-up spermatozoa were incubated with or without PTK or PKA inhibitors (H89 and genistein (GEN)) for 30 min in Ham's F10 and human serum albumin at 37°C in 5% CO₂. Spermatozoa were stimulated further with 1 mmol cAMP l⁻¹ and 1 mmol PTX l⁻¹ for 1 h and motion parameters assessed using a Hamilton-Thorn motion analyser (v.10.8). Control spermatozoa were incubated with medium only. Data are expressed as percentages of the control (90 min without inhibitors) indicated by a dashed line. However, the effect of the inhibitors should be evaluated in comparison with the spermatozoa treated with cAMP and PTK. Wilcoxon's signed-rank test was used to analyse cAMP and PTX data versus control. The rest of the data were compared against cAMP and PTX using Welch's test. Superscripts indicate significance of the data comparison (a: $P < 0.0001$; c: $P < 0.05$) ($n = 10$).

bands (<40 kDa). Almost all motion parameters were significantly reduced, and a tenfold decrease in the percentage of hyperactivated spermatozoa was registered (Table 2, Fig. 7).

Discussion

Regulation of sperm motility is a complex phenomenon mediated by many factors, among which protein tyrosine phosphorylation plays an important role (Yunes *et al.*, 1994; Leclerc *et al.*, 1996; Luconi *et al.*, 1996; Vijayaraghavan *et al.*, 1997b; Tash and Bracho, 1998; Turner *et al.*, 1999). In fact, phosphorylation and dephosphorylation of selected flagellar proteins in tyrosine residues have been shown to be linked to activated and hyperactivated sperm motility (Si and Okuno, 1999; Fujinoki *et al.*, 2001).

Tyrosine phosphorylation in spermatozoa has been associated with capacitation, and it has become the focus of many studies as a critical signal transduction mechanism involved in regulation of sperm function (Visconti and Kopf, 1998). Studying mouse spermatozoa, Visconti *et al.* (1995b) demonstrated that capacitation and protein tyrosine phosphorylation were regulated by a cAMP-dependent pathway. The authors suggested that protein kinase A would be located upstream from

Fig. 8. Effect of kinase inhibitors on protein tyrosine kinase (PTK) and protein kinase A (PKA) activity of spermatozoa stimulated with 1 mmol cAMP l⁻¹ and 1 mmol pentoxifylline (PTX) l⁻¹ for 1 h in Ham's F10 and human serum albumin at 37°C in 5% CO₂. A colorimetric immunoreaction (ELISA) detecting tyrosine- and serine-phosphorylated target peptides was used to measure kinase activity. Data (mean $\pm$ SE, $n = 10$) are expressed as percentage of control (spermatozoa incubated in Ham's F10 and human serum albumin without stimulators or inhibitors), indicated by a dashed line. However, the effect of the inhibitors (H89 and genistein (GEN)) should be evaluated in comparison with spermatozoa treated with cAMP and PTK. Wilcoxon's signed-rank test was used to analyse cAMP and PTX data versus control. The rest of the data were compared against cAMP plus PTX using the Welch's test or paired t test. Superscripts indicate significance of comparisons (c: $P < 0.05$). □: solubilized PTK; ▨: solubilized PKA; ■: PTK in whole sperm extracts.

tyrosine kinase; however, the possibility of a direct influence of capacitation-related changes on sperm protein tyrosine kinase and tyrosine phosphorylation remains. Such an inter-relationship between protein kinase A and tyrosine kinase pathways has also been demonstrated in spermatozoa of other species, including humans (Hayashi *et al.*, 1987; Dey and Brokaw, 1991; Visconti *et al.*, 1995b, 1999; Carrera *et al.*, 1996; Osheroff *et al.*, 1999; Si and Okuno, 1999). Concerning human spermatozoa, Osheroff *et al.* (1999) have demonstrated that cholesterol efflux, a phenomenon known to occur during capacitation, is associated with an increase in tyrosine phosphorylation in spermatozoa, apparently through an upregulation of protein kinase A. On the basis of data from the present study it is proposed that the activity of tyrosine kinase may be associated with motility regulation in a direct manner, effecting capacitation-related changes in the movement of human spermatozoa without the necessary intervention of protein kinase A.

