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A novel signal transduction cascade in capacitating human spermatozoa characterised by a redox-regulated, cAMP-mediated induction of tyrosine phosphorylation

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SUMMARY

Capacitation is a priming event that renders mammalian spermatozoa responsive to signals originating from the cumulus-oocyte complex. The attainment of a capacitated state is dependent upon an increase in tyrosine phosphorylation and results in the acquisition of responsiveness to physiological agonists such as progesterone and ZP3. In this study we have shown that this capacitation-dependent increase in tyrosine phosphorylation is controlled by a unique redox-regulated, cAMP-mediated, signal transduction cascade. Either stimulation of reactive oxygen species generation or elevation of intracellular cAMP induced increases in phosphotyrosine expression by human spermatozoa and enhanced their responsiveness to progesterone. Ultimate convergence of the redox- and cAMP-regulated pathways was indicated by the ability of the protein kinase A inhibitor, H89, to block both modes of signal transduction. Furthermore, the fact that the redox-regulated pathway could be silenced by catalase, while this enzyme had no effect on the cAMP-mediated response, indicated that oxidant generation must lie upstream from cAMP in the reaction sequence. In keeping with this conclusion, a functional association was demonstrated between the redox status of human spermatozoa and their cAMP content. The continuous production of reactive oxygen species was also shown to be necessary for the protein kinase A-tyrosine phosphorylation axis to remain functional. If the generation of oxidising conditions during capacitation was prevented with 2-mercaptoethanol, 2-deoxyglucose or the flavoprotein inhibitor, diphenylene iodonium, then cAMP could no longer trigger tyrosine phosphorylation. These data support a model for human sperm capacitation as a redox-regulated process, involving a unique sequence of interactive events including reactive oxygen species production, elevation of intracellular cAMP, stimulation of protein kinase A and the induction of tyrosine phosphorylation. This is the first report of such a signal transduction cascade and may have implications for the functional significance of reactive oxygen metabolites in other cell types.

Key words: Reactive oxygen species, cAMP, Tyrosine phosphorylation, Spermatozoa

INTRODUCTION

The capacitation of human spermatozoa is a complex priming phenomenon designed to sensitise these cells to signals originating from the oocyte-cumulus complex (Yanagimachi, 1994). The stimuli responsible for activating spermatozoa in the vicinity of the oocyte include the steroid, progesterone (Osman et al., 1989; Blackmore et al., 1990; Aitken et al., 1996a) and the zona glycoprotein, ZP3 (Wassarman, 1988), both of which elicit rapid calcium transients in the acrosomal domain of the sperm head (Osman et al., 1989; Blackmore et al., 1990; Florman, 1994). The sensitization of spermatozoa to such calcium signals during capacitation involves a complex array of changes including a reduction in the cholesterol content of the sperm plasma membrane (Langlais and Roberts, 1985), the removal of coating materials from the sperm surface (Bergamo et al., 1993), the inhibition of membrane ATPase activity (Adeoya-Osiguwa and Fraser, 1996), a submicromolar rise in intracellular calcium levels (Spungin and Breitbart, 1996) and an increase in intracellular cAMP (White and Aitken, 1989; Parrish et al., 1994). However, at the heart of this process lies a spontaneous increase in tyrosine phosphorylation that appears to be an absolute precondition for the attainment of a capacitated state (Leyton and Saling, 1989; Aitken et al., 1995b, 1996b; Visconti et al., 1995).

The stimulation of tyrosine phosphorylation during sperm capacitation is known to be a redox-regulated event stimulated by the cellular generation of reactive oxygen species (ROS) (Aitken et al., 1995b, 1996b; Leclerc et al., 1997). Human spermatozoa were the first cells in which the cellular generation of hydrogen peroxide (H_2O_2) was indicated (MacLeod, 1943) and one of a number of disparate cell types in which a clear association between ROS generation and tyrosine phosphorylation has been demonstrated (Aitken et al.,

1995b; Monteiro and Stern, 1996). However, ROS are not the only molecular species thought to be involved in the control of tyrosine phosphorylation during sperm capacitation. Other lines of evidence have also implicated cAMP in the regulation of this process. Treatments that raise intracellular cAMP, including exposure to phosphodiesterase inhibitors or membrane-permeant cAMP analogues, stimulate tyrosine phosphorylation in capacitating spermatozoa. In contrast, disruption of this signal transduction pathway, using cAMP antagonists or protein kinase A (PKA) inhibitors, has the opposite effect (Visconti et al., 1995; Leclerc et al., 1996; Galantino-Homer et al., 1997).

The relative importance of PKA- and redox-regulated pathways in controlling the tyrosine phosphorylation events associated with sperm capacitation is currently unknown, The present study addresses this question and presents results indicating that the activation of both pathways is essential, if human spermatozoa are to attain a capacitated state. Moreover, a significant convergence of these pathways has been observed via an unusual mechanism involving the redox regulation of intracellular cAMP.

MATERIALS AND METHODS

Spermatozoa

This study was based upon semen samples donated by a panel of healthy, normozoospermic donors (World Health Organization, 1992) after 2 or 3 days abstinence. The samples were produced into sterile containers and left for at least 30 minutes to liquefy before being processed by discontinuous Percoll gradient centrifugation (Aitken et al., 1996a) and suspended at a final concentration of 20×10^6/ml in Hepes-buffered Biggers Whitten and Whittingham medium (BWW; Biggers et al., 1971) supplemented with 0.3% Albuminar (Armour Pharmaceutical Company, Eastbourne, UK). The Percoll-purified sperm suspensions used in this study were more than 80% motile and free of detectable leukocyte contamination (Aitken et al., 1995a). Sperm motility was monitored throughout the experiments described in this paper to ensure that none of the treatments examined affected the viability of these cells. For the assessment of motility, 10 µl of each sperm suspension was placed on a microscope slide pre-warmed to 37°C and covered with a 19×19 mm coverslip. The preparation was then examined at 100× magnification using phase contrast optics and counts of the percentage of motile spermatozoa made with the aid of a grid on an eyepiece graticule. Spermatozoa in WHO categories *a+b+c* were regarded as motile (World Health Organization, 1992).

Sperm-oocyte fusion

In order to monitor the ability of human spermatozoa to exhibit a functional acrosome reaction accompanied by the generation of a fusogenic equatorial segment, zona-free hamster ova were used in a heterologous in vitro fertilization assay (Yanagimachi et al., 1976; Aitken et al., 1993). For this assay, the isolated spermatozoa were diluted 1:1 with the relevant test solution and incubated for 2 hours at 37°C, in an atmosphere of 5% CO_2 in air to effect capacitation. At the end of this incubation period, a calcium transient was generated in the spermatozoa by a 5 minute exposure to 5 µM progesterone. The spermatozoa were then centrifuged at 500 ***g*** for 5 minutes before being resuspended in fresh medium BWW, incubated for a further 30 minutes and finally dispersed as 50 µl droplets under liquid paraffin prior to the introduction of zona-free oocytes. After 3 hours coincubation the degree of sperm-oocyte fusion was assessed and expressed as the mean number of spermatozoa penetrating each oocyte (total number of nuclear decondensations/total number of oocytes) (Aitken et al., 1993). A minimum of 15 ova were used for each replicate and each experiment was replicated at least 3 times, giving a minimum of 45 ova for each treatment.

Analysis of cAMP

200 µl samples of spermatozoa at 20×10^6/ml were pelleted at 500 ***g*** for 5 minutes and then resuspended in ice-cold ethanol:acetic acid (99:1). After thorough mixing the spermatozoa were extracted for 20 minutes before being centrifuged at 500 ***g*** at 4°C for 10 minutes. The supernatant was then pipetted into an assay tube and dried down overnight at ambient temperature. The cAMP content of the extract was subsequently measured using a Biotrak cAMP enzyme immunoassay system (RPN 225; Amersham, Little Chalfont, UK) according to the manufacturer's instructions.

