

1-1-2003

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Recommended Citation

Ecroyd, Heath W.; Jones, Russell C.; and Aitken, Robert J.: Endogenous redox activity in mouse spermatozoa and its role in regulating the tyrosine phosphorylation events associated with sperm capacitation 2003, 347-354.
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Abstract

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Keywords

capacitation, sperm, associated, events, phosphorylation, tyrosine, regulating, role, its, spermatozoa, mouse, activity, endogenous, redox, CMMB

Disciplines

Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences

Publication Details

Ecroyd, H. W., Jones, R. C. & Aitken, R. J. (2003). Endogenous redox activity in mouse spermatozoa and its role in regulating the tyrosine phosphorylation events associated with sperm capacitation. *Biology of Reproduction*, 69 (1), 347-354.

Endogenous Redox Activity in Mouse Spermatozoa and Its Role in Regulating the Tyrosine Phosphorylation Events Associated with Sperm Capacitation¹

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ABSTRACT

We investigated the role of endogenous redox activity in regulating the signal transduction pathway leading to tyrosine phosphorylation in mouse spermatozoa. Endogenous redox activity was monitored using a luminol-peroxidase chemiluminescent probe. Chemiluminescence increased in spermatozoa that were actively undergoing cAMP-mediated tyrosine phosphorylation events associated with capacitation and was inhibited in a dose-dependent manner by addition of catalase or diphenylene iodonium, both of which also inhibited tyrosine phosphorylation within the cell at points downstream of cAMP. Excluding bicarbonate from the incubation medium reduced the redox activity of sperm by 80–90% and dramatically reduced tyrosine phosphorylation. This study provides the first evidence that tyrosine phosphorylation associated with capacitation in mouse spermatozoa is redox regulated by a flavinoid-containing enzyme involving mediation by hydrogen peroxide. Bicarbonate regulated the redox activity of mouse spermatozoa, and this regulation may contribute to the impact of this anion on tyrosine phosphorylation during capacitation of mouse spermatozoa.

gamete biology, signal transduction, sperm capacitation, sperm maturation

INTRODUCTION

Ejaculated mammalian spermatozoa are unable to immediately fertilize ova. Instead, they must first undergo a series of maturational changes within the female tract that confers upon these cells the ability to interact with the oocyte and achieve fertilization. These changes, collectively termed capacitation, were identified independently by Austin and Chang in the 1950s [1, 2]. A number of correlates of the process have been identified, including plasma membrane remodeling [3–5], an increase in sperm metabolic rate [6–9], the expression of hyperactivated motility [10, 11], and the ability to undergo the acrosome reaction on the surface of the zona pellucida [5, 12]. Recent studies suggest that some, if not all, of these processes are driven by a unique cAMP-dependent signaling cascade that leads to a dramatic increase in the tyrosine phosphorylation status of these cells [13]. This signaling pathway is probably ubiquitous among mammals; it has now been identified in spermatozoa from all species that have been studied, including

the human [14], rat [15], mouse [13], bull [16], pig [17], hamster [18, 19], monkey [20], and tammar wallaby (L. Bennetts, unpublished results). The process appears to be a necessary prerequisite for plasma membrane remodeling [21], hyperactivation [18, 20, 22], and sperm-egg binding [23, 24]. A number of key medium constituents required to support the capacitation process *in vitro* exert their action by regulating tyrosine phosphorylation [24–27].

In this context, bicarbonate plays a key role in the capacitation of human [28, 29], hamster [19, 30], and mouse [25] spermatozoa through its ability to control phosphotyrosine expression in these cells [25]. In mouse spermatozoa, the beneficial effects of bicarbonate are independent of intracellular pH; alternative buffers do not reduce the dependence of tyrosine phosphorylation on the presence of bicarbonate. Moreover, bicarbonate's action is thought to be upstream of cAMP because agonists of this system restore phosphotyrosine expression to spermatozoa incubated in the absence of this anion [25]. The current consensus is that bicarbonate acts via direct stimulation of a soluble form of the adenylyl cyclase enzyme present within mouse spermatozoa [25, 31, 32]. However, this model of bicarbonate action may not apply to all species [33].

