Analysis of the mechanism by which calcium negatively regulates the tyrosine phosphorylation cascade associated with sperm capacitation

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Summary

The capacitation of mammalian spermatozoa involves the activation of a cAMP-mediated signal transduction pathway that drives tyrosine phosphorylation via mechanisms that are unique to this cell type. Controversy surrounds the impact of extracellular calcium on this process, with positive and negative effects being recorded in independent publications. We clearly demonstrate that the presence of calcium in the external medium decreases tyrosine phosphorylation in both human and mouse spermatozoa. Under these conditions, a rise in intracellular pH was recorded, however, this event was not responsible for the observed changes in phosphotyrosine expression. Rather, the impact of calcium on tyrosine phosphorylation in these cells was associated with an unexpected change in the intracellular availability of ATP. Thus, the ATP content of both human and mouse spermatozoa fell significantly when these cells were incubated in the presence of external calcium. Furthermore, the removal of glucose, or addition of 2-deoxyglucose, decreased ATP levels within human spermatozoon populations and induced a corresponding decline in phosphotyrosine expression. In contrast, the mitochondrial inhibitor rotenone had no effect on either ATP levels or tyrosine phosphorylation. Addition of the affinity-labeling probe 8-N_3 ATP confirmed our prediction that spermatozoa have many calcium-dependent ATPases. Moreover, addition of the ATPase inhibitor thapsigargin, increased intracellular calcium levels, decreased ATP and suppressed tyrosine phosphorylation. Based on these findings, the present study indicates that extracellular calcium suppresses tyrosine phosphorylation by decreasing the availability of intracellular ATP, and not by activating tyrosine phosphatases or inhibiting tyrosine kinases as has been previously suggested.

Key words: Human spermatozoa, Capacitation, Tyrosine phosphorylation, Calcium, ATP

Introduction

During the process of mammalian fertilization, spermatozoa undergo a priming process, known as capacitation, before they are capable of fertilizing the oocyte. Functional maturation of the spermatozoa is achieved as these cells ascend the female reproductive tract; a process that can take anywhere from 6 to 30 hours depending on the species (Chang, 1951). Originally assumed to be a simple accumulation process, the movement of spermatozoa from the vagina to the upper part of the female reproductive tract is a highly regulated phenomenon, coordinated with oocyte release and culminating in the creation of viable embryos. Using the rabbit as an animal model, Chang (Chang, 1951; Chang, 1955) demonstrated that spermatozoa must spend at least 6 hours in the female reproductive tract before fertilization can be successful. The finite period of time that spermatozoa must spend in the female tract has been termed ‘capacitation’ (Austin, 1952) and involves a variety of post-translational modifications that allow these cells to respond to cues presented in the cumulus-oocyte complex (Aitken et al., 1996).

In the context of assisted reproduction, the capacitation of spermatozoa is performed in vitro. In this procedure, freshly ejaculated or epididymal sperm are washed and incubated in a simple defined medium that mimics oviductal fluid. The latter normally contains electrolytes, metabolic energy sources and a macromolecule such as serum albumin to allow for cholesterol efflux (Yanagimachi, 1969; Yanagimachi and Usui, 1974; Aitken et al., 1983; Yanagimachi and Bhattacharyya, 1988; Yanagimachi, 1994a). Biochemical changes occurring during capacitation have been reported and include a decrease in membrane cholesterol content (Aitken et al., 1983; Cross, 1998), an increase in intracellular pH (Schackmann et al., 1981; Carr and Acott, 1989; Parrish et al., 1989) changes in intracellular Ca^{2+} concentration (Baldi et al., 1991) and the production of reactive oxygen species (Aitken and Clarkson, 1987; Aitken and Buckingham, 1992; Aitken et al., 1992a; Aitken et al., 1992b; Aitken and Fisher, 1994; Aitken et al., 1995; De Lamirande and Gagnon, 1995; Aitken et al., 1997; Aitken et al., 1995; De Lamirande et al., 1998).

A further correlate of capacitation is the ability of spermatozoa to undergo tyrosine phosphorylation (Visconti et al., 1995a; Visconti et al., 1995b; Aitken et al., 1996). In all mammalian species studied thus far, tyrosine phosphorylation in spermatozoa is regulated by the second messenger, cAMP (Furuya et al., 1992; Duncan and Fraser, 1993; Visconti et al., 1995b; Leclerc et al., 1996; Galantino-Homer et al., 1997; Visconti et al., 1997; Aitken

et al., 1998a; Aitken et al., 1998b; Leclerc et al., 1998). Thus, addition of its cell permeable analogue, dibutryl cAMP (dbcAMP), or phosphodiesterase inhibitors including 3-isobutyl-1-methylxanthine (IBMX), increase tyrosine phosphorylation of sperm proteins (Aitken et al., 1998a; Leclerc et al., 1998). It has been proposed that following production of cAMP, tyrosine phosphorylation occurs through a protein kinase A (PKA)-dependent mechanism, since this process can be inhibited by H89, a PKA inhibitor (Thundathil et al., 2002). Furthermore, one of the proteins phosphorylated in spermatozoa is an A-kinase-anchoring protein (AKAP), which anchors PKA and ensures the enzyme is exposed to local changes in cAMP (Pawson and Scott, 1997). As PKA is a serine/threonine kinase, it cannot play a direct role in tyrosine phosphorylation and therefore must activate tyrosine kinases indirectly (Leclerc et al., 1996). Such kinases must be highly cell specific since cAMP-dependent tyrosine phosphorylation has not been reported for any other cell type examined to date.

A powerful, potential regulator of tyrosine phosphorylation during sperm capacitation is Ca^{2+}. Thus, early reports documented that by increasing extracellular Ca^{2+} in the surrounding medium, an increase in tyrosine phosphorylation could be observed in both mouse (Visconti et al., 1995a) and human (Leclerc et al., 1998) spermatozoa. However, this is in direct contrast to others who observed that increasing extracellular Ca^{2+} decreased tyrosine phosphorylation, suggesting that Ca^{2+} negatively regulates phosphotyrosine expression during sperm capacitation (Luconi et al., 1996). Supporting this latter notion, kinetic analysis of both tyrosine kinase and phosphatase activities in the presence and absence of Ca^{2+} suggested that this cation had a negative impact on src kinase activity, but no impact on phosphatase activity (Luconi et al., 1996). Following on from this discovery, Leclerc and Goupil (Leclerc and Goupil, 2002), recently used immunohistochemical and western blot analysis to demonstrate the presence of c-yes kinase (a member of the src family of kinases) in the head of human spermatozoa. This enzyme was activated by cAMP and inhibited with Ca^{2+} (Leclerc and Goupil, 2002). Furthermore, upon addition of IBMX an increase in the activity of c-yes was demonstrated, suggesting this enzyme may be involved in the cAMP/PKA-dependent pathway of sperm capacitation.

With these data in mind, current evidence suggests that Ca^{2+} either positively or negatively regulates a kinase involved in sperm capacitation. The goal of this study was to re-evaluate the impact of extracellular Ca^{2+} on this unique tyrosine phosphorylation cascade in human and mouse spermatozoa and resolve the mechanisms by which this cation exerts its regulatory effect.

Materials and Methods

Materials

Bovine serum albumin (BSA) was purchased from Research Organics (Cleveland, OH). Hepes, penicillin and streptomycin were from Gibco (Grand Island, NY). The anti-phosphotyrosine antibody (clone 4G10) was purchased from Upstate Biotechnology (Lake Placid, NY). The goat anti-mouse antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and the goat serum from Hunter Antisera (Jesmond, NSW). Fura-2AM and BCECF were from Molecular Probes (Molecular Probes, Leiden, The Netherlands). All other reagents were obtained from Sigma (St. Louis, MO).

Medium

BWW medium consisted of 95 mM NaCl, 44 μM sodium lactate, 25 mM $NaHCO_3$, 20 mM Hepes, 5.6 mM D-glucose, 4.6 mM KCl, 1.7 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 0.27 mM sodium pyruvate, 0.3% (w/v) BSA, 5 U/ml penicillin, 5 μg/ml streptomycin, pH 7.4. BWW minus calcium (BWW $-Ca^{2+}$) consisted of normal BWW except that 1.7 mM NaCl replaced the $CaCl_2$. This buffer contained 2.0±0.5 μM Ca^{2+}, as measured by atomic absorption analysis.

Preparation of human spermatozoa

Freshly ejaculated human semen were obtained by masturbation, collected into a sterile plastic container and transported immediately into the laboratory for subsequent analysis according to normal criteria set out by the World Health Organization (1992). Approval for the donation of human semen samples was obtained from the University of Newcastle human ethics committee. Each sample was allowed to liquefy for 45 minutes at 37°C, before being processed. The spermatozoa were separated from seminal plasma on a two step discontinuous Percoll gradient as previously described (Aitken et al., 1997). Following centrifugation, sperm concentration was assessed using a Neubauer haemocytometer. The cells were aliquoted into various treatments at a final concentration of 10×10^6 sperm/ml and then incubated at 37°C under a 5% CO_2, 95% air atmosphere.

Preparation of mouse epididymal spermatozoa

Caudal epididymal spermatozoa were obtained from adult (8-14 week) Swiss mice. The experiments described in this report were approved by the University of Newcastle animal ethics committee. The mice were killed by carbon dioxide asphyxiation and the reproductive tracts removed. Caudal spermatozoa were collected by back-flushing with water-saturated paraffin oil, collecting the perfusate and depositing it under oil at 37°C. Sperm were then activated by addition of BWW that lacked Ca^{2+} (BWW $-Ca^{2+}$). The sperm suspension was left to disperse in the droplet for 10 minutes at 37°C and then the sperm concentration was assessed using a Neubauer hemocytometer. The cells were aliquoted into various treatment groups at a final concentration of 10×10^6 sperm/ml and then incubated at 37°C under a 5% CO_2, 95% air atmosphere. Cell viability was assessed after each treatment using the hypo-osmotic swelling (HOS) test (World Health Organization, 1997).