Tyrosine phosphorylation

Spermatozoa, at a concentration of 20×10^6/ml in 500 µl BWW medium, were centrifuged (500 ***g*** for 5 minutes) and solubilized in 70 µl SDS-solubilization buffer (0.187 M Tris-HCl, pH 6.8, containing 2% SDS, 10% glycerol, 1 mM vanadate, 1 mM PMSF, 0.02 IU aprotinin) for 45 minutes at 25°C with occasional vortexing. The sperm extract was then centrifuged (7500 ***g*** for 30 minutes) and the supernatant added to an equal volume of SDS-PAGE buffer comprising 0.0625 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 2% 2-mercaptoethanol (2ME), and heated to 100°C for 5 minutes. The samples were loaded onto 5% polyacrylamide gels and electrophoresed as described by Aitken et al. (1996a). For each lane of the electrophoresis gel, the protein extracted from 1.5×10^6 spermatozoa was applied. Western blot analysis was subsequently conducted using a monoclonal anti-phosphotyrosine antibody (UBI, TCS Biologicals, Buckingham, UK) and enhanced chemiluminescence detection technique (Amersham, Little Chalfont, UK) (Aitken et al., 1996a).

Reactive oxygen species (ROS) generation

The ability of human spermatozoa to generate reactive oxygen species was monitored by luminol-peroxidase and lucigenin-dependent chemiluminescence as previously described (Aitken et al., 1992). Chemiluminescence was measured on a Berthold 9505 C luminometer (Berthold, Wilbad, Germany) at 37°C and the results expressed in terms of photons counted per minute.

Statistics

The data were analysed by analysis of variance (ANOVA) for repeated measures using the Statview programme (Abacus Concepts, Berkeley, CA) on an Apple Macintosh Centris 650 computer. Differences between individual groups were examined with Fisher's PLSD test and all experiments were repeated at least 3 times on independent samples.

RESULTS

Relative efficacy of redox- and cAMP-induced sperm capacitation

The relative importance of the redox- and cAMP-regulated pathways in stimulating capacitation was assessed by monitoring the responsiveness of the spermatozoa to the calcium signals induced by progesterone. Using this experimental paradigm, a significant stimulation of sperm capacitation was observed when either ROS generation was promoted with NADPH (Aitken et al., 1996a) or intracellular cAMP levels were raised with pentoxifylline ($P<0.001$; Fig.

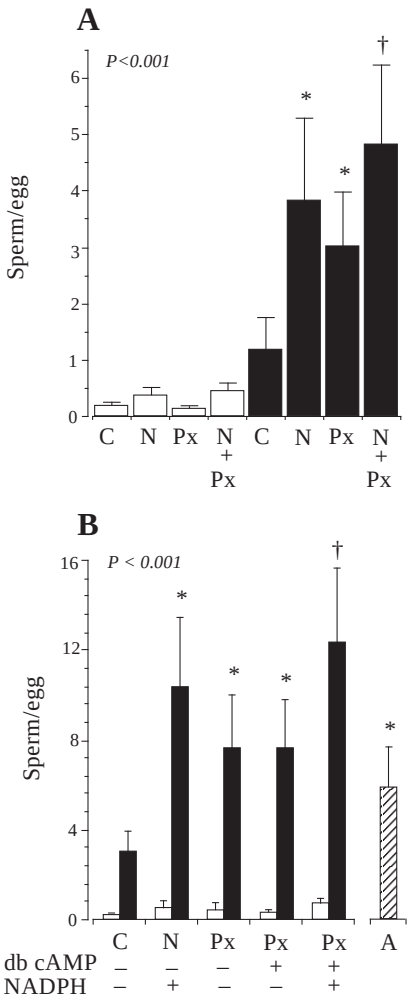

Fig. 1. Induction of sperm capacitation through either cAMP- or redox-regulated pathways. (A) The stimulation of ROS production with NADPH (2.5 mM) or the elevation of cAMP with pentoxifylline (3.0 mM) significantly stimulated the responses of human spermatozoa on addition of 5 μM progesterone (solid bars). Overall significance (ANOVA) for the effect of treatment on sperm-oocyte fusion was $P<0.001$ for 6 replicates; * $P<0.05$ for the enhancing effect of NADPH and pentoxifylline relative to control (Fisher's PLSD); † $P<0.05$ for the enhancing effect of the combination of 2.5 mM NADPH and 3.0 mM pentoxifylline relative to pentoxifylline alone (Fisher's PLSD). (B) Impact of the membrane permeant cyclic AMP analogue, dbcAMP, on the capacitation of human spermatozoa in the presence of NADPH and pentoxifylline. Incubation for 2 hours in the presence of 3.0 mM pentoxifylline and 1 mM dbcAMP gave sperm-oocyte fusion rates that were no different from those observed with pentoxifylline alone on exposure to progesterone (solid bars). However the stimulation of ROS production in such cells with NADPH (2.5 mM) induced a significant increase in oocyte fusion rates relative to the pentoxifylline or pentoxifylline+dbcAMP groups, on addition of progesterone (solid bars), and this rate increase was higher than that induced by A23187 (hatched bar). Overall significance (ANOVA) for the effect of treatment on sperm-oocyte fusion was $P<0.001$ for 6 replicates; * $P<0.05$ for the enhancing effect of treatment on the rates of sperm-oocyte fusion observed relative to the controls; † $P<0.05$ for the enhancing effect of NADPH on the rates of sperm-oocyte fusion observed relative to the groups exposed to pentoxifylline+dbcAMP or pentoxifylline alone (Fisher's PLSD). In the absence of progesterone, none of the above treatments had any effect on sperm-oocyte fusion rates (open bars). C, control; N, NADPH; Px, pentoxifylline; A, A23187.

1A). These metabolic pathways produced equivalent levels of capacitation, although significant additive effects could also be demonstrated when both modes of signal transduction were activated simultaneously (Fig. 1A). Even when the cAMP wing of the signal transduction cascade was maximally stimulated through the combined presence of the cAMP analogue dbcAMP and pentoxifylline, the addition of NADPH resulted in significantly enhanced rates of sperm-oocyte fusion (Fig. 1B). Such results indicated that while both the cAMP- and ROS-regulated pathways were of similar biological importance, the latter induced changes in the spermatozoa that could not be replicated by the elevation of cyclic AMP alone. The potency of the redox pathway was also indicated by the ability of NADPH and progesterone to induce higher rates of sperm-oocyte fusion than A23187 (Fig. 1B), previously recognized as the most powerful agonist available for the activation of human spermatozoa (Aitken et al., 1993; World Health Organization, 1992).

None of treatments evaluated in this section of the study had any impact on percentage motility, which remained uniformly high (>80%) throughout these experiments.