H89 is a specific protein kinase A inhibitor that operates by competing with ATP at the catalytic subunit of the kinase (Engh *et al.*, 1996). There is ample evidence of its protein kinase A-inhibiting properties, both in somatic and sperm cells (Leclerc *et al.*, 1996; Galantino-Homer *et al.*, 1997; Harrison and Miller, 2000; Momura *et al.*, 2000). Visconti *et al.* (1995b)

Fig. 9. Effect of protein kinase A (PKA) and protein tyrosine kinase (PTK) inhibitors (H89 and genistein (GEN)) on the cAMP and pentoxifylline (PTX) enhanced-incidence of phosphotyrosine-immunoreactive sperm tails. Swim-up spermatozoa were incubated with the inhibitors for 30 min and then stimulated with 1 mmol cAMP l⁻¹ and 1 mmol PTX l⁻¹ for 1 h in Ham's F10 plus human serum albumin at 37°C in 5% CO₂. Spermatozoa were then fixed and permeabilized with methanol and labelled with phosphotyrosine monoclonal antibody (4G10) and fluorescein isothiocyanate-conjugated anti-mouse IgG. Data (mean ± SE, *n* = 10) are expressed as percentage of control (spermatozoa incubated in medium alone; dashed line). However, the effect of the inhibitors should be evaluated in comparison with spermatozoa treated with cAMP and PTK. Wilcoxon's signed-rank test was used to compare cAMP and PTX versus control data. Data with inhibitors were compared against cAMP and PTX data using Welch's test or paired *t* test. Superscripts indicate the statistical significance of comparisons (a: *P* < 0.0001; b: *P* < 0.001; c: *P* < 0.05).

showed that mouse spermatozoa treated with H89 displayed decreased tyrosine phosphorylation, acrosome reactivity and IVF potential. In the present study, in which human spermatozoa incubated under capacitating conditions were used, H89 significantly inhibited protein kinase A; however, this inhibition did not translate into a reduction of tyrosine kinase activity. Consequently, phosphotyrosine-immunoreactive proteins were slightly decreased in western blots of both whole and soluble sperm protein extracts, and motion parameters suffered only a small reduction. However, addition of genistein, which inhibited tyrosine kinase but not protein kinase A, produced a significant reduction in phosphotyrosine proteins and motion parameters. Decreased tyrosine phosphorylation of flagellar proteins has been associated with diminished ability of spermatozoa to hyperactivate, probably due to decreased flexibility of the fibrous sheath of the sperm tail (Si and Okuno, 1999). It is worth noting that in the present study, in addition to assessing the tyrosine phosphorylation of human sperm proteins using western blot analysis and their incidence using immunofluorescence, the sperm enzymatic activity specific to tyrosine and protein kinase A was measured, which has not been reported in the studies cited above.

H89 was reported by Leclerc *et al.* (1996) to inhibit capacitation in human spermatozoa, measured by the ability of spermatozoa to acrosome react,

Fig. 10. Effect of protein kinase A and protein tyrosine kinase inhibitors on sperm tyrosine phosphorylation at baseline and after stimulation with 1 mmol cAMP l⁻¹ and 1 mmol pentoxifylline l⁻¹ at 37°C in 5% CO₂ for 1 h. Proteins were extracted from swim-up spermatozoa in SDS-buffer, loaded on to a 7.5% (w/v) polyacrylamide gel (1 × 10⁶ l⁻¹ cells per lane), separated by electrophoresis, transferred to immobilon P sheets and immunolabelled with phosphotyrosine antibody (4G10). Lanes were loaded with proteins extracted from untreated spermatozoa and spermatozoa treated with cAMP and PTX alone or in combination with H89 and genistein (GEN). Images were taken with a SPOT II digital camera. Molecular masses of bands were determined using a Kodak digital imaging system (v 2.1) (*n* = 4).

while decreasing tyrosine phosphorylation only slightly. Moreover, treatment with H89 induced a small decrease in sperm motion parameters. Inclusion of Rp-cAMPS, an inactive analogue of cAMP which competes with cAMP for the RII subunit of protein kinase A, partially inhibited capacitation and tyrosine phosphorylation and showed no effect on motility. These findings are in agreement with those reported in the present study.

As mentioned above, the involvement of protein kinase A in the regulation of sperm tyrosine phosphorylation of sperm proteins has been well documented. However, results of the present study indicate that activation of protein kinase A is not a *sine qua non* condition for capacitation-related upregulation of sperm tyrosine kinase activity, at least for the activity involved in the modulation of human sperm motility. Supporting this contention, Vijayaraghavan *et al.* (1997c) reported that inhibition of protein kinase A catalytic subunit in bovine spermatozoa had little effect on basal or chloro-deoxyadenosine-stimulated sperm motility.