Extraction of sperm proteins

After incubation, spermatozoa were centrifuged (500 ***g***, 3 minutes) and washed in 1 ml of BWW lacking BSA. The spermatozoa were then solubilized (2% w/v SDS, 0.375 M Tris, pH 6.8, 10% sucrose) and heated to 100°C for 5 minutes. Following centrifugation at 20,000 ***g*** for 10 minutes, the supernatant was retained and boiled in SDS-sample buffer containing 2% (v/v) 2-mercaptoethanol. Samples 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, IL) according to the manufacturer's instructions. A minimum of 1 μg of total protein was loaded per lane.

SDS-PAGE and western blotting

SDS-PAGE was conducted on 1 μg solubilized sperm proteins using 7.5 or 10% polyacrylamide gels at 10 mA constant current per gel. The proteins were then transferred onto nitrocellulose Hybond Super-C membrane (Amersham International, Sydney, Australia) at 350 mA constant current for 1 hour. The membrane was blocked for 1 hour at room temperature with Tris buffered saline (TBS; 0.02 M Tris, pH 7.6, 0.15 M NaCl) containing 3% (w/v) BSA. The

membrane was then incubated for 2 hours at room temperature in a 1:4000 dilution of a monoclonal anti-phosphotyrosine antibody (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 minutes with TBS containing 0.01% Tween 20, and then incubated for 1 hour at room temperature with goat anti-mouse immunoglobulin G horseradish peroxidase (HRP) 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, Sydney, Australia) according to the manufacturer's instructions.

Stripping nitrocellulose membranes

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. For this procedure, approximately 30 ml of stripping buffer, consisting of 2% (w/v) SDS, 62.5 mM Tris, pH 6.7, 100 mM 2-mercaptoethanol, was added to the membrane for 1 hour with constant shaking at 60°C. The membrane was then washed (3× 10 minutes in TBS), blocked and probed with the primary antibody as described.

Measurement of intracellular pH in spermatozoa

Spermatozoa were collected and adjusted to 10×10^6 (human) or 1×10^6 (mouse) cells/ml with complete BWW. Cells were then incubated in a 5% CO_2 incubator at 37°C. When needed, 9 μM 2′,7′-bis-2(2-carbosyethyl)-5-(and-6)-carboxyfluorescence, acetoxy-methyl ester (BCECF) was added and the incubation continued for a further 40 minutes. Following this, the cells were pelleted (400 ***g***, 2 minutes) and washed twice to remove free dye. To determine the intracellular pH ($[pH]_i$), a ratiometric analysis of fluorescence data using an excitation wavelength of 440 nm and an emission of 530 nm (5 nm excitation/emission bandpass) was performed. Calibration was achieved by loading uncapacitated spermatozoa with BCECF and washing to remove the free dye as described above. Following the second wash, samples were placed in a range of pH buffers (from 6.4-7.4). 10 μM of the K^+/H^+ ionophore nigericin was added to collapse the pH gradient across the sperm membrane. A 440/490 nm ratio was then plotted against the known pH values. Unknown samples were then analyzed from this standard curve.

Measurement of intracellular Ca^{2+} in sperm

The intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was assessed using the fluorescent Ca^{2+} indicator Fura-2AM. Spermatozoa were loaded with 2 μM Fura-2AM (20 minutes, 37°C), washed in BWW $-Ca^{2+}$ (300 ***g***, 5 minutes) and resuspended at a concentration of 10×10^6 sperm/ml. The $[Ca^{2+}]_i$ was determined by loading 1 ml of sperm into a prewarmed cuvette, and fluorescence intensity recorded using a Shimadzu (Tokyo, Japan) RF-5301PC spectrofluorophotometer following excitation at 340 nm and 380 nm, and emission at 510 nm, and calculating the fluorescence ratio (F). The $[Ca^{2+}]_i$ was calculated using the equation $[Ca^{2+}]_i = K_d\ (F-F_{min})/(F_{max}-F)$, where K_d = 224 nm. F_{max} and F_{min} were recorded at the end of each incubation. F_{max} was determined after the addition of 1% (v/v) Triton X-100 made up in BWW containing 1.7 mM $CaCl_2$, and F_{min} was determined after addition of 5 mM EGTA (pH 10) to the cuvette.

Labeling with 8-azido ATP

In order to label Ca^{2+}-dependent ATP consuming enzymes 2 μCi α-^{32}P-labelled 8-azidoadenosine-5′-triphosphate [8-N_3ATP; 20 Ci/mmol, 2 mCi/ml] was added to 10×10^6 cells. At the time indicated, 1 ml of cells were removed and subjected to UV irradiation (2 minutes at 1200 MW/m^2). The cells were then washed twice with BWW-BSA. Following the second wash, spermatozoa were lysed in 5% (w/v) CHAPS for further analysis.

Measurement of ATP levels

Human (10×10^6 cell/ml) or mouse (1×10^6 cells/ml) spermatozoa were incubated in either BWW or BWW $-Ca^{2+}$. At the times indicated, 50 μl of sample were taken and ATP was measured using an ATP assay kit according to the manufacturer's instructions (Sigma, St Louis, IL). Every effort was made to ensure that loss of ATP did not occur post-lysis. Thus, equal amounts of $CaCl_2$ were added to lysates of sperm incubated in BWW $-Ca^{2+}$ so that both populations had the same amount of Ca^{2+} immediately post-lysis. The manufacturer-supplied lysis buffer was then added, which contained EDTA to chelate this residual Ca^{2+}. Upon lysis, the mixture was immediately placed in a 4°C water ice slush to minimize post-lysis Ca^{2+}-dependent ATP consumption. Finally, before performing luminometry measurements, samples were coded and then randomized.

Acrosome reactions

A sub-sample from each treatment was treated with 2.5 μM A23187 and incubated for 30 minutes. Untreated 'control' samples were treated with vehicle alone (1% DMSO, v/v). After incubation, spermatozoa were fixed with ice-cold methanol and stored at 4°C. Samples were coded and arranged in random order to avoid bias, plated onto poly-L-lysine-coated slides and allowed to dry. The presence of the acrosome was assessed by adding phosphate-buffered saline (PBS; pH 7.4) containing fluorescein-labeled *Arachis hypogea* lectin (1 mg/ml), incubated for 15 minutes at 4°C, rinsed in PBS and viewed under a Zeiss Axioplan 2 microscope equipped with FITC filters. Spermatozoa were examined for the occurrence of the acrosome reaction, and a minimum of 200 cells were scored per treatment.

Statistics

All experiments were replicated at least three times and the statistical significance of any differences observed between group means was determined by analysis of variance. Before testing $[Ca^{2+}]_i$ measurements were log transformed and all percentage data were arcsine transformed. Fisher's Protected Least Significant Difference was used to test the statistical significance of differences between group means; $P<0.05$ was considered to be statistically significant.

Results

Calcium negatively regulates tyrosine phosphorylation in human spermatozoa

In order to clarify the impact of extracellular Ca^{2+} on human spermatozoa, cells were incubated in either BWW or BWW $-Ca^{2+}$ medium and the level of tyrosine phosphorylation was measured via western blot analysis (Fig. 1A). Over a 3-hour time course, human spermatozoa incubated with extracellular Ca^{2+} (BWW) demonstrated only a slight increase in tyrosine phosphorylation. In contrast, spermatozoa incubated in Ca^{2+}-depleted medium (BWW $-Ca^{2+}$) showed not only a marked increase, but a hastening of tyrosine phosphorylation, with the maximal response seen after 140 minutes. As the degree of tyrosine phosphorylation changed in every protein, the membrane was stripped and re-probed with α-tubulin to demonstrate equal amounts of protein in each lane (Fig. 1B).

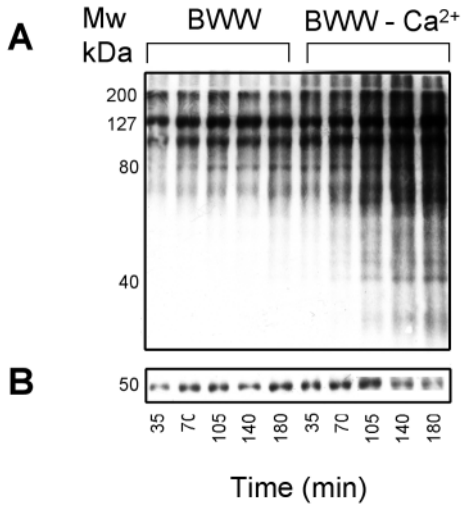

Fig. 1. Impact of calcium on tyrosine phosphorylation in human spermatozoa. Spermatozoa (10×10^6) were incubated in either BWW medium or in calcium-depleted medium (BWW $-Ca^{2+}$) as indicated. (A**)** At the times indicated, the cells were centrifuged, washed and lysed in 2% SDS. 1 μg of each lysate was loaded onto a 10% polyacrylamide gel. Tyrosine phosphorylated proteins were detected with western blot analysis using the anti-phosphotyrosine antibody, 4G10. The positions of the molecular mass markers (kDa) are shown on the left hand side. (B) The nitrocellulose membrane was stripped (2% SDS, 2 mM DTE, 50 mM Tris, pH 8.8) at 65°C with shaking. Following this, the membrane was blocked and re-probed using α-tubulin antibody, as a loading control. The western blot is representative of three independent experiments.

Changes in intracellular pH do not drive tyrosine phosphorylation

It has been reported in bovine spermatozoa, that intracellular pH $[pH]_i$ is a regulator of intracellular calcium $[Ca^{2+}]_i$ concentrations (Vijayaraghavan and Hoskins, 1990). Specifically, alkalinization of the $[pH]_i$ is associated with a decrease in $[Ca^{2+}]_i$, whereas acidification of the $[pH]_i$ is associated with increased $[Ca^{2+}]_i$ (Vijayaraghavan and Hoskins, 1990). Therefore, we examined whether the removal of Ca^{2+} from the incubation medium resulted in rise in $[pH]_i$ in human spermatozoa. Over a 3-hour time course, spermatozoa incubated in BWW $-Ca^{2+}$ showed a significant rise in $[pH]_i$ compared to cells incubated in complete BWW (Fig. 2A). In order to establish whether such a rise in $[pH]_i$, could account for the increase in tyrosine phosphorylation observed in calcium-depleted medium, we manipulated the $[pH]_i$, and examined the impact of this change on tyrosine phosphorylation. To achieve this, spermatozoa were placed into calcium-containing BWW medium buffered to a pH of 8.2. This allowed the $[pH]_i$ of spermatozoa to rise to the same as that seen in cells undergoing tyrosine phosphorylation in calcium-depleted medium (Fig. 2B). However, after 3 hours incubation, those cells in BWW at pH 8.2 (Fig. 2C, lane 3) showed no appreciable increase in tyrosine phosphorylation compared to control cells (BWW; Fig. 2C, lane 2). As a positive control, spermatozoa incubated in medium depleted of Ca^{2+}, demonstrated the anticipated increase in phosphotyrosine expression (Fig. 2B, lane 1). Such an increase was not an artifact of unequal protein loading, as demonstrated by the α-tubulin control (Fig. 2D).

