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A flow cytometric assay for global estimation of tyrosine phosphorylation associated with capacitation of spermatozoa from two marsupial species, the tammar wallaby (*Macropus eugenii*) and the brushtail possum (*Trichosurus vulpecula*)

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Abstract

The phosphorylation of tyrosine residues in cellular proteins is a major signal transduction event during sperm capacitation. In this study protein phosphorylation was monitored using a fluorescein isothiocyanate (FITC)-labeled antiphosphotyrosine monoclonal antibody and a flow cytometric procedure optimized for sperm. Using this technique, the correlation between tyrosine phosphorylation and sperm capacitation was examined in two marsupial species, the brushtail possum and the tammar wallaby and compared with that of ram spermatozoa. The levels of tyrosine phosphorylation in sperm from all three species were increased by the addition of cyclic AMP (cAMP) and vandate, a phosphotyrosine phosphatase inhibitor and were decreased by the addition of the phosphotyrosine kinase inhibitor, staurosporine. Oviductal conditioned media (CM) induced a progressive increase in tyrosine phosphorylation in both marsupial species and also induced morphological transition from a streamlined to a 'T'-shape configuration in brushtail possum spermatozoa but not in tammar wallaby spermatozoa. Transition to the 'T'-shape orientation associated with capacitation in marsupial spermatozoa was observed by 2 h of incubation in both species when tyrosine phosphorylation was increased by higher levels of cAMP i.e. 5 mM dibutyryl cAMP plus 3 mM pentoxyphylline. Thus the tyrosine phosphorylation trigger with CM may differ in these two marsupial species. Ram sperm tyrosine phosphorylation could be increased by addition of lower levels of cAMP (1 mM). These results support the finding that tyrosine phosphorylation is associated with sperm capacitation in marsupials. Similar results were obtained by using SDS PAGE/Western blot analysis of tyrosine phosphorylation in the brushtail possum spermatozoa. The specificity, efficiency and sensitivity of the procedure described here make it applicable for routine assessment of capacitation in large numbers of samples and in other species.

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Introduction

Mammalian spermatozoa acquire fertilization ability during their sojourn in the female reproductive tract by undergoing complex but not yet fully understood biochemical, physiological and molecular alterations that are collectively known as capacitation. In many domestic mammalian species including humans this phenomenon can be reproduced *in vitro* in defined media (for reviews see Sidhu & Guraya 1989, Sidhu, 1991, Yanagimachi, 1994). In Australian marsupials, however, *in vitro* capacitation cannot be achieved in defined media unless sperm are cocultured with oviductal cells and/or their secretions (Sidhu *et al.* 1998, 1999*a*,*b*; Mate *et al.* 2000).

Understanding the mechanism(s) of sperm capacitation has been the subject of many recent investigations. One of the major problems confronting gamete biologists is the lack of any definitive assay for sperm capacitation apart from *in vitro* fertilization. Hyperactive sperm motility, onset of the acrosome reaction (Yanagimachi, 1994) and changes in chlortetracycline (CTC) staining patterns have been used to monitor capacitation in some species (Maxwell & Johnson 1997, Dinkins & Brackett 2000,

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Parker *et al.* 2000, Rathi *et al.* 2001) but are not useful assays for monitoring the process in spermatozoa from Australian marsupials. There are no overt morphological changes associated with capacitation in eutherian spermatozoa, however there is a distinct reorientation of the sperm head (from streamlined to 'T'-shape) during capacitation in marsupials (Bedford & Breed 1994, Molinia *et al.* 1998, Jungnickel *et al.* 1999, Sidhu *et al.* 1998, 1999*a*,*b*). Although the transition of marsupial sperm to the 'T'-shape orientation is a useful indicator it is not definitive, as not all 'T'-shape spermatozoa are capable of fertilization (Bedford & Breed 1994, Mate *et al.* 2000, Sidhu *et al.* 2003).

Several lines of evidence indicate that the control of mammalian sperm function at fertilization is through protein tyrosine phosphorylation (Tesarik *et al.* 1993, Visconti *et al.* 1995, Aitken *et al.* 1998, Flesch *et al.* 1999, Jha & Shivaji 2002). Capacitation in particular appears to be associated with tyrosine phosphorylation of sperm proteins by a cAMP-dependent protein kinase (PKA) in several eutherian species (e.g. Visconti *et al.* 1995, 1999, Naz, 1999, Pavlok *et al.* 2001, Tardif *et al.* 2001). In human spermatozoa at least two second messenger systems utilize amplifier enzymes located on the inner surface of the plasma membrane (de Jonge *et al.* 1991*a*,*b*; Doherty *et al.* 1995). The first enzyme, adenylate cyclase, allows cAMP production and activation of cAMP-dependent protein kinase (PKA). The second, phospholipase C, converts polyphosphoinositide phosphatidylinositol 4,5-biphosphate into inositol 1,4,5-triphosphate and 1,2-diacylyglycerol. The latter activates a phospholipid Ca^{2+}-dependent protein kinase (PKC). The activation of PKC has already been reported during marsupial sperm acrosome reaction, mediated by arachidonic acid derived from phospholipase A2 action on phosphatidylcholine (Sistina & Rodger 1997). The levels of cAMP within spermatozoa result from a balance between reactions catalyzed by adenylate cyclase and phosphodiesterase. Similarly, the phosphorylation on tyrosine residues of proteins is the result of phosphotyrosine kinase (PTK) enzyme and its counteraction by phosphotryosine phosphatase (PTPase) enzyme. Formation of both cAMP and phosphorylation at tyrosine residues of protein can be up-regulated or down-regulated by using stimulators or inhibitors in permeabilized. We have analyzed these pathways in marsupial sperm by using specific inhibitors or stimulators of these enzymes to investigate if these can modify the equilibrium.