ROS- and cAMP-induced changes in tyrosine phosphorylation

The relative significance of the redox- and cAMP-mediated pathways of sperm capacitation was also reflected in the patterns of tyrosine phosphorylation observed in these experiments. Thus, tyrosine phosphorylation was enhanced when intracellular cAMP concentrations were raised with either dbcAMP or pentoxifylline (Fig. 2, lanes 2 and 4). This enhancement of tyrosine phosphorylation primarily involved a complex set of protein bands with an average molecular mass of around 115 kDa (Fig. 2, lanes 2 and 4). However when dbcAMP was used in combination with pentoxifylline then phosphorylation of several additional minor bands at approx. 250 kDa, 200 kDa, 159 kDa, 133 kDa and 82 kDa was observed (Fig. 2, lane 5). An increase in tyrosine phosphorylation was also detected when NADPH was used to stimulate ROS generation during the capacitation period (Fig. 2, lane 3). However, when NADPH was combined with pentoxifylline then a higher level of phosphorylation was observed than with either of these reagents alone (Fig. 2, lane 6 versus lanes 2 and 3). Similarly when NADPH was used to supplement incubations containing both dbcAMP and pentoxifylline, then particularly high levels of tyrosine phosphorylation were observed (Fig. 2, lane 7), in keeping with the bioassay data (Fig. 1B). Fig. 2 also illustrates the profound suppressive effect of 2-deoxyglucose on the patterns of tyrosine phosphorylation in human spermatozoa (Fig. 2, lanes 8-14), the details of which are discussed below.

Since both ROS and cAMP were clearly capable of stimulating the tyrosine phosphorylation events associated with sperm capacitation, the interactions between these pathways were subsequently explored in experiments involving the selective deletion of specific steps in this signal transduction cascade.

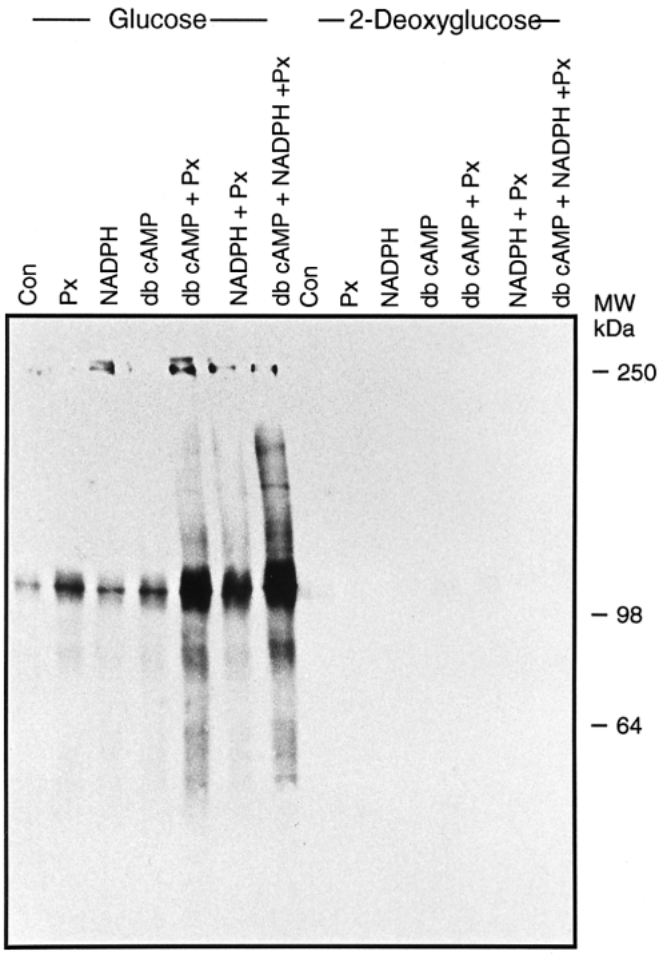

Fig. 2. Patterns of tyrosine phosphorylation observed in populations of human spermatozoa after 2 hours incubation in the presence of 5.5 mM glucose or an equimolar concentration of 2-deoxyglucose. The stimulation of sperm capacitation with 3.0 mM pentoxifylline (Px), 2.5 mM NADPH or 1 mM dbcAMP (lanes 2-4) induced levels of tyrosine phosphorylation that were elevated compared with control incubations in medium BWW alone (Con, lane 1). In keeping with the bioassay data presented in Fig. 1B, maximal levels of tyrosine phosphorylation were observed when NADPH was added to spermatozoa treated with the combination of dbcAMP and pentoxifylline (lane 7). When glucose was replaced with 2-deoxyglucose (lanes 8-14), tyrosine phosphorylation was severely inhibited, regardless of which stimulants were added to the medium. The positions of molecular mass markers are indicated.

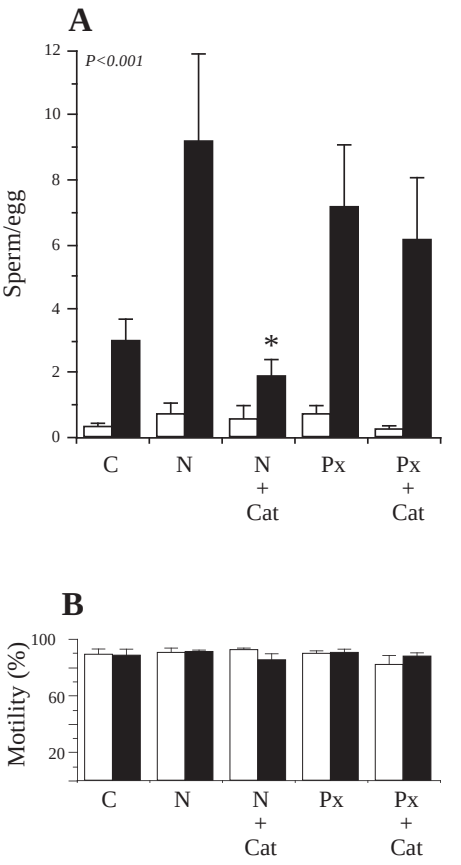

Fig. 3. Influence of catalase on the rates of sperm-oocyte fusion observed in response to the extragenomic action of progesterone. (A) Stimulation of ROS generation for 2 hours with 2.5 mM NADPH (N) resulted in an increase in the responsiveness of human spermatozoa to progesterone (5.0 μM) that was significantly suppressed by the presence of catalase (5 kU). In contrast, the stimulation of sperm capacitation with 3.0 mM pentoxifylline (Px) could not be inhibited by the presence of catalase. Open and solid bars represent samples before and after progesterone addition respectively. Overall significance (ANOVA) for the effect of treatment on sperm-oocyte fusion was $P<0.001$ for 5 replicates; * $P<0.05$ for the suppressive effect of catalase on the rates of sperm-oocyte fusion observed with NADPH (Fisher's PLSD). (B) None of these changes were reflected in the level of sperm motility.

Inhibition of ROS generation with catalase

In view of the proven ability of H_2O_2 to stimulate capacitation and enhance the responsiveness of human spermatozoa to progesterone (Aitken et al., 1996a), the impact of catalase on the cAMP- and redox-regulated pathways of sperm capacitation was examined. Catalase significantly suppressed the ability of NADPH to enhance sperm capacitation (Fig. 3A), without affecting sperm motility (Fig. 3B). In contrast, catalase had no effect on the stimulation of sperm function observed with the phosphodiesterase inhibitor, pentoxifylline (Fig. 3A).

This difference in the susceptibility of the redox- and cAMP-mediated pathways to catalase inhibition was reflected in the patterns of tyrosine phosphorylation observed in presence of this enzyme. Thus, catalase suppressed the spontaneous expression of tyrosine phosphorylation during sperm capacitation (Fig. 4, left panel, lanes 1 and 2) and also inhibited the stimulation of tyrosine phosphorylation observed in the presence of NADPH (Fig. 4, right panel, lanes 2 and 3), in concert with the loss of biological responsiveness observed under these conditions (Fig. 3A). In contrast, the failure of catalase to disrupt the capacitation-promoting effects of pentoxifylline (Fig. 3A) was reflected in its inability to disrupt the tyrosine phosphorylation observed in the presence of this phosphodiesterase inhibitor (Fig. 4 left panel, lanes 3 and 4). Thus while the redox-regulated wing of the phosphotyrosine-mediated capacitation process was dependent on the availability of H_2O_2, the cAMP-regulated wing was not.