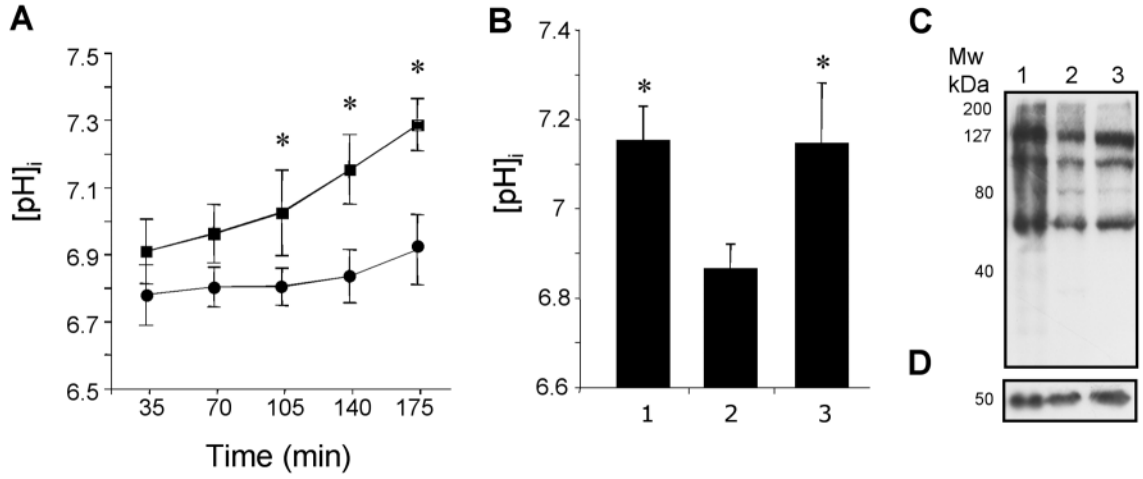

Fig. 2. Impact of extracellular calcium on intracellular pH. (A) Spermatozoa (10×10^6/ml) were incubated in either complete BWW (circles) or BWW $-Ca^{2+}$ (squares). Approximately 40 minutes before the time shown, 1 ml of sperm was harvested, washed and incubated with 9 μM BCECF. Following 40 minutes incubation, the cells were washed to remove free dye. A ratiometric analysis of 440/530 nm was performed and plotted against known standards to determine the $[pH]_i$. The results are presented as the average of three experiments performed in triplicate; $^*P<0.05$. (B) Spermatozoa (10×10^6/ml) were incubated in either BWW $-Ca^{2+}$ (lane 1), complete BWW (lane 2) or BWW with a pH of 8.2 (lane 3). After 2 hours, 1 ml of sperm from each treatment was harvested, washed and incubated with 9 μM BCECF. Following 40 minutes incubation, the cells were washed to remove free dye. A ratiometric analysis of 440/530 nm was performed and plotted against known standards to determine the $[pH]_i$. The results are presented as the average of three experiments performed in triplicate; $^*P<0.05$.
(C) Spermatozoa (10×10^6/ml) were incubated in either BWW $-Ca^{2+}$ (lane 1), complete BWW (lane 2) or BWW with a pH of 8.2 (lane 3). Following a 3-hour incubation the cells were lysed (2% SDS) and subject to anti-phosphotyrosine western blot analysis as described in Materials and Methods. (D) The nitrocellulose membrane was stripped at 65°C with shaking. Following this, the membrane was blocked and re-probed using α-tubulin antibody. The western blot is representative of three independent experiments.

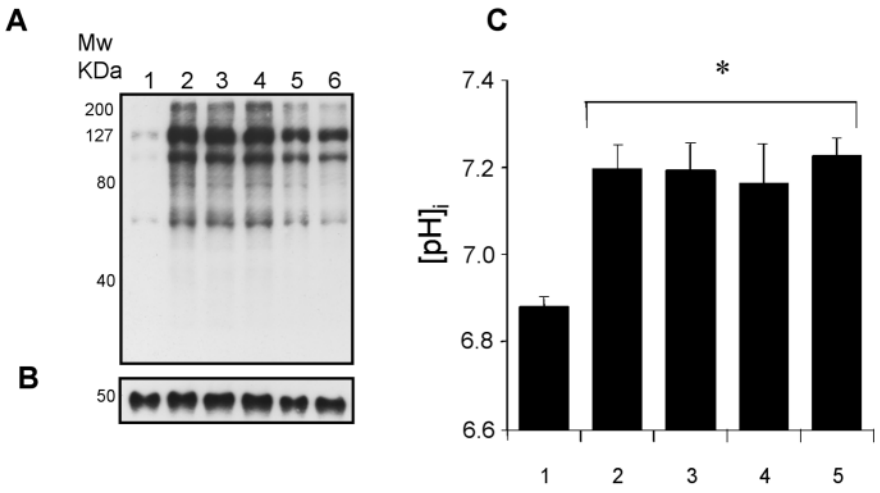

Fig. 3. Impact of tyrosine phosphorylation on intracellular pH. (A) Spermatozoa were incubated for 3 hours in either complete BWW (lane 1) BWW –Ca^{2+} (lane 2), or BWW –Ca^{2+} containing 1 (lane 3) 10 (lane 4) 20 (lane 5) or 50 (lane 6) μM H89. The cells were centrifuged, washed and lysed in 2% SDS. 1 μg of each lysate was loaded onto a 10% polyacrylamide gel. Tyrosine phosphorylated proteins were identified by western blot analysis using the antiphosphotyrosine antibody 4G10. The positions of the molecular mass markers (kDa) are shown on the left. (B) The nitrocellulose membrane was stripped at 65°C with shaking. Following this, the membrane was blocked and re-probed using α-tubulin antibody. The western blot is representative of three similar experiments. (C) Spermatozoa were incubated for 3 hours in BWW (lane 1) or BWW –Ca^{2+} (lane 2), or BWW –Ca^{2+} containing 10 (lane 3), 20 (lane 4) or 50 (lane 5) μM H89. The cells were then harvested, and the $[pH]_i$ was determined as described. The graph is the average of three independent experiments; *P<0.05.

Although, under these experimental conditions, $[pH]_i$ had no impact on tyrosine phosphorylation, it was of interest to determine whether tyrosine phosphorylation regulated $[pH]_i$. Upon addition of increasing concentrations of H89 to human spermatozoa bathed in BWW –Ca^{2+}, a dose-dependent inhibition of tyrosine phosphorylation was seen (Fig. 3A), which is not an artifact of protein loading (Fig. 3B). However, the $[pH]_i$ under these conditions continued to rise (Fig. 3C), suggesting that changes in tyrosine phosphorylation and $[pH]_i$ observed in calcium-depleted medium are independent events.

Calcium homeostasis and ATP levels in human spermatozoa

Given the profound impact that extracellular Ca^{2+} had on phosphotyrosine expression by human spermatozoa, it was of interest to determine how the level of $[Ca^{2+}]_i$ changed under these conditions. In order to investigate this, we measured the level of $[Ca^{2+}]_i$ over a 2-hour time course using the fluorescent probe Fura-2AM (Fig. 4). This experiment revealed similar levels of $[Ca^{2+}]_i$ in spermatozoa incubated in either BWW or BWW –Ca^{2+} (approx. 200 nM; Fig. 4). Since it is established that Ca^{2+} can diffuse through the sperm plasma membrane (Vijayaraghavan and Hoskins, 1990), $[Ca^{2+}]_i$ homeostasis must be maintained by pumping this cation into Ca^{2+} stores within the spermatozoa, or across the plasma membrane, or both (for a review, see Breitbart, 2002). This being the case, the level of ATP in spermatozoa incubated in complete BWW, would expected to be lower than that incubated in BWW –Ca^{2+}. To examine this hypothesis, spermatozoa were placed in either BWW or BWW –Ca^{2+} and the amount of ATP measured (Fig. 5). Spermatozoa incubated in BWW showed no increase in ATP levels over a 2-hour incubation. Cells incubated in BWW –Ca^{2+} had similar levels of ATP after 10 minutes incubation as those incubated in BWW. However, this level increased by ~15% over the next 20 minutes and remained significantly higher (P<0.05) for the rest of the incubation. Thus, after 120 minutes, spermatozoa incubated in BWW –Ca^{2+} had 59 μg ATP/10^6 cells, compared with 47 μg ATP/10^6 cells in complete BWW. Every effort was made to ensure that the decrease of ATP was not due to an artificial post-lysis decrease in ATP levels. Thus, 4 mM EDTA was present in the lysis buffer to chelate any excess Ca^{2+}. Furthermore, we also standardized the amount of Ca^{2+} (post-lysis) in each sample by including $CaCl_2$ into the BWW –Ca^{2+} lysis buffer.

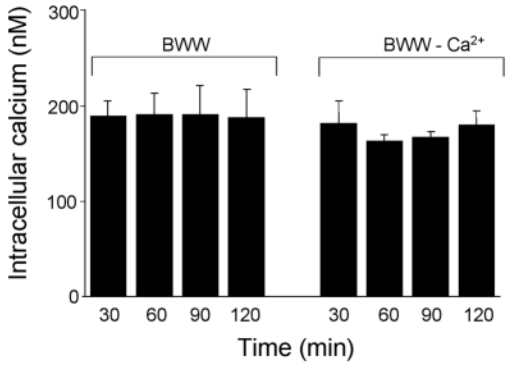

Fig. 4. Relationship between intracellular and extracellular calcium. Spermatozoa were incubated in either complete BWW or BWW –Ca^{2+} as indicated. Approximately 40 minutes before the time shown, 10×10^6 cells were harvested and loaded with 2 μM FURA 2. Following a 20-minute incubation, the cells were centrifuged, washed and the $[Ca^{2+}]_i$ was determined as described in Materials and Methods. This graph represents the data from three experiments performed in duplicate.