In addition to bicarbonate, endogenously produced reactive oxygen species (ROS) have been identified as key regulators of sperm capacitation [34, 35]. Studies of human [14, 36, 37] and rat [15] sperm employing oxidizing conditions, enzymatic scavengers of ROS such as catalase, and flavoprotein inhibitors such as diphenylene iodonium (DPI) have shown that the cAMP-signalling pathway leading to tyrosine phosphorylation is redox regulated in these species. ROS play important roles in cell signaling cascades, and hydrogen peroxide in particular is able to regulate tyrosine phosphorylation through both the stimulation of tyrosine kinases [38] and the inhibition of tyrosine phosphatase activity [39].

Although mouse spermatozoa are able to generate free radicals [40, 41], the impact of this redox activity on capacitation remains to be established. The aim of the present study was to examine this question with particular emphasis on the role of redox activity in mediating the effect of bicarbonate on the tyrosine phosphorylation observed during the capacitation of mouse spermatozoa.

MATERIALS AND METHODS

Reagents

BSA was purchased from Research Organics (Cleveland, OH). Hepes, penicillin, and streptomycin were from Gibco (Grand Island, NY). Catalase and superoxide dismutase (SOD) were purchased from Calbiochem (San Diego, CA). Anti-phosphotyrosine antibody (clone 4G10) was pur-

¹This study was funded by an Australian Research Council project grant (A099180338).

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Received: 24 October 2002.

First decision: 25 November 2002.

Accepted: 7 March 2003.

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ISSN: 0006-3363. <http://www.biolreprod.org>

chased from Upstate Biotechnology (Lake Placid, NY). The goat anti-mouse antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and the goat serum was from Hunter Antisera (Jesmond, NSW, Australia). All other reagents were obtained from Sigma (St. Louis, MO).

Sperm Preparation

Spermatozoa from the cauda epididymides were obtained from adult Swiss mice (CD45) 8–14 wk of age. Mice were killed by carbon dioxide asphyxiation. Spermatozoa were collected by backflushing the cauda epididymides with water-saturated paraffin oil and then storing under oil at 37°C. Sperm were then activated by addition of modified Biggers, Whitten, and Whittingham medium (BWW) [42] in which the BSA had been replaced with 1 mg/ml polyvinyl alcohol (BWW/PVA). This medium was used because it minimizes the loss of cholesterol from the sperm plasma membrane [3, 4] and slows tyrosine phosphorylation [13]. Bicarbonate-free BWW/PVA was also used to activate sperm in some experiments. Spermatozoa from the caput epididymides were collected by dissecting the region free of fat, blotting it free of blood, and immersing it in 500 μ l of BWW/PVA under water-saturated paraffin oil. The cells were released by piercing the duct five to eight times with a 30-ga needle. The sperm suspensions were allowed to disperse for 10 min at 37°C before determining their concentration using a Neubauer hemocytometer. Unless otherwise stated, the cells were then diluted into various treatments in BWW (i.e., the medium contained BSA instead of PVA) at a final concentration of 10^6 sperm/ml and then incubated at 37°C under an atmosphere of 5% CO₂ in 95% air. The time at which spermatozoa were diluted into BWW was taken as $t = 0$ min. Viability was assessed at the end of the incubation using the hypo-osmotic swelling test [43]. No effect on cell viability was found following the treatments reported here.

Redox Activity

The endogenous redox activity of mouse spermatozoa was monitored following dilution into various treatments at a concentration of 10^6 sperm/ml unless otherwise stated. This spontaneous redox activity was monitored by chemiluminescence using luminol (25 μ M) and horseradish peroxidase (4.4 U) as the detection system [44] in a LB953 Autolumat luminometer (Berthold, Wilbad, Germany). All measurements were conducted at 37°C.