The measurement of protein tyrosine phosphorylation in spermatozoa under conditions that permit capacitation thus gives a quantitative estimation of this event. In general, there are two methods used for estimating tyrosine phosphorylation of spermatozoa: (i) incorporation of radioactive phosphate on tyrosine residues, followed by precipitation with anti-phosphotyrosine antibodies or phosphoamino-acid determination (Naz, 1999) and (ii) Western blot using anti-phosphotyrosine monoclonal antibodies (Kalab *et al.* 1998). These methods are time consuming, cannot be applied to large numbers of samples and do not provide information on sub-populations within a sample. In this study, we have optimized the use of anti-phosphotyrosine antibodies for monitoring tyrosine phosphorylation in spermatozoa by flow cytometry. The global levels of sperm tyrosine phosphorylation under different *in vitro* conditions that induce capacitation were detectable in permeabilized cells using fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies against tyrosine phosphoproteins. The cell-associated fluorescence could be quantitated on a flow cytometer. With this technique we could estimate the effects of oviduct conditioned media and other intracellular modulators i.e. cAMP and phosphodiesterase inhibitors on Australian marsupial sperm tyrosine phosphorylation vis-à-vis capacitation.

Materials and Methods

Animals

Adult brushtail possums were from the Landcare Research Animal Facility at Lincoln, New Zealand. Possums were housed individually in cages as described previously (Sidhu *et al.* 1999*a*) and fed a diet of cereal pellets supplemented with fruit and vegetables. Tammar wallabies were from the Macquarie University Fauna Park, Sydney, Australia. Wallabies were housed in outside yards and fed a diet of cereal pellets. All animals had access to water available *ad libitum*. This research was approved by the Animal Ethics Committees at Macquarie University and Landcare Research.

Collection of sperm

Adult male possums were killed at Landcare Research by using a mask with CO_2:O_2 (21:11/min) followed by an intracardial injection of sodium barbiturate (125 mg/kg). Testes and epididymides were removed and sperm were recovered by back-flushing the vas deferens with 0.5–1.0 ml EMEM. Sperm samples from several males were pooled and pellet-frozen according to the method of Molinia & Rodger (1996) and were transported to Macquarie University for use after thawing at 37 °C. Sperm samples from tammar wallabies were collected by electro-ejaculation according to the method of Rodger & White (1978). Sperm samples from rams (frozen and fresh) were a gift from Dr Chis Maxwell's Laboratory at the University of Sydney, Australia.

In vitro capacitation of sperm using oviduct cultures and a co-culture system

Oviduct explants cultures and conditioned media (CM) from oviduct explants were prepared from pregnant mare's serum gonadotropin/luteinizing hormone-primed females as described previously (Sidhu *et al.* 1999*a*). Briefly, 3–4 mm^2 pieces of oviduct tissue were prepared from oviducts that were slit open longitudinally keeping

the epithelium, stroma and musculature associations intact so as to simulate *in vivo*-like conditions. These explants were washed twice with PBS and cultured in EMEM, pH 7.4 containing polyvinyl alcohol (PVA) (1 mg/ml), 100 IU penicillin/ml, 100 μg streptomycin/ml and growth factors (10 ng epidermal growth factor/ml, 5 μg insulin/ml, 5 ng transferrin/ml, 50 IU selenium/ml) (Sigma Chemical Co., St Louis, MO, USA). Oviduct explants (5 mg tissue per 250 μl medium) were cultured in 35-mm petri dishes at 36 °C under 5% CO_2 in humidified air. After 4 h of culture, the medium was replaced and the explants were cultured for another 20 h. Explant-conditioned medium (CM) was collected after centrifugation at 4000 ***g*** for 15 min at 4 °C and supernatants (CM) were preserved at − 20 °C.