To ensure that extracellular Ca^{2+} was regulating intracellular ATP levels, a dose-dependent study was performed in which spermatozoa were incubated in BWW –Ca^{2+}, supplemented with 0-600 μM $CaCl_2$. We took into consideration that the amount of Ca^{2+} present in BWW –Ca^{2+} was approximately 2 μM as measured by atomic absorption analysis (data not shown). Spermatozoa were incubated for 120 minutes and subjected to western blot analysis (Fig. 6). This analysis revealed a clear decrease in tyrosine phosphorylation when more than 100 μM Ca^{2+} was added to the buffer (Fig. 6; lane 3).

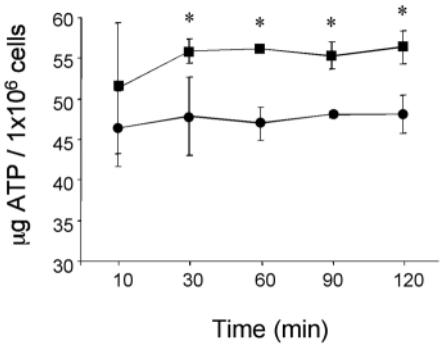

Fig. 5. Impact of extracellular calcium on intracellular ATP. Spermatozoa (10×10^6/ml) were incubated in either complete BWW (circles) or BWW $-Ca^{2+}$ (squares). At the times indicated, 50 µl of the cell suspension was taken for measurement of ATP, as described in Materials and methods. The graph represents the data from three experiments performed in quadruplicate; $*P<0.05$.

Even though external Ca^{2+} caused a decrease in intracellular ATP, it could still be argued from the data presented thus far, that Ca^{2+} itself may negatively regulate kinase activity. To confirm that ATP levels are the critical factor, we sought to alter the amount of ATP production in the sperm populations. This was achieved by incubating sperm in BWW (with or without Ca^{2+}), which was devoid of glucose (to inhibit glycolysis), or contained 1 µM rotenone (to inhibit the mitochondria) or contained 2 mM deoxyglucose (which is phosphorylated by hexokinase to deoxyglucose-6-phosphate, a non-metabolizable substrate). When spermatozoa were incubated in either complete BWW (Fig. 7A, open bars) or Ca^{2+}-depleted BWW (Fig. 7A, closed bars), both the absence of glucose (lane 2) and the presence of 2-deoxyglucose (lane 3) caused a decrease in the amount of ATP over the vehicle control (lane 1). In contrast, the addition of rotenone did not decrease the level of ATP production in both cases. Western-blot analysis of tyrosine phosphorylated proteins in sperm incubated under these conditions is shown in Fig. 7B. Spermatozoa incubated in BWW devoid of glucose (Fig. 7B, lane 2) demonstrated a decrease in the amount of tyrosine phosphorylation compared to the vehicle control (Fig. 7B, lane 1) in keeping with the observed decline in intracellular ATP. Furthermore, the level of tyrosine phosphorylation was further decreased in the presence of 2-deoxyglucose (Fig. 7B, lane 3), a phenomenon that was again highly correlated with the low level of ATP observed in these samples. In contrast, the addition of rotenone (Fig. 7B, lane 4), a potent inhibitor of the mitochondrial complex 1, had little effect on either ATP production or tyrosine phosphorylation in these cells (Fig. 7B, lane 1 versus 4).

Spermatozoa incubated in BWW $-Ca^{2+}$ demonstrated the same pattern. Thus, in the absence of glucose (Fig. 7B lane 6), a decrease in tyrosine phosphorylation occurred compared to that of the vehicle control (Fig. 7B, lane 5). Moreover, in the presence of 2-deoxyglucose (Fig. 7B, lane 7), a further decrease in tyrosine phosphorylation was observed, perfectly matching the low levels of ATP observed in these cells (Fig. 7B, lanes 3 and 7). Finally, rotenone (Fig. 7B, lane 8), which did not affect intracellular ATP levels (Fig. 7A, lane 4), also had no affect on the high levels of tyrosine phosphorylation observed in calcium-depleted medium.

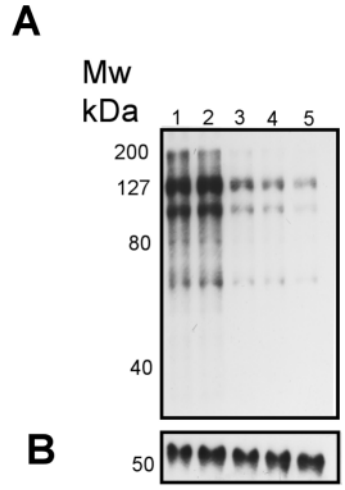

Fig. 6. Relationship between extracellular calcium and tyrosine phosphorylation. (A) Spermatozoa were incubated in BWW $-Ca^{2+}$ (lane 1), or BWW $-Ca^{2+}$ supplemented with 50 (lane 2), 100 (lane 3), 300 (lane 4) or 600 (lane 5), µM $CaCl_2$. After 120 minutes incubation, the cells were centrifuged, washed and lysed in 2% SDS. 1 µg of each lysate was loaded onto a 10% polyacrylamide gel. Tyrosine phosphorylated proteins were detected by western blot analysis using the anti-phosphotyrosine antibody 4G10. The positions of the molecular mass markers (kDa) are shown on the left hand side. (B) The nitrocellulose membrane was stripped at 65°C with shaking. Following this, the membrane was blocked and re-probed using α-tubulin antibody. The western blot is representative of three similar experiments.

Extracellular calcium decreases ATP levels in mouse epididymal spermatozoa

The above data demonstrated that the presence of extracellular Ca^{2+} causes a decrease in intracellular ATP, and consequent tyrosine phosphorylation, in human spermatozoa. In order to establish that this model was not specific to human, we attempted to look for the same response in an animal model.

Mouse caudal epididymal preparations were placed in either complete BWW medium or medium depleted of Ca^{2+}, and their intracellular ATP levels were measured over time. As shown (Fig. 8A), an increase in the amount of ATP was observed in spermatozoa incubated in calcium-depleted medium (squares) compared with cells cultured in complete BWW (circles) after only 30 minutes incubation ($P<0.05$). This change in ATP levels was also reflected in the intensity of tyrosine phosphorylation (Fig. 8B). Thus, as in the human, mouse spermatozoa incubated in complete BWW for 90 minutes (Fig. 8B, lane 1) had lower levels of tyrosine phosphorylation compared to cells incubated in BWW $-Ca^{2+}$ (Fig. 8B, lane 2). The presence of a constitutively phosphorylated hexokinase (molecular mass 116 kDa) demonstrated that equal protein loading had occurred between samples. These data suggest that the ability of extracellular Ca^{2+} to regulate tyrosine phosphorylation through a decrease in ATP levels is not specific to human sperm, but occurs across species.

Calcium deficient medium promotes the acrosome reaction through a H89-dependent process

Recently, Luconi et al. (Luconi et al., 1996) have suggested that tyrosine phosphorylation negatively regulates the acrosome reaction. By incubating spermatozoa in Ca^{2+}-free medium, they demonstrated that the acrosome reaction was inhibited following progesterone challenge, even though tyrosine phosphorylation in these cells was high. That acrosomal exocytosis was inhibited in calcium-free medium,

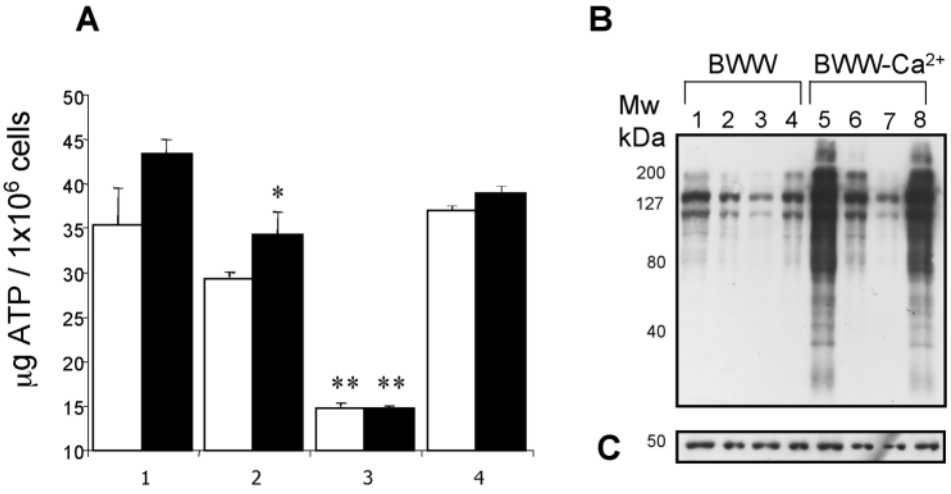

Fig. 7. Relationship between ATP and tyrosine phosphorylation. (A) Human spermatozoa were incubated for 120 minutes in either complete BWW (open bars) or BWW $-Ca^{2+}$ (closed bars). The four treatments consisted of the vehicle control BWW (lane 1), BWW-glucose (lane 2) BWW +2-deoxyglucose (lane 3) and BWW +1 μM rotenone (lane 4). 50 μl of cell suspension was taken for ATP measurements, as described in Materials and Methods. The graph represents the data from three experiments performed in quadruplicate; $*P<0.05$, $**P<0.01$. (B) Cells were incubated in either complete BWW (lanes 1-4) or BWW $-Ca^{2+}$ (lanes 5-8) as shown. Following a 120-minute incubation, cells incubated in complete BWW (lanes 1, 5), BWW –glucose (lanes 2, 6), BWW +2-deoxyglucose (lanes 3, 7) or BWW +1 μM rotenone (lanes 4, 8) were harvested, washed and lysed in 2% SDS. 1 μg of each lysate was loaded onto a 10% polyacrylamide gel. Tyrosine phosphorylated proteins were detected by western blot analysis using the anti-phosphotyrosine antibody 4G10. The positions of the molecular mass markers (kDa) are shown on the left. (C) The nitrocellulose membrane was stripped at 65°C with shaking. Following this, the membrane was blocked and re-probed using α-tubulin antibody. The western blot is representative of three independent experiments.