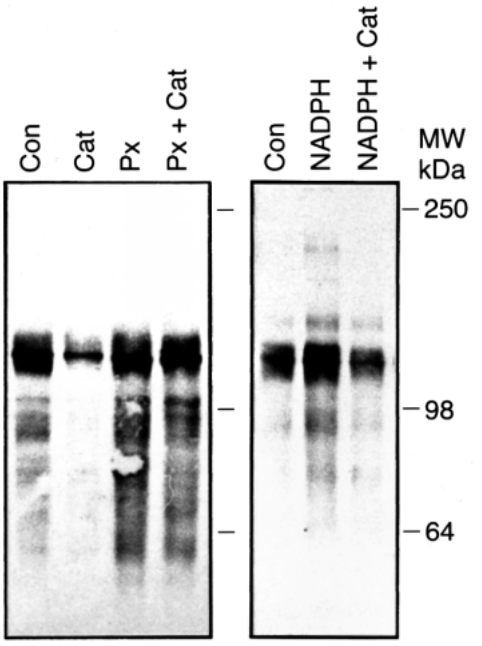

Fig. 4. Influence of catalase on tyrosine phosphorylation in human spermatozoa. (Left) Comparison of the ability of catalase to inhibit tyrosine phosphorylation observed during spontaneous sperm capacitation or following stimulation with pentoxifylline (Px). Catalase (Cat) suppressed the tyrosine phosphorylation observed after 2 hours incubation in control medium (Con) but could not influence phosphorylation in the presence of pentoxifylline (Px). (Right) Catalase could inhibit the tyrosine phosphorylation observed following the stimulation of ROS generation with NADPH. The positions of molecular mass markers are indicated.

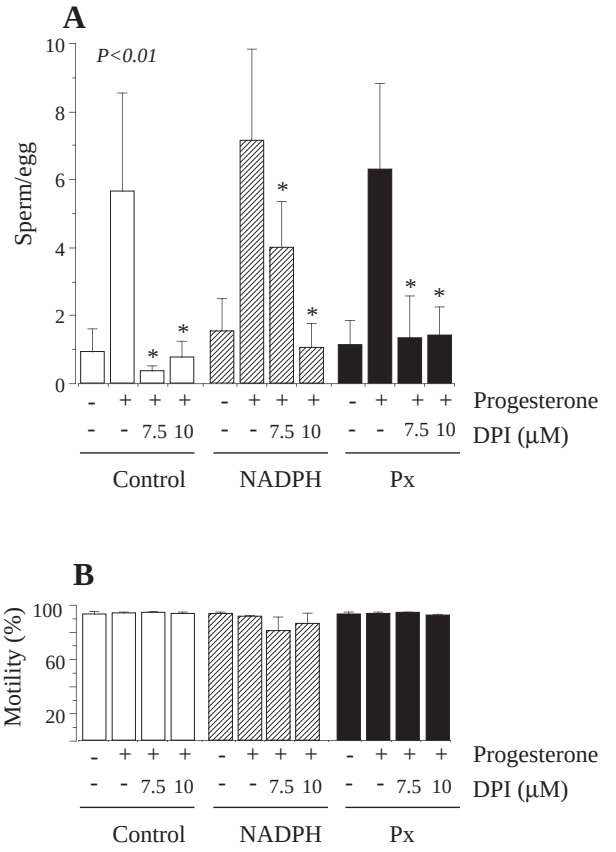

Fig. 5. Influence of the flavoprotein inhibitor DPI on the biological responsiveness of human spermatozoa to progesterone. (A) DPI (7.5 μM and 10 μM) significantly suppressed the levels of sperm-oocyte fusion observed regardless of whether capacitation was spontaneous, or promoted with 2.5 mM NADPH or 3.0 mM pentoxifylline (Px). Overall significance (ANOVA) for the effect of treatment on sperm-oocyte fusion was $P<0.01$ for 4 replicates; * $P<0.05$ for the suppressive effect of DPI on the responses of human spermatozoa to 5.0 μM progesterone (Fisher's PLSD). (B) None of these changes affected motility. Open bars, samples incubated in BWW; hatched bars, samples incubated in the presence of 2.5 mM NADPH; solid bars, samples incubated in the presence of 3.0 mM pentoxifylline (Px).

Inhibition of ROS generation with DPI

Since the NADPH oxidase responsible for ROS generation by human spermatozoa can be blocked by the flavoprotein inhibitor, DPI (Aitken et al., 1997a), the impact of this reagent on the ability of the cAMP- and redox-regulated pathways to promote sperm capacitation was examined. Surprisingly, the results of this experiment demonstrated that the presence of DPI (7.5 and 10.0 μM) significantly inhibited ($P<0.01$) the responses of human spermatozoa to the extragenomic action of progesterone regardless of whether NADPH or pentoxifylline had been used to promote capacitation (Fig. 5A) in the absence of any effect on motility (Fig. 5B). In keeping with these biological observations, DPI was also found to suppress the high levels of tyrosine phosphorylation induced by both NADPH and pentoxifylline (Fig. 6, lanes 3 and 4).

Sperm incubation in the presence of 2-deoxyglucose

The spontaneous generation of ROS by human spermatozoa is thought to involve the transfer of electrons from NADPH to ground state oxygen to produce the superoxide anion, which then dismutates to H_2O_2 under the influence of superoxide dismutase (Aitken et al., 1997a). The rate limiting step in this sequence of reactions is the availability of NADPH, generated as a result of glucose flux through the hexose monophosphate shunt (HMS) (Gomez et al., 1996). In order to investigate the significance of ROS in the stimulation of tyrosine phosphorylation, experiments were designed in which glucose flux through the HMS was prevented, in order to deprive the putative NADPH oxidase of substrate, while maintaining the cells' potential to generate ATP, and hence cAMP. This objective was achieved by replacing the glucose in medium BWW with 2-deoxyglucose. The latter is transported into cells at the same rate as glucose (Traxinger and Marshall, 1990) but inhibits NADPH generation by the HMS, and hence ROS production (Naftalin and Rist, 1993). This inhibition centres on data generated with human red blood cells indicating that the rate of utilization of 2-deoxyglucose-6-phosphate by glucose-6-phosphate dehydrogenase (G6PDH) is less than 5% the utilization of glucose-6-phosphate. Human spermatozoa possess a splice variant of this enzyme (Hirono and Beutler, 1989) and although the interaction between 2-deoxyglucose-6-phosphate and sperm G6PDH has not been characterized, it is known that human granulocytes, which share the same splice variant (Hirono and Beutler, 1989), exhibit a suppression of

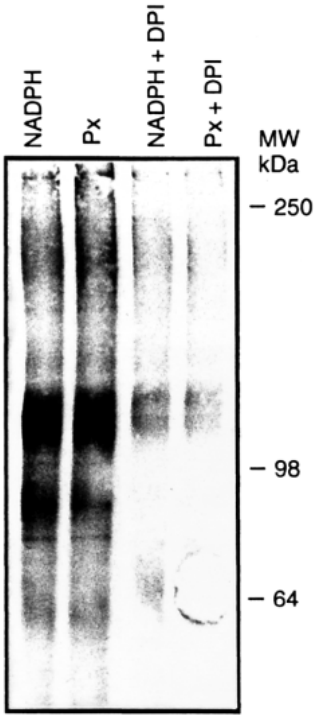

Fig. 6. Impact of DPI on the patterns of tyrosine phosphorylation observed in response to NADPH and pentoxifylline. The high levels of tyrosine phosphorylation observed with 2.5 mM NADPH and 3.0 mM pentoxifylline (Px; lanes 1 and 2) were effectively suppressed by the concomitant presence of DPI (10 μM; lanes 3 and 4). The positions of molecular mass markers are indicated.