CM was used to induce sperm capacitation as described previously (Sidhu *et al.* 1999*a*). Highly motile sperm samples were obtained after swim up in EMEM medium plus PVA from 4–5 frozen–thawed epididymal sperm pellets in the case of possums, and from ejaculated wallaby semen. Spermatozoa were incubated in CM (diluted 1:1) at a final concentration of 2×10^6/ml at 36 °C in 5% CO_2 and humidified air in 1.5 ml plastic tubes. Ram spermatozoa were incubated under *in vitro* conditions that induce capacitation as described by Gillan *et al.* (1997).

The percentage of motile sperm, progressive motility and percentage 'T'-shape (sperm head–tail orientation) were assessed subjectively using a phase contrast inverted microscope at 0, 2 and 4 h. The onset of 'T'-shape orientation in sperm is an indicator of sperm capacitation in marsupials (Sidhu *et al.* 1998, 1999*b*). Sperm viability was also assessed using a Fertilight Sperm Viability Kit according to the instructions of the supplier (Molecular Probes Inc., Eugene, OR, USA) and as described previously (Sidhu *et al.* 1999*a*).

Flow cytometric analysis

Sperm aliquots (40×10^6) were taken at the times indicated above and fixed in ice-cold 1% formaldehyde in PBS, pH 7.2 for 30 min at 4 °C. After centrifugation ($1000 \times g$, 3 min), spermatozoa were permeabilized in 0.05% saponin (Sigma Chemical Co.) for 10 min at room temperature. Permeabilization of spermatozoa is a critical step in staining, as the kinase enzyme is most likely localized on the inside of sperm plasma membrane. Permeabilization had no apparent effect on sperm morphology detectable by phase contrast microscopy. Non-specific binding sites were blocked by incubating in 0.1% BSA and 0.1% Tween 20 in PBS for 30 min at room temperature. Tyrosine phosphoproteins were localized using a monoclonal antibody (mAb) (10 μg/ml) against human tyrosine phosphoprotein conjugated with FITC (clone PT-66-FITC, Sigma Chemical Co.) in PBS containing 0.1% BSA for 30 min at room temperature. A non-specific FITC-conjugated mAb, MOPC-21 (Sigma Chemical Co.) was used as a control for non-specific binding. After two washes in PBS, labeled sperm were resuspended in PBS containing 0.1% BSA for flow cytometric analysis and examination by fluorescence microscopy.

Stained samples were analyzed using a BD FACS-Calibur fitted with a fluorescence-activated cell sorting module (Becton Dickinson, Sydney, Australia) equipped with a 15 mW argon laser emitting light at 488 nm. Sheath fluid was Osmosol (LabAids Pty. Ltd, Sydney, Australia). The cytometer was equipped with forward-angle light scatter (FSC; $<15°$), side-angle light scatter (SSC; $>15°$), and three fluorescence detectors: FL1 515–565 nm, FL2 565–605 nm, and FL3 >605 nm. The green fluorescence due to FITC was detected in FL1. Logarithmic amplification was used throughout, and fluorescence acquisition was gated by light scatter parameters. The detection threshold was adjusted in FL1 to eliminate particles emitting green fluorescence significantly below that of spermatozoa. Compensation was set so as to remove FL2 fluorescence from the FL1 channel and FL1 from the FL2 channel. The typical flow rate was 1000 cells/s for immunofluorescence measurement. At least 10 000 cells were analyzed for each sample and data were stored in a histogram mode on a log scale.

Activation/inhibition of tyrosine phosphorylation in sperm

In order to determine the pathways of tyrosine phosphorylation activation in sperm by CM, dibutyryl cAMP (dbcAMP – a cAMP analog) and inhibitors of phosphodiesterase (3-isobutyl-1-methylxanthine (IBMX), caffeine, pentoxyphylline) and the tyrosine phosphorylation pathway (staurosporine, phosphotyrosine) were used prior to flow cytometric analysis. Media containing varying concentrations of dbcAMP, IBMX, caffeine, and pentoxyphylline alone or in combination were made up as 100 × concentrated stocks. Concentrations of dbcAMP (1 mM), caffeine (2 mM) or IBMX (0.1 mM) that were effective in ram spermatozoa had no effect on tyrosine phosphorylation of marsupial spermatozoa (data not shown). These preliminary experiments demonstrated that marsupial sperm require higher levels of cAMP for activation of the tyrosine phosphorylation pathway than eutherian sperm. In subsequent experiments, 5 mM dbcAMP + 3 mM pentoxyphylline were used to optimally activate tyrosine phosphorylation in marsupial spermatozoa.

SDS PAGE and Western blotting

In order to validate this novel flow cytometric assay for sperm tyrosine phosphorylation, SDS PAGE/Western blotting assay was also run for comparison using only the brushtail possum spermatozoa with similar treatments.