Fig. 11. Effect of protein kinase A and protein tyrosine kinase inhibitors on tyrosine phosphorylation of soluble (Triton-X100) sperm proteins stimulated by 1 mmol cAMP l^{-1} and 1 mmol pentoxifylline (PTX) l^{-1} at 37°C in 5% CO_2 during 1 h of incubation. Proteins were extracted from swim-up spermatozoa in SDS-buffer, solubilized with 0.05% Triton-X100, loaded on to a 7.5% (w/v) polyacrylamide gel ($1 \times 10^6 \text{ l}^{-1}$ cells per lane), separated by electrophoresis, transferred on to Immobilon P sheets and immunolabelled with phosphotyrosine antibody (4G10). Lanes were loaded with proteins extracted from untreated spermatozoa and spermatozoa treated with cAMP and PTX alone or in combination with H89 and genistein (GEN). Images were taken with a SPOT II digital camera. Molecular masses of bands were determined using a Kodak digital imaging system (v 2.1) ($n=4$). Arrowheads indicate the approximate molecular mass of the main visible protein bands.

Other factors, besides activation of the protein kinase A pathway, may modulate tyrosine kinase activity in the sperm tail. The redox status of the sperm cells has a profound effect in tyrosine phosphorylation (Aitken *et al.*, 1995; Krzyzosiak *et al.*, 2000). Sperm tyrosine kinase activity has also been shown to be influenced by Ca^{2+} as well as other cations (Berruti, 1994; Aitken *et al.*, 1995; Luconi *et al.*, 1996). Ca^{2+} can activate phosphatases, which may indirectly affect tyrosine phosphorylation (Tash and Bracho, 1994; Leclerc *et al.*, 1996; Luconi *et al.*, 1996; Smith *et al.*, 1996). Travis *et al.* (2001) demonstrated that ATP specifically produced by a glycolytic pathway in the principal piece of the flagellum, acting downstream from cAMP, was strictly required, and represented an important regulator for protein tyrosine phosphorylation events at the sperm tail.

In the present study, contrary to H89, a less specific protein kinase A inhibitor, H8, which competes with ATP at the catalytic subunit, inhibited both sperm protein

kinase A and tyrosine kinase activity, reducing tyrosine phosphorylation. A more marked reduction was observed when staurosporin, a wide-spectrum kinase inhibitor, was used. Protein kinase A inhibition caused by H89, H8 or staurosporin was similar in magnitude (58, 59 and 52% of control, respectively). However, the magnitude of the decrease in tyrosine phosphorylation induced by these compounds was different, and directly related to their extent of inhibition of tyrosine kinase activity. Most studies citing an upstream regulation of tyrosine phosphorylation by protein kinase A have not measured specific kinase activity and the impact of the used inhibitors on this parameter. Results of the present study show no correlation between protein kinase A inhibition and downregulation of human sperm tyrosine phosphorylation. Interestingly, inhibition of both tyrosine and serine-threonine kinase activities at the same time had a differential impact on motion parameters, with a net increase in hyperactivated spermatozoa. Similar changes have been reported as a result of increased PP_2B phosphatase activity (Tash *et al.*, 1988; Tash and Bracho, 1994).

The involvement of a protein kinase A and tyrosine kinase cross-talk in the regulation of human sperm motility parameters was explored further by incubating spermatozoa with a permeable analogue of cAMP, dibutyryl cAMP, and a phosphodiesterase inhibitor, pentoxifylline, to prevent cAMP intracellular degradation. The stimulation of the protein kinase A transduction pathway resulted in a selective increase in the magnitude of certain sperm motion parameters such as velocity (VAP and VCL) and ALH as well as a slight reduction in linearity. Higher curvilinear velocity with lower linearity and greater amplitude of head displacement defines hyperactivated motility in human spermatozoa (Mortimer *et al.*, 1984; Burkman, 1991). The percentage of hyperactivated spermatozoa in the aliquots incubated with cAMP plus pentoxifylline was five times more than in the control (no protein kinase A stimulation). Such enhancement has been reported by Tesarik *et al.* (1992), Pang *et al.* (1993), Lewis *et al.* (1994) and Nassar *et al.* (1999). In parallel with those modifications in the motion characteristics of spermatozoa, an increase in the protein kinase A activity of the soluble fraction as well as in the tyrosine kinase activity of both soluble and whole-cell extracts was recorded in the present study. Phosphotyrosine-containing proteins were increased in both fractions.