even though phosphotyrosine expression was high is not surprising since Ca^{2+}-dependent enzymes, such as actin severing proteins, are involved in the acrosome reaction (Bielfeld et al., 1994; Breitbart, 2002). Therefore, we revisited the question of the importance of tyrosine phosphorylation for the acrosome reaction. To achieve this, cells were incubated in either BWW for 3 hours (Fig. 9A, lane 1), BWW $-Ca^{2+}$ for 3 hours (Fig. 9A, lane 2) or BWW $-Ca^{2+}$ for 2.5 hours before being transferred to calcium-containing BWW for 30 minutes (Fig. 9A, lane 3). Following this, the level of acrosomal exocytosis was assessed (Fig. 9A). As shown, spermatozoa incubated under tyrosine phosphorylation-promoting, calcium-depleted conditions demonstrated a significant increase in the level of A23187-induced acrosome reaction, provided calcium was re-introduced into the system (Fig. 9A, lane 2 versus 3). To ensure phosphotyrosine expression remained high in this system, cells incubated in BWW (Fig. 9B, lane 1), BWW $-Ca^{2+}$ (Fig. 9B, lane 2) or BWW $-Ca^{2+}$ for 2.5 hours then BWW for 30 minutes (Fig. 9B, lane 3) were probed for phosphotyrosine expression. As shown (Fig. 9B), tyrosine phosphorylation is not reduced when the cells are re-introduced into calcium medium.

The importance of tyrosine phosphorylation to capacitation was further validated when we inhibited the rise in phosphotyrosine expression using 30 μM H89, and re-assessed the level of acrosomal exocytosis. Under these conditions, we found a 20% decrease in the level of A23187-induced acrosomal loss compared with untreated controls (data not shown).

Human spermatozoa contain a number of Ca^{2+}-dependent ATP-utilizing enzymes

The impact of extracellular calcium on intracellular ATP suggested the presence of Ca^{2+}-dependent, ATP-consuming enzymes, which can include ATPases, Ca^{2+} pumps and kinases. It is therefore possible that extracellular Ca^{2+} did not have a direct effect on kinase activity, but rather an indirect effect through ATP levels. In order to look for the presence of Ca^{2+} sensitive kinases (such as c-yes) we employed a

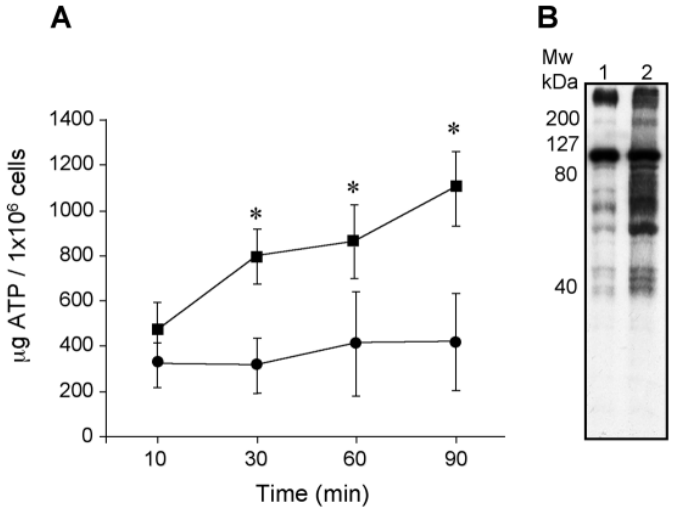

Fig. 8. Extracellular calcium ATP and tyrosine phosphorylation in mouse spermatozoa. (A) Mouse spermatozoa obtained from the cauda epididymides were incubated in either BWW (circles) or BWW $-Ca^{2+}$ (squares). At the times indicated, 50 μl of each cell suspension was taken for ATP measurements as described in Materials and methods. The graph represents data from three experiments performed in duplicate; $*P<0.05$. (B) Caudal mouse preparations were harvested and incubated in either complete BWW (lane 1) or BWW $-Ca^{2+}$ (lane 2). Following a 90-minute incubation, the cells were lysed and 1 μg of each lysate was loaded onto a 10% polyacrylamide gel. Tyrosine phosphorylated proteins were detected by western blot analysis using the anti-phosphotyrosine antibody, 4G10. The positions of the molecular mass markers (kDa) are shown on the left hand side. This western blot is representative of four independent experiments.

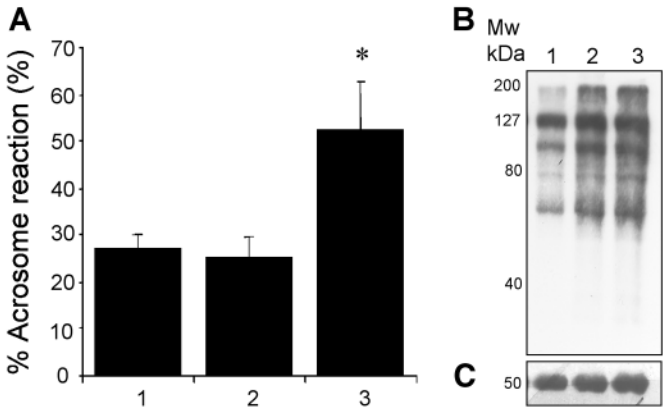

Fig. 9. Sperm function correlates with tyrosine phosphorylation. (A) Cells were placed into either complete BWW (lane 1) or BWW –Ca^{2+} (lane 2). Approximately 2.5 hours later, an aliquot of cells in BWW –Ca^{2+} was placed into complete BWW (lane 3) and challenged with 2.5 μM A23187 for a further 30 minutes. The level of acrosome reaction was then counted as described in Materials and Methods. The graph represents the mean and standard deviation of three independent experiments; **P*<0.05. (B) Phosphotyrosine expression in the treatments illustrated in A. (C) The nitrocellulose membrane was stripped at 65°C with shaking. Following this, the membrane was blocked and re-probed using α-tubulin antibody. The western blot is representative of three independent experiments.

photoaffinity-labeling agent, 8-azido ATP (8-N_3ATP). This radio-labeled compound will covalently bind to the active site of ATP-dependent enzymes, and under UV excitation will photoaffinity label the active site.

Spermatozoa were lysed in BWW medium with (Fig. 10A, lane 1) or without (Fig. 10A, lane 2) Ca^{2+}. Of the proteins that became photoaffinity labeled all but one was dependent on Ca^{2+} being present in the lysis buffer. We found no evidence for an enzyme that was negatively regulated by Ca^{2+} under these conditions. A combination of experiments in which we included capacitated and non-capacitated spermatozoa and lysed them with or without Ca^{2+}, demonstrated that the process of capacitation did not activate any kinase; it was simply the presence of Ca^{2+} in the lysis buffer that allowed for photoaffinity labeling (data not shown).

Inhibition of a thapsigargin sensitive ATPase increases intracellular calcium levels and decreases phosphotyrosine expression

The data presented herein suggest that $[Ca^{2+}]_i$ homeostasis is maintained in mammalian spermatozoa by Ca^{2+}-dependent ATPases. One prime candidate for this process is a thapsigargin sensitive Ca^{2+}-ATPase, whose role appears to involve the storage of calcium within the acrosome. Previous reports have demonstrated that upon addition of thapsigargin, an increase in $[Ca^{2+}]_i$ occurs in human spermatozoa (Spungin and Breitbart, 1996; Dragileva et al., 1999; Dorval et al., 2003). In keeping with the data presented in this paper, elevation of intracellular calcium through the addition of thapsigargin, caused a general decrease in the level of ATP, which became significant at 10 μM (Fig. 10B; ***P*<0.01). At this dose, tyrosine phosphorylation became significantly suppressed, further reinforcing the concept that calcium negatively regulates tyrosine phosphorylation through the ATP status of these cells (Fig. 10C,D).

Discussion

Contrasting reports exist on the impact of extracellular Ca^{2+} on tyrosine phosphorylation in spermatozoa. On one hand, analyses of both mouse (Visconti et al., 1995a) and human (Leclerc et al., 1998) spermatozoa have documented that increasing amounts of extracellular Ca^{2+} increase tyrosine phosphorylation. On the other hand, others have demonstrated the opposite effect (Luconi et al., 1996; Carrera et al., 1996). The aim of this work was to resolve the impact of extracellular Ca^{2+} upon tyrosine phosphorylation in mammalian spermatozoa and to elucidate the mechanism(s) by which it exerts its cellular effects.

It has been demonstrated that during capacitation of both bull (Vredenburgh-Wilberg and Parrish, 1995) and mouse (Zeng et al., 1996) spermatozoa, cytosolic alkalinization occurs. Furthermore, such changes have been suggested to regulate $[Ca^{2+}]_i$ levels and consequently, tyrosine phosphorylation levels. In other biological systems, changes in $[pH]_i$ have been shown to regulate various cellular functions including Ca^{2+} homeostasis and the status of ion channels (Busa et al., 1985), gene expression (Isfort et al., 1993) and cell death (Reynolds et al., 1996). In our hands, spermatozoa incubated in Ca^{2+}-depleted medium also displayed cytosolic alkalinization, which rose simultaneously with tyrosine phosphorylation. However, the changes in $[pH]_i$ associated with Ca^{2+} removal in human spermatozoa, did not regulate tyrosine phosphorylation in these cells.

Furthermore, changing the tyrosine phosphorylation status of the cells did not change $[Ca^{2+}]_i$. Such a phenomenon has been reported in neonatal rat cardiomyocytes, where Fyn, a member of the src family of tyrosine kinases, has been shown to associate with, and induce phosphorylation of, the anion Cl^-/HCO_3^- exchanger, AE1 (Puceat et al., 1988). Moreover, in alveolar type II cells, stimulation of PKC with 80 nM phorbol 12-myristate 13-acetate appeared to regulate the Na^+/H^+ exchanger and cause an increase in $[pH]_i$ (Wadsworth et al., 1996). However, in spermatozoa, even when tyrosine phosphorylation was inhibited by the addition of up to 50 μM H89, cytosolic alkalinization still occurred, demonstrating that these events are completely independent of one another.