The spermatozoa were incubated in a total volume of 150 μl in Eppendorf tubes at a final concentration of 2.4 million sperm/ml in different sets of four tubes each. Each treatment group comprised 5 mM dbcAMP + 3 mM

pentoxyphylline at 0 h and 2 h and in the presence of the phosphotyrosine kinase inhibitors staurosporine (400 nM) and genistein (400 nM) respectively. At each time point, spermatozoa were washed through 500 μl of 1 mM orthovandate at 8000 × ***g*** for 7 min and pellets were extracted by boiling for 7 min in 50 μl 2% w/v SDS, 0.375 M Tris, pH 6.8, 3% w/v mercaptoethanol, 1 mM orthovandate plus a cocktail of protease inhibitors (Sigma Chemical Co.). The supernatants were then stored at −20 °C until required. In order to ensure that equal amounts of protein were loaded into the gels, protein estimations were performed on each sample using a bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. A minimum of 1 mg total protein was loaded per lane. In order to confirm equal loading of protein, blots that had been probed for phosphotyrosine proteins were stripped and reprobed with an antibody against α-tubulin (Aitken *et al.* 1998). For this procedure, approximately 30 ml stripping buffer, consisting of 2% (w/v) SDS, 62.5 mM Tris pH 6.7 and 100 mM 2-mercaptoethanol, were added to the membrane for 1 h with constant shaking at 60 °C. The membrane was then washed for 3 × 10 min in Tris-buffered saline (TBS; 0.02 M Tris pH 7.6, 0.15 M NaCl), blocked and probed with the primary antibody.

SDS-PAGE was conducted on 1 μg solubilized sperm proteins using 7.5 or 10% polyacrylamide gels at 20 mA constant current per gel according to the method of Aitken *et al.* (1998). The proteins were then transferred onto a nitrocellulose hybond super-C membrane (Amersham International, Sydney, Australia) at 350 mA constant current for 1 h. The membrane was blocked for 1 h at room temperature with TBS containing 3% (w/v) BSA. The membrane was then incubated for 2 h at room temperature in a 1:4000 dilution of a monoclonal anti-phosphotyrosine (clone 4G10) or anti-α-tubulin (clone B-5-1-2) in TBS containing 1% (w/v) BSA and 0.1% (v/v) Tween. After incubation, the membrane was washed four times for 5 min with TBS containing 0.01% Tween-20, and then incubated for 1 h at room temperature with goat anti-mouse immunoglobulin G horseradish peroxidase conjugate, at a concentration of 1:3000 in TBS containing 1% (w/v) BSA and 0.1% (v/v) Tween-20. The membrane was again washed as described above and then the phosphorylated proteins were detected using an enhanced chemiluminescence (ECL) kit (Amersham International) according to the manufacturer's instructions.

Statistical analysis

The log scale data from the flow cytometric analyses were transformed to linear scale as per instructions on a BD FACS-Calibur machine and the mean linear values of the treatment groups were compared using the Student's *t*-test (SigmaStat for Windows, Jandel Scientific, San Rafael, CA, USA).

Results

Specificity of the assay

Increasing the levels of cAMP in marsupial sperm using dbcAMP (5 mM) and the phosphodiesterase inhibitor, pentoxyphylline (3 mM), significantly ($P < 0.01$) increased phosphorylation at tyrosine residues of sperm proteins in both the tammar wallaby and the brushtail possum as shown by this assay (Figs 1, 2). Staurosporine (400 nM), a PTK inhibitor added to permeabilized sperm prior to incubation with antibody, significantly ($P < 0.01$) decreased the phosphorylation at tyrosine residues of sperm proteins in both the tammar wallaby and the brushtail possum (Figs 1, 2). Addition of 10 mM phosphotyrosine significantly ($P < 0.01$) decreased the level of cell-associated fluorescence. In both the marsupial species, relatively high levels of cAMP (i.e. 5 mM dbcAMP plus 3 mM pentoxyphylline) were required for induction of tyrosine phosphorylation. In ram spermatozoa, however, tyrosine phosphorylation occurred in the presence of lower concentrations of cAMP (1 mM) and caffeine (2 mM) (Fig. 3).

No fluorescence was observed when FITC-PT66 mAb was added to live or non-permeabilized fixed sperm cells due to the intracellular localization of PTK. Observation of PT66 mAb-FITC-labeled brushtail possum and the tammar wallaby spermatozoa by fluorescence microscopy indicated localization of fluorescent antibody on the sperm head and tail with more intensity on the mid-piece. In ram spermatozoa, however, the localization was more intense at the post nuclear ring with a faint reaction at the post nuclear cap and mid-piece. A non-specific mAb, MOPC-21 conjugated with FITC, used to assess non-specific binding gave a very low fluorescence. This low fluorescence intensity was eliminated in flow cytometric analyses using appropriate gating. Thus the PT66 antibody reacted specifically with phosphotyrosine residues and the assay described here appears suitable for measuring sperm total cellular phosphotyrosine levels.