Cyclic AMP Assays

Spermatozoa were centrifuged at $500 \times g$ for 3 min, then cAMP was extracted by adding ice-cold 6% trichloroacetic acid and incubating for 10 min. The extracted sample was centrifuged at $20,000 \times g$ for 10 min, and the supernatant was washed three times with 10 volumes of water-saturated butanol and then dried under nitrogen at 50°C. Samples were stored at -20°C . The cAMP content of the cells was measured using a Biotrak cAMP enzyme immunoassay system (Amersham International, Buckinghamshire, U.K.) according to the manufacturer's instructions.

Immunocytochemical Localization of Tyrosine Phosphorylation

Spermatozoa were fixed for 10 min with 1% paraformaldehyde, centrifuged ($500 \times g$ for 3 min), resuspended in PBS (pH 7.4)/glycine containing 0.01% sodium azide, and stored at 4°C. Before plating, samples were arranged in a random order to avoid bias in the counts. Cells were plated onto poly-L-lysine-coated slides and permeabilized by exposure to 0.2% Triton X-100 in PBS at room temperature for 10 min. The slides were then washed three times with PBS, and nonspecific sites were blocked with 10% goat serum and 1% BSA in PBS for 1 h at 37°C in a humid chamber. After incubation, the slides were washed three times with PBS, and anti-phosphotyrosine antibody (1:100) was applied. The slides were again incubated for 1 h at 37°C in a humid chamber, washed three times with PBS-Tween, and then incubated in the presence of fluorescein isothiocyanate (FITC)-labeled goat anti-mouse antibody (1:200) for an additional hour at 37°C in a humid chamber. After washing, slides were allowed to air dry before mounting in mowiol and viewing the fluorescence with an Axioplan 2 microscope equipped with FITC filters (Zeiss, Oberkochen, Germany). The percentage of cells displaying positive fluorescence over the tail, partial + complete fluorescence according to the classification by Urner et al. [24], was scored (minimum of 200 cells/treatment). Negative controls were samples that did not receive the primary antibody and samples treated with the anti-phosphotyrosine antibody preincubated for 1 h with 20 mM *O*-phospho-L-tyrosine at room temperature.

Tyrosine Phosphorylation by SDS-PAGE and Western Blotting

After incubation, spermatozoa were centrifuged ($500 \times g$ for 3 min) and washed in PBS. The spermatozoa were then solubilized with 2% SDS (0.375 M Tris-HCl, pH 6.8, 2% SDS, 10% sucrose) and heated to 100°C for 5 min. After centrifugation at $20,000 \times g$ for 10 min, the supernatant was retained and boiled in SDS-sample buffer [45] containing 2% 2-mercaptoethanol. Samples were then stored at -20°C until required. To ensure equal protein loading onto gels of the various samples, protein estimations were performed on each sample using a bicinchoninic acid kit (Pierce, Rockford, IL) and the microtiter plate protocol according to the manufacturer's instructions.

SDS-PAGE was conducted on sperm proteins using 7.5% polyacrylamide gels at 10 mA constant current per gel. The proteins were then transferred onto a nitrocellulose hybrid super-C membrane (Amersham) at 350 mA constant current for 1 h according to the method of Towbin et al. [46]. The membrane was blocked for 1 h at room temperature with Tris-buffered saline (TBS; 0.02 M Tris-HCl, 0.15 M NaCl, pH 7.6) containing 3% BSA. The membrane was then incubated for 2 h at room temperature in a 1:4000 dilution of a monoclonal anti-phosphotyrosine antibody (clone 4G10) in TBS containing 1% BSA and 0.1% Tween-20. After incubation, the membrane was washed four times for 5 min each with TBS containing 0.01% Tween-20 and then incubated for 1 h at room temperature with goat anti-mouse immunoglobulin G, horseradish peroxidase conjugate at a concentration of 1:3000 in TBS containing 1% BSA and 0.1% Tween-20. The membrane was again washed as described above, and the phosphorylated proteins were detected using an enhanced chemiluminescence kit (Amersham) according to the manufacturer's instructions. To ensure antibody specificity, membranes were also incubated with anti-phosphotyrosine antibody that was first absorbed with 20 mM *O*-phospho-L-tyrosine for 1 h with constant rotation at room temperature prior to use in immunoblotting.