Inhibition of the catalytic activity of protein kinase A by addition of H89 blunted the enhancement of sperm motility and tyrosine phosphorylation, highlighting participation of kinase A in the modulation of these phenomena. However, the reduction in cAMP and pentoxifylline-stimulated motion parameters was much more marked when genistein was included in the medium. The changes in sperm motion correlated with a significant reduction in tyrosine kinase activity and the

tyrosine-phosphorylated proteins of the soluble sperm fraction.

Both soluble and insoluble phosphotyrosine-containing proteins have been associated with sperm motility. Notably, the tyrosine phosphorylation status of a 55 kDa protein isolated from the soluble supernatant of bovine spermatozoa has been shown to be associated with the presence and stimulation of motility (Vijayaraghavan *et al.*, 1997b). The degree of tyrosine phosphorylation of this protein was much higher in motile caudal than in immotile caput epididymal spermatozoa. Motility activators, isobutylmethyl xanthine and 8-bromo-cAMP, increased the phosphotyrosine content of the 55 kDa protein. This protein was later identified as a form of glycogen synthase kinase, GSK-3 (Vijayaraghavan *et al.*, 2000). In the present study, a 55 kDa protein band identified in the soluble fraction of human spermatozoa clearly correlated with motility changes imposed by the capacitation process, cAMP and kinase inhibitors.

Soluble cytosolic proteins may connect capacitation-associated changes to structural proteins involved in sperm movement. A 37 kDa protein soluble in Triton-X100, antigenically related to calpactin II, has been identified in human sperm extracts (Berruti, 1988). Calpactins comprise a family of Ca^{2+} -binding proteins that in somatic cells mediate plasma membrane-cytoskeleton interactions. Another group of proteins with Ca^{2+} -binding properties has recently been characterized in human spermatozoa (Naaby-Hansen *et al.*, 2002). The main Ca^{2+} -binding isoform is an acidic, 86 kDa protein, known as CABYR, the ability of which to bind Ca^{2+} is associated with its tyrosine-phosphorylated status. Both properties of this protein increase during capacitation. Interestingly, CABYR has been localized to the principal piece of human spermatozoa, over the fibrous sheath area, with a very similar distribution to that of A kinase anchoring proteins (AKAPs) 4 and 3 (Johnson *et al.*, 1997; Mandal *et al.*, 1999). Changes in the sperm plasma membrane that occur during capacitation might be linked to tail cytoskeletal components, such as the fibrous sheath, through Ca^{2+} -binding proteins such as calpactins or CABYR.

In the present study, the tyrosine phosphorylation of proteins with a molecular mass similar to that of AKAP4 and CABYR (81–86 kDa) was significantly affected by both tyrosine and A kinase inhibition. AKAPs have been identified in spermatozoa of different species (Carrera *et al.*, 1994, 1996; Moss *et al.*, 1999; Vijayaraghavan *et al.*, 1999). They are present in the insoluble fraction of sperm extracts and some of them have been identified as the major tyrosine-phosphorylated proteins of the fibrous sheath, a cytoskeletal structure that extends all along the principal piece of the spermatozoon and is involved in modulating flagellar bending (Si and Okuno, 1993). More interesting is the role of AKAPs as scaffolding proteins that bring into close interaction several signal

transduction molecules such as kinases, phosphatases, Ca^{2+} -binding proteins, Ca^{2+} channels and membrane receptors (for a review, see Dodge and Scott, 2000). Anchoring proteins located in the sperm tail might act as multimeric transductional complexes integrating signals coming from plasma membrane and cytosol, and efficiently transferring them to axonemal and para-axonemal proteins that are the ultimate regulators of sperm movement.

In conclusion, tyrosine phosphorylation in human spermatozoa appears to be directly influenced by capacitation-related changes that occur during incubation in protein-supplemented complete medium *in vitro*. The same or similar changes also stimulate cAMP-dependent kinase activation, which besides phosphorylating proteins in serine and threonine residues facilitates tyrosine phosphorylation. This stimulatory pathway appears to activate certain sperm motility parameters preferentially, such as velocity and amplitude of lateral head displacement, increasing the proportion of spermatozoa that display hyperactivated motility. These changes occur in parallel to an increase in soluble tyrosine kinase activity and its resulting soluble phosphoproteins. Insoluble tyrosine kinase activity is also augmented when the protein kinase A pathway is stimulated, increasing tyrosine phosphorylation of structural proteins such as AKAPs and axonemal proteins. Both systems are closely interrelated at the sperm tail and are critically involved in regulation of human sperm motility.

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