Sperm capacitation and tyrosine phosphorylation

Effects of CM

Incubating marsupial spermatozoa (tammar wallaby and brushtail possum) in oviduct CM (1:1) maintained higher sperm percentage motility, progressive motility and percentage viability than in controls, and in the possum, a significantly higher percentage of motile spermatozoa were transformed to a 'T'-shape orientation by 2-h incubation as reported earlier (Sidhu *et al.* 1998, 1999*a*). Aliquots of sperm taken from CM showed an increase in phosphorylation at tyrosine residues of sperm proteins at 2 h in both the tammar wallaby and the brushtail possum (Figs 4, 5). In the brushtail possum, the increase in tyrosine phosphorylation caused by oviduct CM was correlated with the onset of 'T'-shape orientation in sperm at 2 h of incubation. In the tammar wallaby sperm the

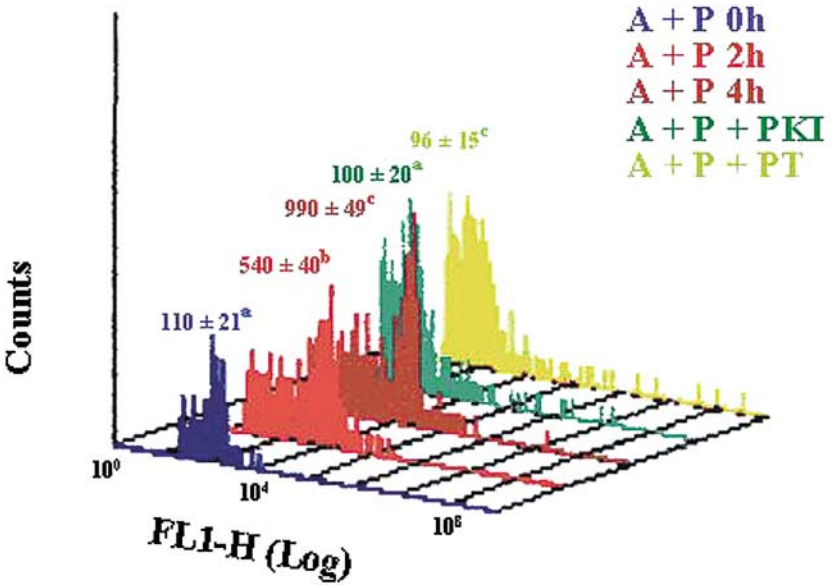

Figure 1 A flow cytometric analysis of the effects of 5 mM cAMP (A) + 3 mM pentoxyphylline (P), the phosphotyrosine kinase inhibitor (PKI) staurosporine (400 nM), and phosphotyrosine (PT) (10 mM) on phosphotyrosine levels in the tammar wallaby spermatozoa at different incubation times (0, 2, 4 h). Spermatozoa were incubated in the presence of cAMP and pentoxyphylline and aliquots taken for flow cytometric analyses as described under Materials and Methods. Staurosporine was added at the start of incubation and phosphotyrosine was added during the blocking step. The original relative fluorescence intensities on the X-axis are shown on a log scale. The sperm counts are on the Y-axis. The mean linear values for fluorescent intensities on an arbitrary scale (0–1200) are represented at the top of each peak. The experiments were repeated four times on different samples with similar results. The mean linear values with different superscripts differ significantly ($P < 0.01$).