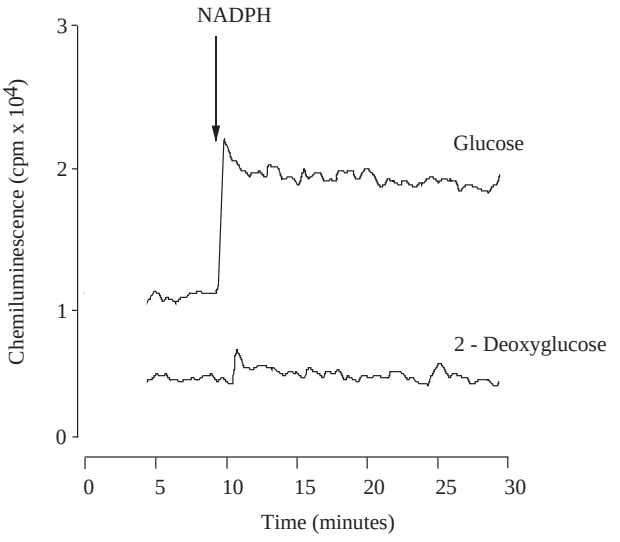

Fig. 7. Impact of 2-deoxyglucose on ROS generation by human spermatozoa. Spermatozoa were incubated in BWW supplemented with lactate (25 mM) but in which the glucose (5.5 mM) had been replaced with equimolar 2-deoxyglucose. The presence of the latter suppressed both the basal levels of superoxide generation and the response to NADPH, as monitored by lucigenin-dependent chemiluminescence.

ROS generation in the presence of 2-deoxyglucose (Kleinveld et al., 1991). Although the futile phosphorylation of 2-deoxyglucose could place a strain on the cells' ATP balance, the maintenance of cell viability was assured by the lactate (25 mM) present in BWW, because human spermatozoa possess functional mitochondria that utilize a lactate/pyruvate shuttle to sustain ATP levels and cell viability in the absence of glucose (Suter et al., 1979).

In keeping with these principles, incubation of human spermatozoa in BWW supplemented with 2-deoxyglucose suppressed the steady-state generation of superoxide anion (Fig. 7) while sperm motility, and hence cell viability, was fully sustained. Furthermore, the ability of these cells to exhibit an oxidative burst in the presence of 100 nM 12-myristate, 13-acetate, phorbol ester was also suppressed under these conditions (data not shown). Although the inhibition of HMS activity by 2-deoxyglucose should have been circumvented by the direct addition of NADPH to the sperm suspensions (Aitken et al., 1996a, 1997a) the generation of superoxide anion was still suppressed under these conditions (Fig. 7), indicating that the oxidase itself was inhibited in the presence of this reagent.

Assessment of the tyrosine phosphorylation status of human spermatozoa in the presence of 2-deoxyglucose precisely mirrored the analysis of ROS generation. Thus, the spontaneous expression of phosphotyrosine residues during sperm capacitation was inhibited in the presence of 2-deoxyglucose via mechanisms that could not be reversed by the presence of NADPH (Fig. 2, lanes 8 and 10). Furthermore, the inability of human spermatozoa to undergo tyrosine phosphorylation in the presence of 2-deoxyglucose was, in turn, reflected by their failure to exhibit a biological response to progesterone in terms of sperm-oocyte fusion despite the full maintenance of sperm motility (Fig. 8A,B). Thus, in the presence of 2-deoxyglucose, sperm-oocyte fusion rates following progesterone treatment were significantly suppressed relative to the glucose-supplemented controls (Fig. 8A). This suppression of sperm-oocyte fusion was evident even when NADPH was present to compensate for the inhibition of the HMS by 2-deoxyglucose (Fig. 8A).

Unexpectedly, increasing cAMP availability with pentoxifylline or dbcAMP also failed to support tyrosine phosphorylation in the presence of 2-deoxyglucose (Fig. 2, lanes 9, 11, 12 and 14). Moreover a biological response to progesterone could not be elicited under these conditions, giving sperm-oocyte fusion rates in the presence of pentoxifylline and deoxyglucose that were no different from those observed in the presence of 2-deoxyglucose alone (Fig. 8A).

These results suggested that ROS were not only involved in mediating the stimulatory effects of NADPH on sperm capacitation but also appeared to be necessary for cAMP to exert its control over this process. In order to ensure that this effect was due to a specific interference with the redox regulation of human sperm function, and not an artefact created by the use of 2-deoxyglucose, additional experiments were conducted using an alternative approach to counteract the action of ROS. The membrane permeant reducing agent, 2ME, has been shown to disrupt sperm-oocyte fusion by preventing the development of oxidizing conditions during capacitation and thereby suppressing tyrosine phosphorylation (Aitken et al., 1996a). In Fig. 9, the ability of 2ME to suppress the spontaneous increase in tyrosine phosphorylation observed in capacitating human spermatozoa was confirmed (Fig. 9, lanes 1 and 2). However, the presence of 2ME was also shown to inhibit the induction of tyrosine phosphorylation when this process was promoted by cAMP, even when the latter was supplied directly as a membrane permeant cAMP analogue in the presence of pentoxifylline (Fig. 9, lanes 2, 5, 6 and 8).

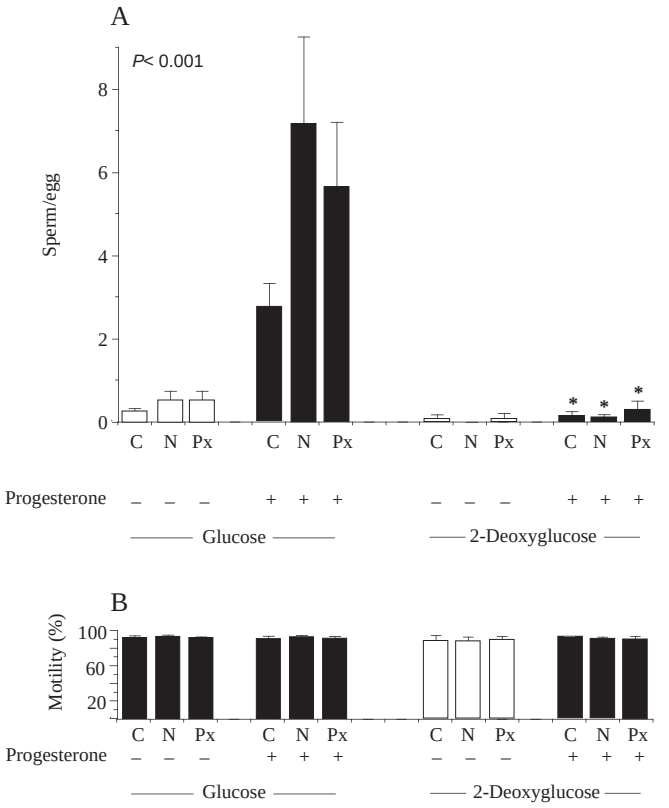

Fig. 8. Influence of 2-deoxyglucose on the rates of sperm-oocyte fusion observed in response to the extragenomic action of progesterone. (A) Following incubation for 2 hours in the presence of 5.5 mM 2-deoxyglucose, the addition of progesterone (solid bars) did not stimulate sperm-oocyte fusion. The inhibitory effect of 2-deoxyglucose was maintained even when the spermatozoa were exposed to 2.5 mM NADPH or 3.0 mM pentoxifylline for 2 hours prior to progesterone addition, despite the stimulatory effects of these treatments in the glucose-supplemented controls. Overall significance (ANOVA) for the effect of treatment on sperm-oocyte fusion was $P<0.001$ for 3 replicates; * $P<0.05$ for the inhibitory effect of 2-deoxyglucose on the responses of human spermatozoa to progesterone (Fisher's PLSD). (B) The presence of 2-deoxyglucose did not influence motility.