In general, mammalian cells are able to maintain $[Ca^{2+}]_i$ at submicromolar levels (Bootman and Berridge, 1995) and in the case of human spermatozoa, $[Ca^{2+}]_i$ is normally around 200 nM (Irvine and Aitken, 1986). As Ca^{2+} readily crosses the plasma membrane (Vijayaraghavan and Hoskins, 1990) it would be necessary for the spermatozoa to either pump excess Ca^{2+} out of the cell, or store this cation in subcellular locations such as the acrosome (Dorval et al., 2002). In either case, homeostatic regulation of Ca^{2+} would be an energy-dependent process. In support of this proposal, spermatozoa incubated under Ca^{2+}-depleted conditions were shown to have significantly higher amounts of ATP than those incubated in Ca^{2+}-supplemented medium. These results suggest that in spermatozoa, homeostatic maintenance of $[Ca^{2+}]_i$ in Ca^{2+}-supplemented medium is an energy-dependent process requiring consumption of internal ATP. A profound

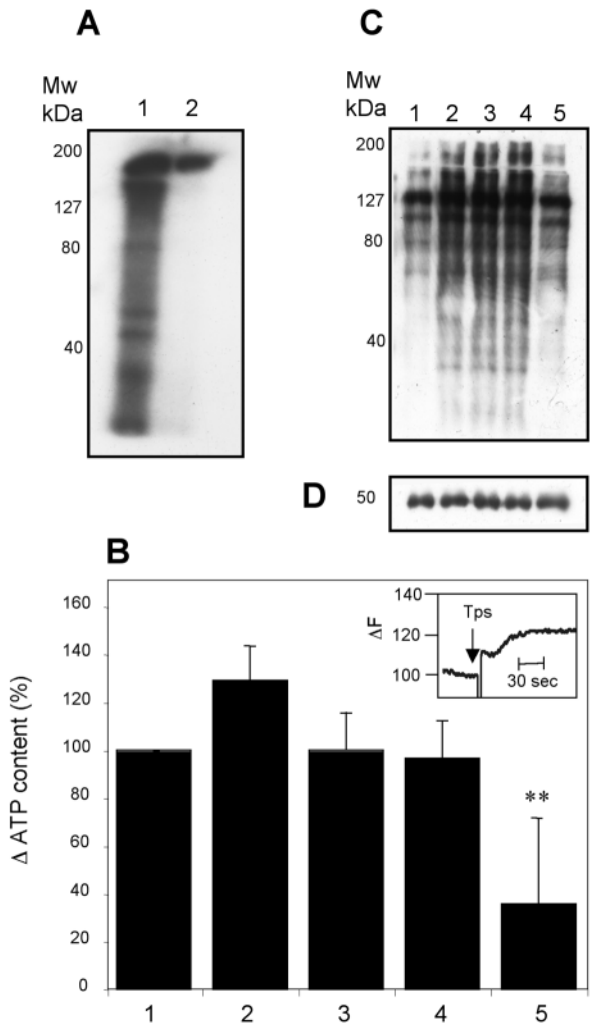

Fig. 10. Sperm function and calcium-dependent ATP binding proteins. (A) Approximately 10×10^6 human spermatozoa were harvested, washed and lysed in either 5% CHAPS, with 0.6 mM $CaCl_2$ (lane 1) or without $CaCl_2$ (lane 2). Approximately 2 μCi α-^{32}P-labeled 8-azidoadenosine-5′-triphosphate [8-N_3ATP; 20 Ci/mmol, 2 mCi/ml] was added. Following 10 minutes incubation, the cells lysates were UV irradiated (2 minutes at 1200 MW/m^2). An equal amount of loading dye was then added to 1 μg of protein, which was subject to 10% SDS-PAGE. Autoradiography was performed using X-ray film according to the manufacturer's instructions. (B) Cells were incubated in BWW $-Ca^{2+}$, (1) or BWW medium (vehicle control, 2), or BWW medium containing 1 (3), 5 (4) or 10 (5) μM thapsigargin. After 3 hours, 50 μl of cell suspension was taken and ATP measured as described in Materials and Methods. Inset shows relative increase in $[Ca^{2+}]_i$ following the addition of thapsigargin (Tps, 10 μM). The graph represents data from three experiments performed in duplicate; $^{**}P<0.01$. (C) Cells were incubated in BWW medium (vehicle control, lane 1,), or BWW medium containing 1 (lane 3), 5 (lane 4) or 10 (lane 5) μM thapsigargin. As a positive control, cells were incubated in BWW $-Ca^{2+}$ (lane 2). After 3-hours incubation, cells were harvested, washed and lysed in 2% SDS. 1 μg of each lysate was loaded onto a 10% polyacrylamide gel. Tyrosine phosphorylated proteins were detected by western blot analysis using the anti-phosphotyrosine antibody, 4G10. The positions of the molecular mass markers (kDa) are shown on the left. (D) The nitrocellulose membrane was stripped at 65°C with shaking. Following this, the membrane was blocked and re-probed using α-tubulin antibody. The western blot is representative of three independent experiments.

consequence of this activity is a significant decrease in the tyrosine phosphorylation events associated with sperm capacitation.

The relationship between Ca^{2+} homeostasis and ATP consumption in human spermatozoa was also evidenced by 8 N_3-ATP labeling. This compound contains a photoactive azido ($-N_3$) group substitution in the base ring of the nucleotide. Upon photolysis, a reactive nitrene is generated that covalently binds to the interacting protein allowing permanent tagging. Since the 8 N_3-ATP is radiolabeled on the γ phosphate, only ATP-dependent enzymes will be labeled, and not the substrates phosphorylated as a consequence of kinase activation. Successful labeling of many enzymes, including creatine kinase (Olcott et al., 1994), adenylate kinase (Chuan et al., 1989) fructose-6-phosphate, 2-kinase (Sakakibara et al., 1984) and ATPases including the mitochondrial ATPase (Murataliev, 1995) the Na^+/K^+ ATPase (Murataliev, 1995) and the thapsigargin sensitive ATPase (Hua and Inesi, 1997) have been performed using this compound.

Upon UV irradiation, the proteins that became photoaffinity labeled were all (bar one) dependent on Ca^{2+} being present in the lysis buffer. In this system, we found no evidence that Ca^{2+} negatively regulates protein kinase activity in human spermatozoa (Leclerc and Goupil, 2002). When capacitated cells were compared to non-capacitated cells, no difference in the protein labeling profile occurred, further suggesting that capacitation does not activate a specific kinase.

A similar mechanism was also demonstrated to exist in mouse spermatozoa. Thus, in the presence of external Ca^{2+}, both ATP levels and tyrosine phosphorylation decreased. The kinetics of ATP loss between the mouse and human spermatozoa appear similar. However, a comparative analysis of both human and mouse ATP levels reveals that mouse sperm contain approximately 4 times as much ATP. The increased amount of ATP may result from the larger head, mid- and principal-piece that mouse spermatozoa possess compared with human cells (Yanagamachi, 1994b).

Although the data presented support the idea that the presence of external Ca^{2+} leads to the consumption of ATP and a consequent decrease in kinase activity, the possibility that Ca^{2+} may directly inhibit kinases in mammalian spermatozoa cannot be discounted. To address this issue, the level of intracellular ATP was adjusted by altering the availability of metabolizable energy substrates in the incubation medium. Thus, when glucose was removed, both a decrease in ATP and tyrosine phosphorylation occurred, regardless of whether cells were in BWW or BWW $-Ca^{2+}$. Furthermore, when 2-deoxyglucose was added to the medium, ATP levels dramatically decreased, and again, tyrosine patterns were highly reduced. These data confirm those of Urner et al. (Urner et al., 2001) who demonstrated that removal of glucose also decreased tyrosine phosphorylation in mouse spermatozoa. They also reflect the importance of glycolysis as the major source of ATP production, as rotenone, a well-documented inhibitor of the mitochondrial complex 1, had no effect on ATP production.

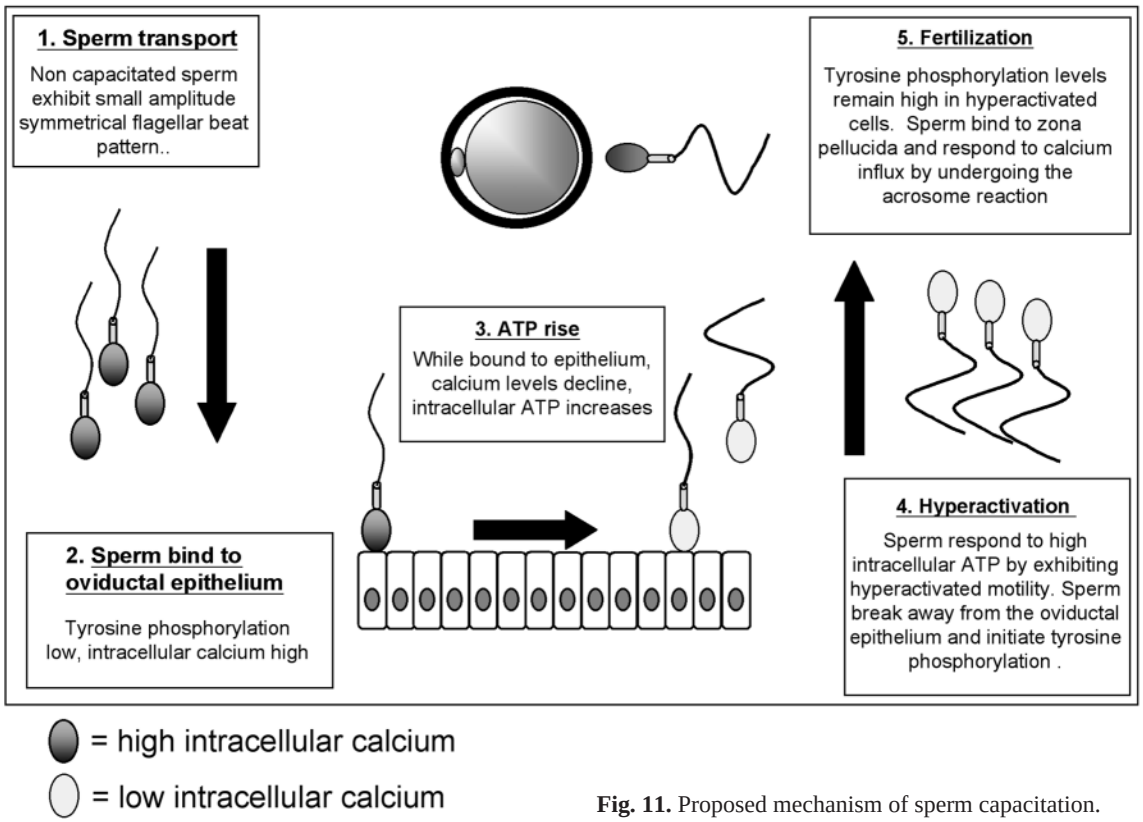

Fig. 11. Proposed mechanism of sperm capacitation.