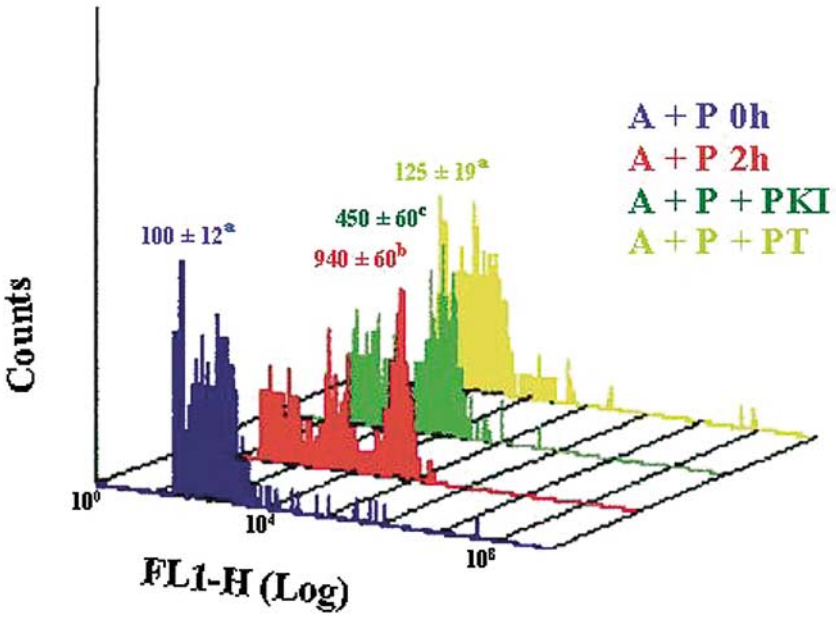

Figure 2 A flow cytometric analysis of the effects of 5 mM cAMP (A) + 3 mM pentoxyphylline (P), phosphotyrosine kinase inhibitor (PKI) staurosporine (400 nM), and phosphotyrosine (PT) (10 mM) on phosphotyrosine levels in the brushtail possum spermatozoa at different incubation times (0, 2 h). Spermatozoa were incubated in the presence of cAMP and pentoxyphylline and aliquots were taken for flow cytometric analyses as described under Materials and Methods. Staurosporine was added at the start of incubation and phosphotyrosine was added during the blocking step. The original relative fluorescence intensities on the X-axis are shown on a log scale. The sperm counts are on the Y-axis. The mean linear values for fluorescent intensities on an arbitrary scale (0–1200) are represented at the top of each peak. The experiments were repeated four times on different samples with similar results. The mean linear values with different superscripts differ significantly ($P < 0.01$).

increase in tyrosine phosphorylation was not correlated with transformation of sperm to 'T'-shape orientation. Preincubation of brushtail possum spermatozoa with staurosporine or phosphotyrosine (inhibitors of phosphotyrosine kinase) prior to exposure to CM maintained tyrosine phosphorylation at basal levels (Figs 4, 5) and prevented transformation to the 'T'-shape orientation.

Effects of inhibitors and stimulators

Caffeine (2 mM) or IBMX (0.1 mM) alone or in combination with 1 mM dbcAMP stimulated percentage motility immediately but had no significant effect on percentage 'T'-shape in the brushtail possum or the tammar wallaby spermatozoa during 0–4 h of incubation. However, 5 mM dbcAMP plus 3 mM pentoxyphylline in addition to stimulating percentage sperm motility immediately also transformed >80% of sperm to the 'T'-shape orientation in both these species within 2 h of incubation. Aliquots of spermatozoa from these incubation conditions also showed a significant progressive increase in tyrosine phosphorylation with time (Figs 1, 2). The addition of staurosporine and phosphotyrosine inhibited the increase in tyrosine phosphorylation.

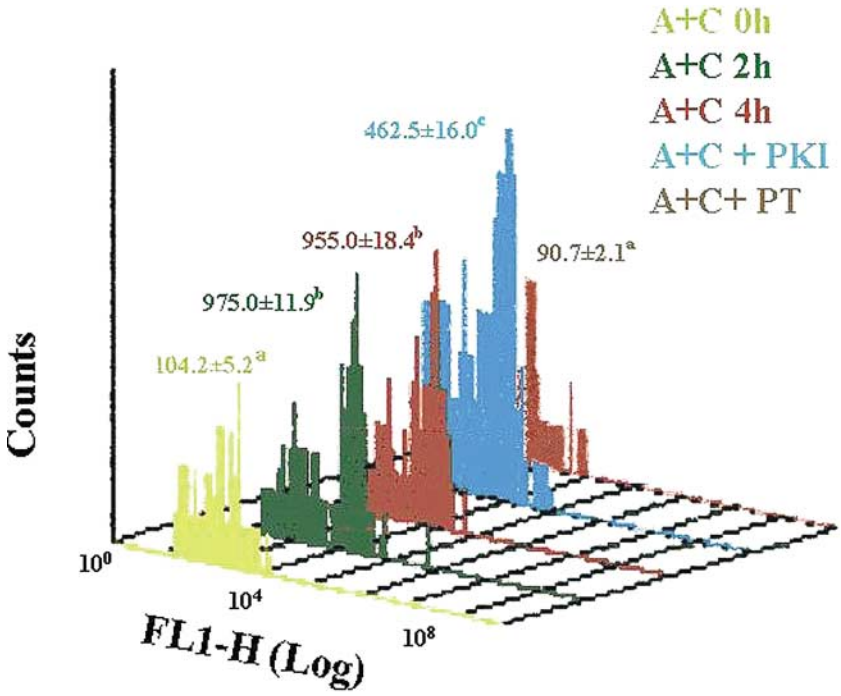

Figure 3 A flow cytometric analysis of the effects of 1 mM cAMP (A) plus 2 mM caffeine (C), the phosphotyrosine kinase inhibitor (PKI) staurosporine (400 nM), and phosphotyrosine (PT) (10 mM) on phosphotyrosine levels in the ram spermatozoa at different incubation times (0, 2, 4 h). Spermatozoa were incubated in the presence of A + C and aliquots were taken for flow cytometric analyses as described under Materials and Methods. The pathway for phosphorylation was assessed by using an inhibitor of phosphotyrosine kinase enzyme, staurosporine (400 nM), at the start of incubation and phosphotyrosine was added during the blocking step to saturate the system. The original relative fluorescence intensities on the X-axis are shown on a log scale. The sperm counts are on the Y-axis. The mean linear values for fluorescent intensities on an arbitrary scale (0–1200) are represented at the top of each peak. The experiments were repeated four times on different samples with similar results. The mean linear values with different superscripts differ significantly ($P < 0.01$).