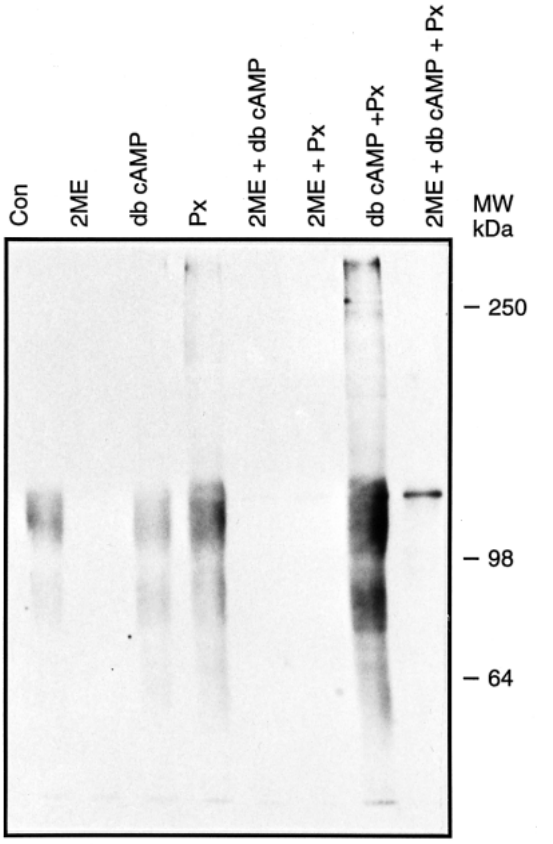

Fig. 9. Influence of the reducing agent 2-mercaptoethanol (2ME) on the patterns of tyrosine phosphorylation observed in human spermatozoa. Incubation of spermatozoa for 2 hours in BWW (Con, lane 1) induced a low level of tyrosine phosphorylation that could be inhibited by the presence of 2ME (0.05%; lane 2). Stimulation of the cAMP-mediated wing of the capacitation process with 3.0 mM pentoxifylline (Px), with (lane 7) or without (lane 4) 1 mM dbcAMP, enhanced tyrosine phosphorylation via mechanisms that were still suppressed by the presence of 2ME (lanes 5, 6 and 8). The positions of molecular mass markers are indicated.

Influence of PKA inhibitor

The above results indicated that the cAMP- and redox-regulated pathways for sperm capacitation were heavily interdependent. In order to determine whether the activation of PKA represented a point of convergence for these two pathways, experiments were conducted using the PKA inhibitor, H89. At doses ranging from 10-100 μM (Fig. 10) this reagent was found to suppress the induction of tyrosine phosphorylation by both the ROS- and cAMP-mediated wings of the signal transduction cascade driving capacitation (Fig. 10).

The impaired tyrosine phosphorylation status of the spermatozoa observed under these conditions was mirrored by their lack of responsiveness to progesterone. In the presence of 50 and 100 μM H89, the ability of progesterone to stimulate sperm-oocyte fusion was significantly inhibited even when 2.5 mM NADPH or 3.0 mM pentoxifylline was present during the preincubation period ($P<0.001$; Fig. 11A). The inhibitory effects of H89 were not due to non-specific effects on the viability of the spermatozoa, because this reagent had no significant effect on motility (Fig. 11B).

Interaction between cAMP and ROS

The fact that H89 could suppress the ability of both ROS and cAMP to stimulate sperm capacitation, suggested a linkage between these two pathways at the level of PKA. Since cAMP is the only known stimulus for PKA, a possible stimulatory action of ROS on the cAMP content of human spermatozoa was investigated. The results of this analysis demonstrated that the stimulation of ROS generation by human spermatozoa had a significant stimulatory effect on the intracellular levels of cAMP, which was greater than that observed with the phosphodiesterase inhibitor, pentoxifylline (Fig. 12A; $P<0.05$). However neither NADPH nor pentoxifylline could induce an increase in intracellular cAMP in the presence of 2-deoxyglucose.

While ROS generation could increase intracellular cAMP levels, the converse was not true. The addition of pentoxifylline or dbcAMP to human spermatozoa had no effect on their basal

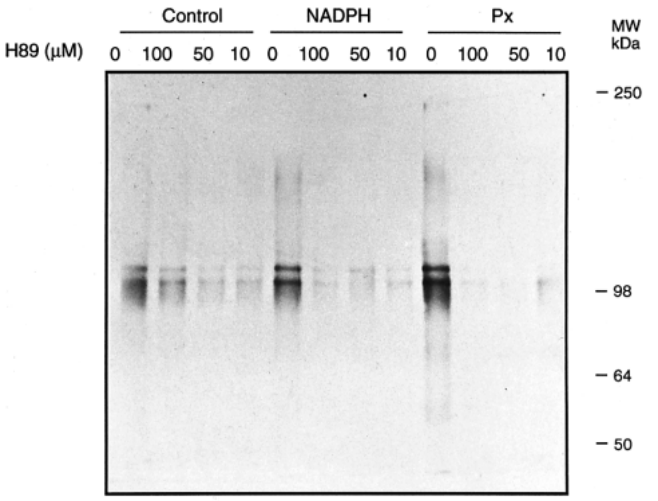

Fig. 10. Influence of the PKA inhibitor H89 on the patterns of tyrosine phosphorylation exhibited by human spermatozoa. The presence of this reagent at doses over the range 10-100 μM suppressed tyrosine phosphorylation even if the spermatozoa were simultaneously exposed to 2.5 mM NADPH or 3.0 mM pentoxifylline during the 2 hour capacitation period. The positions of molecular mass markers are indicated.

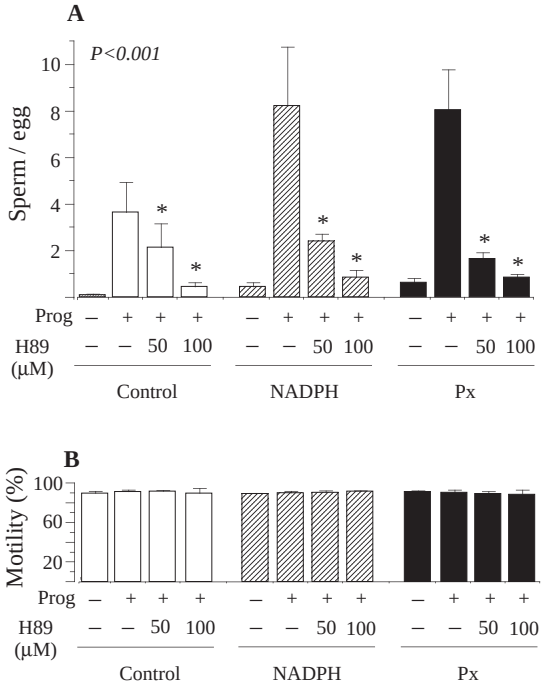

Fig. 11. Impact of the PKA inhibitor H89 on the rates of sperm-oocyte fusion observed in response to the extragenomic action of progesterone. (A) H89 inhibited sperm-oocyte fusion even when the spermatozoa had been incubated for 2 hours in the presence of 2.5 mM NADPH or 3.0 mM pentoxifylline prior to progesterone stimulation. Overall significance (ANOVA) for the effect of treatment on sperm-oocyte fusion was $P<0.001$ for 3 replicates; * $P<0.05$ for the inhibitory effect of H89 on the responses of human spermatozoa to progesterone (Fisher's PLSD). (B) The presence of H89 did not influence sperm motility. Open bars, samples incubated in BWW; hatched bars, samples incubated in the presence of 2.5 mM NADPH; solid bars, samples incubated in the presence of 3.0 mM pentoxifylline (Px).