These data suggest that Ca^{2+} does not directly suppress tyrosine kinase activity, but rather inhibits the availability of ATP. However, the notion that Ca^{2+} negatively regulates tyrosine kinase activity in human spermatozoa has previously been proposed. By measuring the level of both kinase and phosphatase activity (after leaving sperm for 1 hour in the presence and absence of Ca^{2+}), Luconi et al. (Luconi et al., 1996) reported no change in phosphatase activity, but a doubling in kinase activity in calcium-deficient medium. They have suggested that this is due to Ca^{2+} negatively regulating (a) kinase(s). In light the results present herein, we suggest that the apparent increase in kinase activity observed in these experiments when spermatozoa were incubated in Ca^{2+}-deficient medium, was simply due to the increased amount of ATP.

The notion that extracellular Ca^{2+} decreases tyrosine kinase activity has also been supported by the discovery of the tyrosine kinase enzyme, c-*yes*, in human spermatozoa (Leclerc and Goupil, 2002). This protein is located in the acrosomal region of these cells, and is negatively regulated by Ca^{2+}. Its activity was demonstrated to increase in the presence of IBMX, a phosphodiesterase inhibitor. However, recently Thundathil et al. (Thundathil et al., 2002) demonstrated two different signal transduction pathways involved in human sperm capacitation. In the presence of fetal cord serum, follicular fluid ultrafiltrate or progesterone, tyrosine phosphorylation can be inhibited with the tyrphostin, A47, PP2 (tyrosine kinase inhibitors) and PD98059 (MEK-like kinase inhibitor). However, H89, the PKA inhibitor had no effect at 10 μM under these conditions. In contrast, capacitation induced by IBMX could be inhibited with this same concentration of H89, but not any of the other aforementioned compounds. In our system, tyrosine phosphorylation induced by Ca^{2+} withdrawal was sensitive to H89, however, the dose used (20-50 μM) does not suggest the involvement of PKA. In light of these results, it is possible that enzymes such as c-*yes* are only involved in cAMP/PKA-dependent pathways.

A protein(s) central to the role of calcium homeostasis in eukaryotic cells is a thapsigargin-sensitive Ca^{2+}-ATPase(s). Thapsigargin, a specific inhibitor of sarcoplasmic and endoplasmic reticulum Ca^{2+}-ATPase (SERCa) family of proteins (Thastrup et al., 1990) binds to the acrosome of mammalian spermatozoa (Spungin and Breitbart, 1996; Dragileva et al., 1999; Dorval et al., 2003). The ability of 10 μM thapsigargin to elicit an increase in $[Ca^{2+}]_i$ level confirms the data presented by Dorval et al. (Dorval et al., 2003). In our hands, this concentration of thapsigargin induced a decrease in both ATP and phosphotyrosine expression. These data reinforce the concept that Ca^{2+} negatively regulates tyrosine phosphorylation via ATP depletion and further suggests the importance of SERCa-like enzymes and their ability to fill the acrosome with Ca^{2+}.

The biological significance of the tyrosine phosphorylation changes induced on exposure to calcium-depleted medium was demonstrated when these cells were subsequently shown to exhibit an enhanced capacity for undergoing the acrosome reaction, providing sufficient extracellular calcium was present to support the membrane fusion events associated with exocytosis. A dose response analysis revealed that around 100 μM external Ca^{2+} was sufficient to inhibit tyrosine phosphorylation in human spermatozoa. Intriguingly, human seminal plasma typically contains 170 μM Ca^{2+} (Arver and

Sjoberg, 1983). Therefore, in the presence of seminal fluid, ATP levels would be kept low. Under such conditions, the spermatozoa would not tyrosine phosphorylate to a large extent, and premature acrosomal loss would be avoided. On reaching the oviduct, spermatozoa are retained in the isthmic region until ovulation occurs. Recently, Petrunkina et al. (Petrunkina et al., 2001) have demonstrated that upon binding to oviductal epithelial cells the $[Ca^{2+}]_i$ of mammalian spermatozoa is reduced. This may be the 'priming' event, which allows a build up of ATP in mammalian spermatozoa during sperm transport. This increased ATP availability may then lead to the initiation of hyperactivated movement (a critical endpoint of capacitation) allowing these cells to break away from the oviductal epithelium and continue their journey towards the site of fertilization (Suarez, 1996). The high ATP levels present in these cells on release from the oviductal epithelium might then promote a rapid increase in phosphotyrosine expression that would be sustained (see Fig. 9) as the spermatozoa migrate towards the ampullary region of the Fallopian tube, where free ionic calcium concentrations would approximate to those in blood serum (~1.7 mM). In this condition, the spermatozoa would be perfectly primed to exhibit acrosomal exocytosis on binding to the zona pellucida. This hypothesis for sperm capacitation in vivo is summarized in Fig. 11 and is clearly amenable to experimental verification.

In summary, our results clearly resolve previous conflicts concerning the impact of extracellular calcium on a unique tyrosine phosphorylation, signal transduction cascade found only in the male germ line. Extracellular Ca^{2+} has a profound negative impact of phosphotyrosine expression by mammalian spermatozoa by virtue of an associated decrease in intracellular ATP availability. Resolving the nature of the calcium-dependent enzymes involved in ATP depletion may shed light on the etiology of male infertility given the association between defective sperm function and intracellular ATP content (Comhaire et al., 1987).

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References

Aitken, J. and Fisher, H. (1994). Reactive oxygen species generation and human spermatozoa: the balance of benefit and risk. *BioEssays* **16**, 259-267.

Aitken, R. J. and Buckingham, D. (1992). Enhanced detection of reactive oxygen species produced by human spermatozoa with 7-dimethyl amino-naphthalin-1,2-dicarbonic acid hydrazide. *Int. J. Androl.* **15**, 211-219.

Aitken, R. J. and Clarkson, J. S. (1987). Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. *J. Reprod. Fertil.* **81**, 459-469.

Aitken, R. J., Wang, Y. F., Liu, J., Best, F. and Richardson, D. W. (1983). The influence of medium composition, osmolarity and albumin content on the acrosome reaction and fertilizing capacity of human spermatozoa: development of an improved zona-free hamster egg penetration test. *Int. J. Androl.* **6**, 180-193.

Aitken, R. J., Buckingham, D., West, K., Wu, F. C., Zikopoulos, K. and Richardson, D. W. (1992a). Differential contribution of leucocytes and spermatozoa to the generation of reactive oxygen species in the ejaculates of oligozoospermic patients and fertile donors. *J. Reprod. Fertil.* **94**, 451-462.

Aitken, R. J., Buckingham, D. W. and West, K. M. (1992b). Reactive oxygen species and human spermatozoa: analysis of the cellular mechanisms involved in luminol- and lucigenin-dependent chemiluminescence. *J. Cell. Physiol.* **151**, 466-477.

Aitken, R. J., Paterson, M., Fisher, H., Buckingham, D. W. and van Duin, M. (1995). Redox regulation of tyrosine phosphorylation in human spermatozoa and its role in the control of human sperm function. *J. Cell Sci.* **108**, 2017-2025.

Aitken, R. J., Buckingham, D. W., Harkiss, D., Paterson, M., Fisher, H. and Irvine, D. S. (1996). The extragenomic action of progesterone on human spermatozoa is influenced by redox regulated changes in tyrosine phosphorylation during capacitation. *Mol. Cell. Endocrinol.* **117**, 83-93.

Aitken, R. J., Fisher, H. M., Fulton, N., Gomez, E., Knox, W., Lewis, B. and Irvine, S. (1997). Reactive oxygen species generation by human spermatozoa is induced by exogenous NADPH and inhibited by the flavoprotein inhibitors diphenylene iodonium and quinacrine. *Mol. Reprod. Dev.* **47**, 468-482.

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

Aitken, R. J., Harkiss, D., Knox, W., Paterson, M. and Irvine, S. (1998b). On the cellular mechanisms by which the bicarbonate ion mediates the extragenomic action of progesterone on human spermatozoa. *Biol. Reprod.* **58**, 186-196.

Arver, S. and Sjoberg, H. E. (1983). Ionized calcium in human seminal plasma. *Scand. J. Clin. Lab. Invest.* **Supplement 165**, 123-126.

Austin, C. R. (1952). The "capacitation" of the mammalian sperm. *Nature* **170**, 326.

Baldi, E., Casano, R., Falsetti, C., Krausz, C., Maggi, M. and Forti, G. (1991). Intracellular calcium accumulation and responsiveness to progesterone in capacitating human spermatozoa. *J. Androl.* **12**, 323-330.

Bielfeld, P., Anderson, R. A., Mack, S. R., De Jonge, C. J. and Zaneveld, L. J. D. (1994). Are capacitation or calcium ion influx required for the human sperm acrosome reaction? *Feril. Steril.* **62**, 1255-1261.

Bootman, M. D. and Berridge, M. J. (1995). The elemental principles of calcium signaling. *Cell* **83**, 675-678.

Breitbart, H. (2002). Intracellular calcium regulation in sperm capacitation and acrosomal reaction *Mol. Cell. Endocrinol.* **187**, 139-144.

Busa, W. B., Ferguson, J. E., Joseph, S. K., Williamson, J. R. and Nuccitelli, R. (1985). Activation of frog (*Xenopus laevis*) eggs by inositol trisphosphate. I. Characterization of Ca^{2+} release from intracellular stores. *J. Cell Biol.* **101**, 677-682.