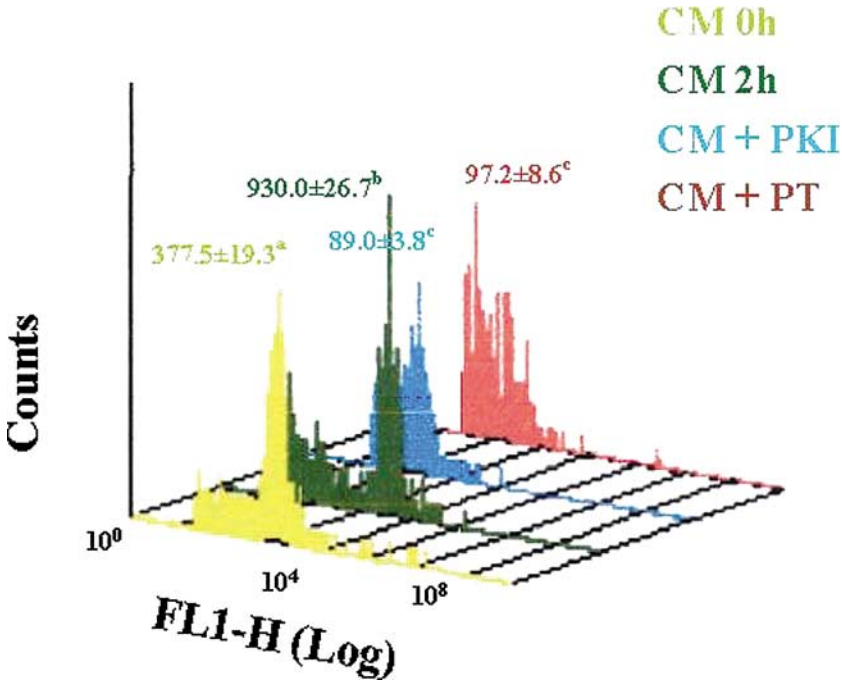

Figure 4 A flow cytometric analysis of the effects of oviduct CM on phosphotyrosine levels in the tammar wallaby spermatozoa at different incubation times (0, 2 h). Spermatozoa were incubated in the presence of CM (1:1) and aliquots taken for flow cytometric analyses as described under Materials and Methods. The pathway of CM-induced phosphorylation was assessed by using an inhibitor of phosphotyrosine kinase enzyme, staurosporine (400 nM), at the start of incubation and phosphotyrosine was added during the blocking step to saturate the system. The original relative fluorescence intensities on the X-axis are shown on a log scale. The sperm counts are on the Y-axis. The mean linear values for fluorescent intensities on an arbitrary scale (0–1200) are represented at the top of each peak. The experiments were repeated four times on different samples with similar results. The mean linear values with different superscripts differ significantly ($P < 0.01$).

A comparison of the flow cytometric assay with the SDS PAGE/Western blot assay for estimating tyrosine phosphorylation using the brushtail possum sperm

In order to validate the flow cytometric assay for sperm tyrosine phosphorylation, an SDS PAGE/Western blot assay was also run for comparison using the brushtail possum sperm. Cyclic AMP/pentoxyphylline stimulated tyrosine phosphorylation at 2 h of incubation and the same was inhibited by using two phosphorylation inhibitors, staurosporine and genistein (Fig. 6). Staurosporine appeared to be more potent than genistein in inhibiting phosphorylation, as was also observed in the flow cytometric assay.