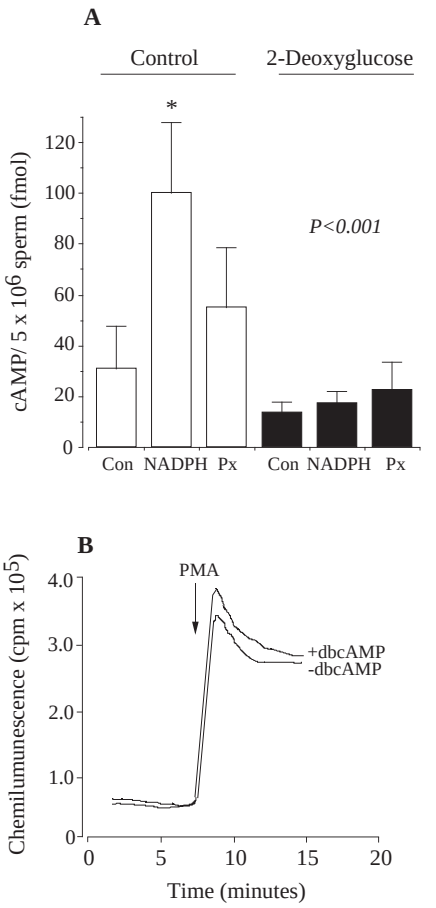

Fig. 12. Interaction between the redox status of human spermatozoa and cAMP generation. (A) Addition of 2.5 mM NADPH to human spermatozoa induced a significant increase ($P<0.05$) in intracellular cAMP levels that was greater than those induced by 3.0 mM pentoxifylline (Px) (open bars). If glucose in the incubation medium was replaced by an equimolar (5.5 mM) concentration of 2-deoxyglucose (solid bars) then cAMP levels remained basal regardless of treatment. Overall significance (ANOVA) for the effect of treatment was $P<0.001$ for 4 replicates; * $P<0.05$ for the stimulatory effect of NADPH on intracellular cAMP relative to control (Con) and pentoxifylline (Px) incubations (Fisher's PLSD). (B) Addition of 1 mM dbcAMP had no effect on the basal levels of ROS generated by human spermatozoa or the stimulatory effects of PMA (100 nM).

ROS generating activity or the response of these cells to PMA stimulation (Fig. 12B).

DISCUSSION

Elucidation of the biochemical mechanisms underlying the process of sperm capacitation is central to our understanding of reproductive pathologies affecting the male gamete and the development of novel approaches to fertility control. In biological terms, capacitation can be thought of as a priming process that renders the spermatozoa exquisitely sensitive to stimuli originating from the cumulus-oocyte complex. A key element of this sensitisation process is the spontaneous increase in tyrosine phosphorylation observed during sperm

capacitation (Visconti et al., 1995; Aitken, 1997; Aitken et al., 1996a). If this tyrosine phosphorylation event is suppressed, then sperm capacitation does not occur and the spermatozoa lose their ability to respond to a variety of physiological (progesterone, ZP3) and non-physiological (A23187) agonists (Aitken et al., 1995b, 1996a; Meizel and Turner, 1996).

The identity of the proteins tyrosine phosphorylated during the capacitation of human spermatozoa is still only poorly understood. When extensive SDS extraction is used, the dominant pattern is of a complex of tyrosine-phosphorylated proteins migrating with a molecular mass of around 100 kDa (Aitken et al., 1996a; Carrera et al., 1996). The electrophoretic profiles are complicated by the fact that several different tyrosine-phosphorylated bands are located in this region of the gel (Carrera et al., 1996). Moreover, many of these phosphorylated proteins appear to be associated with the cytoskeleton, giving rise to some heterogeneity in the precise phosphorylation patterns observed, according to the physiological status of the spermatozoa and the efficiency with which individual proteins have been extracted. Despite such variability, several independent studies have demonstrated the existence of a relationship between the stimulation of tyrosine phosphorylation in human spermatozoa and the attainment of a capacitated state (Aitken et al., 1996a; Leclerc et al., 1997).

The nature of the proteins that are tyrosine phosphorylated during capacitation is currently under investigation. Two of the bands in the complex of approx. 100 kDa have already been identified as the human homologues of mouse sperm AKAP82 and pro-AKAP82, its precursor polypeptide. These molecules are A Kinase Anchor Proteins, which bind protein kinase A to the fibrous sheath (Carrera et al., 1996). In terms of the control mechnisms involved in regulating tyrosine phosphorylation during capacitation, two separate pathways have been proposed. The first advocates a pivotal role for cAMP through a unique PKA/tyrosine phosphorylation signal transduction cascade (Visconti et al., 1995). The second holds that tyrosine phosphorylation is a redox-regulated process stimulated by the inherent capacity of mammalian spermatozoa to generate ROS (de Lamirande and Gagnon, 1993; Griveau et al., 1995; Aitken et al., 1995b, 1996a; Leclerc et al., 1997). The present study has established that these mechanisms for the control of tyrosine phosphorylation are not mutually exclusive and, in the case of human spermatozoa, both pathways are operative, interactive and essential.

The importance of redox status was demonstrated by experiments in which the endogenous production of ROS by human spermatozoa was stimulated by NADPH (Aitken et al., 1996a, 1997a). Under these circumstances, the enhanced production of ROS was accompanied by increased levels of tyrosine phosphorylation (Fig. 2) and elevated rates of sperm-oocyte fusion following treatment with progesterone (Fig. 1). Conversely, when ROS generation was suppressed by the presence of 2-deoxyglucose or DPI, then tyrosine phosphorylation was inhibited (Figs 2 and 7) and the spermatozoa were unable to exhibit a functional response to progesterone (Figs 5 and 8). Such results are consistent with earlier studies indicating that 2-deoxyglucose is capable of suppressing the capacitation of mouse and hamster spermatozoa (Ahuja, 1985; Fraser and Herod, 1990). The importance of H_2O_2 as a key mediator of these changes was demonstrated by the inhibitory action of catalase, which not only suppressed the spontaneous increase in tyrosine phosphorylation during sperm capacitation but also inhibited the stimulatory effects of NADPH (Fig. 4).

The stimulation of tyrosine phosphorylation by ROS has been demonstrated in many different cell types (Monteiro and Stern, 1996) and is generally thought to involve a direct interaction between reactive oxygen metabolites and the tyrosine kinase/phosphatase system. Tyrosine phosphatases possess a conserved cysteine residue in their catalytic domain that must be in a reduced state for full activity to be expressed. Exposure of this class of enzyme to H_2O_2 leads to the inhibition of phosphatase activity (Hecht and Zick, 1992) and a resultant enhancement of phosphotyrosine expression. Direct stimulation of tyrosine kinase activity by H_2O_2 has also been demonstrated for many different kinds of target molecule. For example, studies with the insulin-sensitive cell line, Fao hepatoma cells, have demonstrated the ability of this oxidant to potentiate the tyrosine phosphorylation induced by insulin (Heffetz and Zick, 1989). Similarly, H_2O_2 has been implicated in controlling the tyrosine phosphorylation status of molecules involved in signalling pathways, such as Lck, PLCγ or NF-κB (Monteiro and Stern, 1996). Thus the ability of human spermatozoa to control their tyrosine phosphorylation status through a redox-regulated pathway is in keeping with an emerging body of data emphasizing the general importance of such mechanisms in the control of cellular function. The inhibitory effect of catalase on tyrosine phosphorylation in human spermatozoa may have involved interference with just such a direct effect of H_2O_2 on the tyrosine kinase/phosphatase system. However this study has also revealed that the ROS generated by these cells can utilize additional mechanisms for controlling tyrosine phosphorylation, through their interaction with a unique signal transduction cascade mediated by cAMP.