Carr, D. W. and Acott, T. S. (1989). Intracellular pH regulates bovine sperm motility and protein phosphorylation. *Biol. Reprod.* **41**, 907-920.

Carrera, A., Moos, J., Ning, X. P., Gerton, G. L., Tesarik, J., Kopf, G. S. and Moss, S. B. (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.

Chang, M. C. (1951). Fertilizing capacity of spermatozoa deposited into the fallopian tubes. *Nature* **168**, 697-698.

Chang, M. C. (1955). Development of fertilizing capacity of rabbit spermatozoa in the uterus. *Nature* **175**, 1036-1037.

Chuan, H., Lin, J. and Wang, J. H. (1989). 8-Azido-2′-O-dansyl-ATP. A fluorescent photoaffinity reagent for ATP-binding proteins and its application to adenylate kinase. *J. Biol. Chem.* **264**, 7981-7988.

Comhaire, F. H., Vermeulen, L. and Schoonjans, F. (1987). Reassessment of the accuracy of traditional sperm characteristics and adenosine triphosphate (ATP) in estimating the fertilizing potential of human semen in vivo. *Int. J. Androl.* **10**, 653-662.

Cross, N. L. (1998). Role of cholesterol in sperm capacitation. *Biol. Reprod.* **59**, 7-11.

De Lamirande, E. and Gagnon, C. (1995). Capacitation-associated production of superoxide anion by human spermatozoa. *Free Radical Biol. Med.* **18**, 487-495.

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

Dorval, V., Dufour, M. and Leclerc, P. (2002). Regulation of phosphotyrosine content of human sperm proteins by intracellular Ca^{2+}: Role of Ca^{2+}-adenosine triphosphatases. *Biol. Reprod.* **67**, 1538-1545.

Dorval, V., Dufour, M. and Leclerc, P. (2003). Role of protein tyrosine phosphorylation in the thapsigargin-induced intracellular Ca^{2+} store depletion during human sperm acrosome reaction *Mol. Human Reprod.* **3**, 125-131.

Dragileva, E., Rubinstein, S. and Breitbart, H. (1999). Intracellular Ca^{2+}-

Mg^{2+}-ATPase regulates calcium influx and acrosomal exocytosis in bull and ram spermatozoa. *Biol. Reprod.* **61**, 1226-1234.

Duncan, A. E. and Fraser, L. R. (1993). Cyclic AMP-dependent phosphorylation of epididymal mouse sperm proteins during capacitation in vitro: identification of an M(r) 95,000 phosphotyrosine-containing protein. *J. Reprod. Fertil.* **97**, 287-299.

Furuya, S., Endo, Y., Oba, M., Nozawa, S. and Suzuki, S. (1992). Effects of modulators of protein kinases and phosphatases on mouse sperm capacitation. *J. Assist. Reprod. Genet.* **9**, 391-399.

Galantino-Homer, H. L., Visconti, P. E. and Kopf, G. S. (1997). Regulation of protein tyrosine phosphorylation during bovine sperm capacitation by a cyclic adenosine 3′5′-monophosphate-dependent pathway. *Biol. Reprod.* **56**, 707-719.

Hua, S. and Inesi, G. (1997). Synthesis of a radioactive azido derivative of thapsigargin and photolabeling of the sarcoplasmic reticulum ATPase. *Biochemistry* **36**, 11865-11872.

Irvine, D. S. and Aitken, R. J. (1986). Measurement of intracellular calcium in human spermatozoa. *Gamete Research* **15**, 57-72.

Isfort, R. J., Cody, D. B., Asquith, T. N., Ridder, G. M., Stuard, S. B. and LeBoeuf, R. A. (1993). Induction of protein phosphorylation, protein synthesis, immediate-early-gene expression and cellular proliferation by intracellular pH modulation. Implications for the role of hydrogen ions in signal transduction. *Eur. J. Biochem.* **213**, 349-357.

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. (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.

Leclerc, P. and Goupil, S. (2002). Regulation of the human sperm tyrosine kinase c-yes. Activation by cyclic adenosine 3′,5′-monophosphate and inhibition by Ca(2+). *Biol. Reprod.* **67**, 301-307.

Luconi, M., Krausz, C., Forti, G. and Baldi, E. (1996). Extracellular calcium negatively modulates tyrosine phosphorylation and tyrosine kinase activity during capacitation of human spermatozoa. *Biol. Reprod.* **55**, 207-216.

Murataliev, M. B. (1995). Interaction of mitochondrial F1-ATPase with trinitrophenyl derivatives of ATP. Photoaffinity labeling of binding sites with 2-azido-2′,3′-O-(4,6-trinitrophenyl)adenosine 5′-triphosphate. *Eur. J. Biochem.* **232**, 578-585.

Olcott, M. C., Bradley, M. L. and Haley, B. E. (1994). Photoaffinity labeling of creatine kinase with 2-azido- and 8-azidoadenosine triphosphate: identification of two peptides from the ATP-binding domain. *Biochemistry* **33**, 11935-11941.

Parrish, J. J., Susko-Parrish, J. L. and First, N. L. (1989). Capacitation of bovine sperm by heparin: inhibitory effect of glucose and role of intracellular pH. *Biol. Reprod.* **41**, 683-699.

Pawson, T. and Scott, J. D. (1997). Signaling through scaffold, anchoring, and adaptor proteins. *Science* **278**, 2075-2080.

Petrukina, A. W., Friedrich, J., Drommer, W., Bicker, G., Waberski, D. and Töpfer-Peterson, E. (2001). Kinetic characterization of the changes in protein tyrosine phosphorylation in membrane, cytosolic Ca2+ concentration and viability in boar sperm populations selected by binding to oviductal epithelial cells. *Reproduction* **122**, 469-480.

Puceat, M., Roche, S. and Vassort, G. (1998). Src family tyrosine kinase regulates intracellular pH in cardiomyocytes. *J. Cell Biol.* **141**, 1637-1646.

Reynolds, J. E., Li, J. and Eastman, A. (1996). Detection of apoptosis by flow cytometry of cells simultaneously stained for intracellular pH (carboxy SNARF-1) and membrane permeability (Hoechst 33342). *Cytometry* **25**, 349-357.

Sakakibara, R., Kitajima, S. and Uyeda, K. (1984). Limited proteolysis and photoaffinity labeling with 8-azido-ATP of fructose-6-phosphate, 2-kinase and fructose-2,6-bisphosphatase. *J. Biol. Chem.* **259**, 8366-8371.

Schackmann, R. W., Christen, R. and Shapiro, B. M. (1981). Membrane potential depolarization and increased intracellular pH accompany the acrosome reaction of sea urchin sperm. *Proc. Natl. Acad. Sci. USA* **78**, 6066-6070.

Spungin, B. and Breitbart, H. (1996). Calcium mobilization and influx during sperm exocytosis. *J. Cell Sci.* **267**, 350-355.

Suarez, S. S. (1996). Hyperactivated motility in sperm. *J. Androl.* **17**, 331-335.

Thastrup, O., Cullen, P. J., Drobak, B. K., Hanley, M. R. and Dawson, A. P. (1990). Thapsigargin, a tumour promoter, disharges intracellular Ca^{2+} stores by specific inhibiton of the endoplasmic reticulum Ca^{2+} ATPase. *Proc. Natl. Acad. Sci. USA* **87**, 2466-2470.

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.

Urner, F., Leppens-Luisier, G. and Sakkas, D. (2001). Protein tyrosine phosphorylation in sperm during gamete interaction in the mouse: the influence of glucose. *Biol. Reprod.* **64**, 1350-1357.

Vijayaraghavan, S. and Hoskins, D. D. (1990). Changes in the mitochondrial calcium influx and efflux properties are responsible for the decline in sperm calcium during epididymal maturation. *Mol. Reprod. Dev.* **25**, 186-194.

Visconti, P. E., Bailey, J. L., Moore, G. D., Pan, D., Olds-Clarke, P. and Kopf, G. S. (1995a). Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. *Development* **121**, 1129-1137.

Visconti, P. E., Moore, G. D., Bailey, J. L., Leclerc, P., Connors, S. A., Pan, D., Olds-Clarke, P. and Kopf, G. S. (1995b). Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. *Development* **121**, 1139-1150.

Visconti, P. E., Johnson, L. R., Oyaski, M., Fornes, M., Moss, S. B., Gerton, G. L. and Kopf, G. S. (1997). Regulation, localization, and anchoring of protein kinase A subunits during mouse sperm capacitation. *Dev. Biol.* **192**, 351-363.

Vredenburgh-Wilberg, W. L. and Parrish, J. J. (1995). Intracellular pH of bovine sperm increases during capacitation. *Mol. Reprod. Dev.* **40**, 490-502.

Wadsworth, S., Wu, A. M., Spitzer, A. and Chander, A. (1996). Protein kinase C in intracellular pH regulation in alveolar type II cells. *Am. J. Physiol.* **271**, L106-113.

World Health Organisation (1992). *WHO Laboratory Manual for the Examination of Human Semen and semen-Cervical Mucus Interaction.* Cambridge, UK: Cambridge University Press.

Yanagimachi, R. (1969). *In vitro* capacitation of hamster spermatozoa by follicular fluid. *J. Reprod. Fertil.* **18**, 275-286.

Yanagimachi, R. (1994a). Fertility of mammalian spermatozoa: its development and relativity. *Zygote* **2**, 371-372.

Yanagimachi, R. (1994b). Mammalian fertilization. In *The Physiology of Reproduction* Second Edition (ed. E. Knobil and J. D. Neill), pp. 189-317. New York: Raven Press.

Yanagimachi, R. and Bhattacharyya, A. (1988). Acrosome-reacted guinea pig spermatozoa become fusion competent in the presence of extracellular potassium ions. *J. Exp. Zool.* **248**, 354-360.

Yanagimachi, R. and Usui, N. (1974). Calcium dependence of the acrosome reaction and activation of guinea pig spermatozoa. *Exp. Cell Res.* **89**, 161-174

Zeng, Y., Oberdorf, J. A. and Florman, H. M. (1996). pH regulation in mouse sperm: identification of Na(+)-, Cl(–)-, and HCO_3(–)-dependent and arylaminobenzoate-dependent regulatory mechanisms and characterization of their roles in sperm capacitation. *Dev. Biol.* **173**, 510-520.