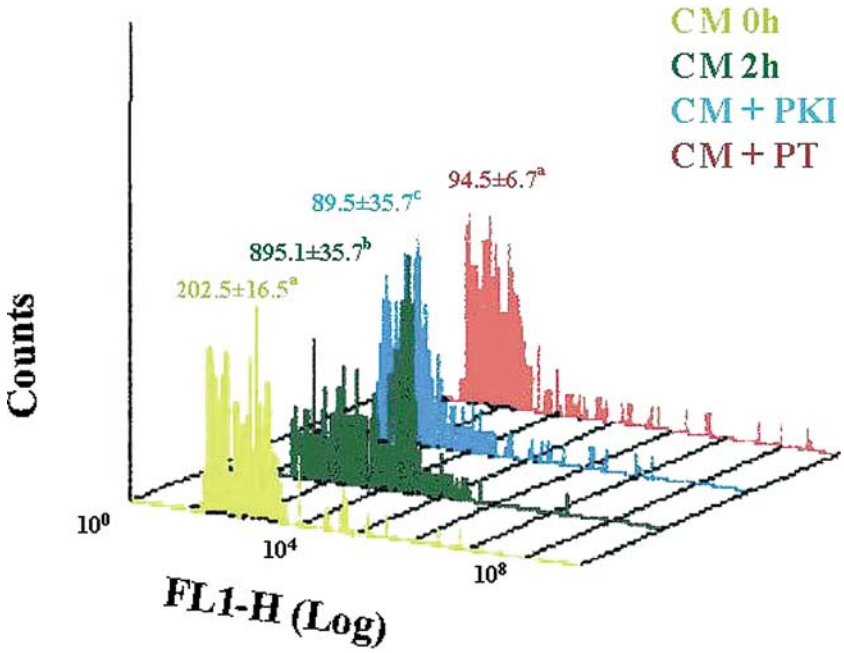

Figure 5 A flow cytometric analysis of the effects of oviduct CM on phosphotyrosine levels in the brushtail possum spermatozoa at different incubation times (0, 2 h). Spermatozoa were incubated in the presence of CM (1:1) and aliquots taken for flow cytometric analyses as described under Materials and Methods. The pathway of CM-induced phosphorylation was assessed by using an inhibitor of phosphotyrosine kinase enzyme, staurosporine (400 nM), at the start of incubation and phosphotyrosine (PT) was added during the blocking step to saturate the system. The original relative fluorescence intensities on the X-axis are shown on a log scale. The sperm counts are on the Y-axis. The mean linear values for fluorescent intensities on an arbitrary scale (0–1200) are represented at the top of each peak. The experiments were repeated four times on different samples with similar results. The mean linear values with different superscripts differ significantly ($P < 0.01$).

Figure 6 Western blot analysis of tyrosine phosphorylation in the brushtail possum spermatozoa. Effects of cAMP (5 mM) + pentoxyphylline (3 mM) at 0 h (lane 1), 2 h (lane 2), plus staurosporine (400 nM) (lane 3), and plus genistein (400 nM) (lane 4).

Discussion

Capacitation is a complex and poorly understood maturational process that prepares spermatozoa for fertilization. Several recent studies have found a correlation between sperm capacitation and phosphorylation on tyrosine residues of specific sperm proteins suggesting that measurement of protein tyrosine phosphorylation may give a quantitative estimate of capacitation. This study has described a new method for estimating levels of tyrosine phosphorylation in spermatozoa undergoing capacitation. The global levels of sperm tyrosine phosphorylation under different *in vitro* conditions that induce capacitation were estimated in permeabilized cells using FITC-conjugated mAbs against tyrosine phosphoproteins and flow cytometric analysis. This technique is rapid, simple and reliable. The specificity of the technique was demonstrated by specific displacement of FITC-labeled PT66 mAb by phosphotyrosine and the failure of PT66 to label non-permeabilized intact sperm owing to the intracellular localization of phosphoproteins. Staurosporine, a strong inhibitor of the phosphotyrosine kinase enzyme specifically inhibited sperm tyrosine phosphorylation. With this technique we were able to estimate the effects of oviduct conditioned media and other intracellular modulators, i.e. cAMP and phosphodiesterase inhibitors, on sperm tyrosine phosphorylation and capacitation in two Australian marsupial species – the brushtail possum and the tammar wallaby. Similarly, the increase in tyrosine phosphorylation in ram spermatozoa under *in vitro* conditions that promote capacitation (Gillan *et al.* 1997) indicates the applicability of this technique to other species. Similar

results were obtained by using SDS PAGE/Western blot as an assay under similar conditions for estimating tyrosine phosphorylation in the brushtail possum sperm.

In the brushtail possum there was a correlation between the onset of 'T'-shape orientation (capacitation) and tyrosine phosphorylation during incubation in oviductal CM. Our data suggest that this CM-induced transformation of possum spermatozoa to the 'T'-shape orientation may be directly related to tyrosine phosphorylation, as the addition of the phosphotyrosine kinase inhibitor, staurosporine, prevented both the increase in tyrosine phosphorylation and 'T'-shape morphology. Although the onset of 'T'-shape orientation was not observed in the tammar wallaby during incubation in oviductal CM, there was an increase in tyrosine phosphorylation with cAMP and time. Interestingly, the addition of stimulators of phosphotyrosine phosphorylation pathways (i.e. dbcAMP and pentoxyphylline) did induce 'T'-shape orientation (capacitation) in the tammar wallaby within 2 h of incubation indicating that CM alone is not sufficient or is slower to induce capacitation and head re-orientation in this species.

In summary, the flow cytometric technique described provides rapid and reliable estimates of global cellular phosphotyrosine levels in spermatozoa. The use of intact cells in this process makes it a very attractive technique for studying the effects of various modulators of tyrosine phosphorylation under *in vitro* conditions. Estimating tyrosine phosphorylation using flow cytometry also provides an opportunity to quantitate sub-populations within samples. It is particularly relevant for the study of sperm capacitation as in any given sample there are sub-populations of spermatozoa at various stages of capacitation that could be estimated by this technique. The only limitation of this technique is that it gives only the global estimates of tyrosine phosphorylation in sperm, and phospho-residues cannot be localized to specific sperm proteins. However, the rapidity of this technique makes it attractive for physiological study of sperm capacitation under different conditions.

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