The involvement of cAMP in the control of tyrosine phosphorylation was indicated by the stimulatory effects of pentoxifylline and dbcAMP on phosphotyrosine expression by human spermatozoa (Fig. 2). Involvement of PKA in this response was also indicated by the inhibitory effects observed with the PKA inhibitor, H89, on both phosphotyrosine expression and sperm-oocyte fusion (Figs 10 and 11). These findings are in keeping with the existence of an unusual signal transduction cascade in mammalian spermatozoa, involving the stimulation of tyrosine phosphorylation via a cAMP/PKA mediated pathway (Visconti et al 1995; Leclerc et al., 1996; Galantino-Homer et al., 1997). Several lines of evidence obtained in this study suggested significant cross-talk between this unique signal transduction cascade and the cellular generation of ROS.

Ultimate convergence of the cAMP- and redox-regulated pathways was demonstrated by the ability of the PKA inhibitor, H89, to suppress the stimulation of tyrosine phosphorylation and sperm-oocyte fusion by both of these signal transduction cascades (Figs 10 and 11). Convergence was also suggested by the fact that the substrates tyrosine phosphorylated under the influence of cAMP and ROS appear to be similar, if not identical (Fig. 6). The fact that catalase could not inhibit tyrosine phosphorylation or sperm-oocyte fusion in the presence of pentoxifylline, but could suppress NADPH-induced responses (Figs 3 and 4), also suggested that any interaction between the cAMP- and ROS-mediated pathways must lie downstream of H_2O_2 production. An important

element of this cross-talk was found to involve the redox regulation of intracellular cAMP availability. Stimulation of ROS production with NADPH resulted in a significant increase in the cAMP content of human spermatozoa while the suppression of ROS generation with 2-deoxyglucose negated this response (Fig. 12A). Thus although ROS generation by human spermatozoa clearly stimulates aspects of sperm physiology unrelated to cAMP (Fig. 1), a major function of the spermatozoon's redox system is to control the availability of this nucleotide.

The only other reported link between cAMP and ROS in the literature concerned the control of adenylyl cyclase activity in A10 cells, a murine vascular smooth muscle cell line (Tan et al., 1995). In this study, oxidative stress was shown to enhance adenylyl cyclase activity via mechanisms that could be disrupted by catalase but not superoxide dismutase. These data are consistent with the present study in suggesting that H_2O_2 is heavily involved in regulating the cAMP/PKA/tyrosine phosphorylation cascade. The possibility that the H_2O_2 exerts its effects following conversion to secondary radical species such as the hydroxyl radical, as suggested for A10 cells (Tan et al., 1995), is consistent with the inhibitory effects of hydroxyl radical scavengers such as dimethylthiourea on the capacitation status of human spermatozoa (Aitken et al., 1996a). However, the precise mechanisms by which such radical species might control cAMP levels in human spermatozoa have not yet been elucidated. It is unlikely that ROS influence sperm adenylyl cyclase through an effect on G-protein regulation, since mammalian spermatozoa do not possess G_s. Alternatively, the stimulatory effects of ROS on the cAMP content of human spermatozoa might involve the suppression of phosphodiesterase activity or stimulation of the adenylyl cyclase catalytic subunit, either directly, or indirectly through the induction of second messengers such as calcium or pH (Hyne and Garbers, 1979). Whatever mechanism is involved, these studies are the first to identify a signal transduction pathway involving the redox regulation of a cAMP/PKA-mediated tyrosine phosphorylation cascade.

In addition to the direct effects of H_2O_2 on the tyrosine kinase/phosphatase system and the redox regulation of cAMP availability, this study suggested a third point in the signal transduction cascade dependent on ROS. The continuous generation of reactive oxygen metabolites also appeared to be necessary for the PKA/tyrosine phosphorylation pathway to remain functional. Thus if ROS production was completely suppressed by the presence of 2-deoxyglucose or DPI, then both tyrosine phosphorylation (Figs 2 and 6) and sperm-oocyte fusion (Figs 5 and 11) were disrupted via mechanisms that could not be rescued by the addition of pentoxifylline. Even if saturating concentrations of cAMP were available to cells incubated in the presence of 2-deoxyglucose then tyrosine phosphorylation was still suppressed (Fig. 2, lanes 8-14). The suppression of PKA-induced tyrosine phophorylation was not the only aspect of sperm biochemistry to become uncoupled in the absence of ROS. In the presence of 2-deoxyglucose, the NADPH oxidase responsible for the cellular generation of ROS also lost activity, even when exogenous substrate was supplied (Fig. 7). Such inhibition was not related to any loss of viability in the presence of DPI and 2-deoxyglucose, since sperm motility was maintained at a high level throughout these experiments. It is also extremely unlikely that DPI was exerting its effects on ROS generation through the non-specific disruption of the mitochondrial electron transport chain, because high concentrations of mitochondrial inhibitors have no effect on ROS generation by human spermatozoa (Aitken and Clarkson, 1987; Aitken et al., 1997a). Moreover, if the redox status of the cell was manipulated by an alternative mechanism, such as the inclusion of a membrane permeant reducing agent (2ME) in the incubation medium, an identical suppression of tyrosine phosphorylation was observed that could not be rescued by the presence of high concentrations of cAMP (Fig. 9, lane 8). These results suggest that the continuous generation of ROS is not only necessary to maintain cAMP production but is also involved in creating an intracellular environment within which all the various elements of this redox-regulated, PKA/tyrosine phosphorylation cascade can remain functional during capacitation.

A key player in this context may be intracellular pH. Recent studies examining the absolute dependence of progesterone on the presence of bicarbonate ions for its biological activity, have emphasized the sensitivity of the cAMP/PKA/tyrosine phosphorylation pathway to changes in intracellular pH (Aitken et al., 1997b). When bicarbonate was omitted from the medium a situation developed closely resembling that observed with DPI, 2-deoxyglucose and 2-mercaptoethanol, in that sperm function and ROS production were suppressed via mechanisms that could not be reversed with cAMP or NADPH. However when intracellular pH was buffered back into the normal range every aspect of sperm biochemistry and function returned to normal, despite the continued absence of bicarbonate. There are precedents for superoxide anion acting as a signal for increased intracellular pH via mechanisms such as the enhancement of Na^+/H^+ antiporter activity (Shibanuma et al., 1988; Demaurex et al., 1996) or the stimulation of specific H^+ conductive pathways (Nanda et al., 1994; Henderson et al., 1995). Localized electron pumping by the oxidase or compartmentalized superoxide dismutase activity could also effect the alkalinization of specific subcellular compartments such as the acrosome, in a similar fashion to that reported for the alkalinization of the phagocytic vacuole of neutrophils (Segal, 1995). Given the pH sensitivity of the ROS-cAMP-PKA-tyrosine phosphorylation pathway, a possible role for sperm NADPH oxidase-like activity in the regulation of intracellular proton balance would be in keeping with the properties of this system in other cell types and the importance of pH as a key regulator of mammalian sperm function (Zeng et al., 1996).

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