Statistics

All experiments were replicated at least three times, and the significance of any differences observed between group means was determined by ANOVA. Before analyses, percentage data were arcsine transformed and cAMP data were log transformed. Subsequent post hoc testing of differences between group means was accomplished using the Tukey test; differences were considered significant at $P < 0.05$.

RESULTS

Patterns of Tyrosine Phosphorylation

Only one protein band (116 kDa) showed significant phosphorylation throughout the 3-h incubation of mouse spermatozoa in BWW (Fig. 1). This protein corresponds to a previously identified hexokinase [47] that is unique to spermatozoa and is constitutively phosphorylated. It was therefore used in this study to confirm equal protein loading of samples. Tyrosine phosphorylation of a range of other proteins (37–220 kDa) increased during 90 min of incubation in BWW but then declined slightly after 180 min. No protein bands were detected by Western blot following preincubation of the antibody with 20 mM *O*-phospho-L-tyrosine (data not shown), indicating that the antibody was specific for tyrosine phosphoproteins.

Immunocytochemical analyses showed an increase in the proportion of cells displaying tyrosine phosphorylation-associated fluorescence (partial + complete) over time (Fig. 2). These levels reached a maximum after 90–120 min when >50% of sperm tails displayed evidence of tyrosine phosphorylation. The increase in cells displaying fluorescence ($P < 0.001$) corresponded to the time when maximum tyrosine phosphorylation was evident, as assessed by Western blot analysis (Fig. 1). No phosphotyrosine-associated fluorescence was observed when the primary antibody was either not included or was preincubated with 20 mM *O*-phospho-L-tyrosine (data not shown).

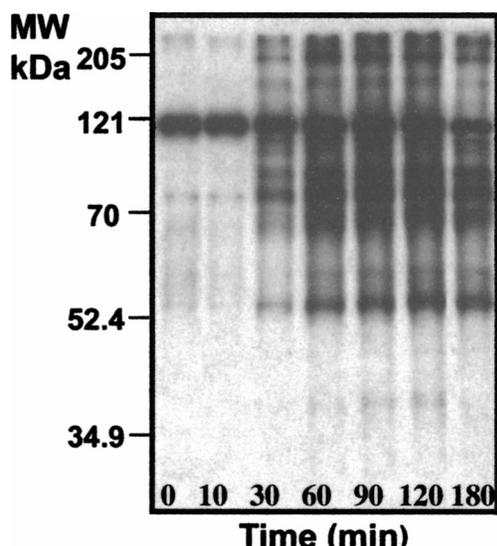


FIG. 1. Protein tyrosine phosphorylation of mouse spermatozoa incubated in BWW. Following incubation for the times indicated, proteins were extracted from the sperm with 2% SDS, and phosphotyrosine residues were detected by Western blot analysis. The result shown is representative of five independent experiments.

Intracellular cAMP

The relationship between cAMP production and tyrosine phosphorylation was examined during incubation for 180 min. Intracellular cAMP concentrations were initially high in the preparations, decreased over the next hour ($P < 0.05$) (Fig. 3), and then increased during the next 30 min of incubation when tyrosine phosphorylation levels were maximal (Fig. 1), before decreasing after 3 h incubation.

Redox Activity

During incubation in vitro, caudal spermatozoa produced a dose-dependent luminol-peroxidase-dependent chemiluminescent signal, which was not evident in the control preparations containing no sperm (Fig. 4A). This signal did not arise from leukocytes, as indicated by the lack of response of samples to N-Formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) provocation and the failure to detect a

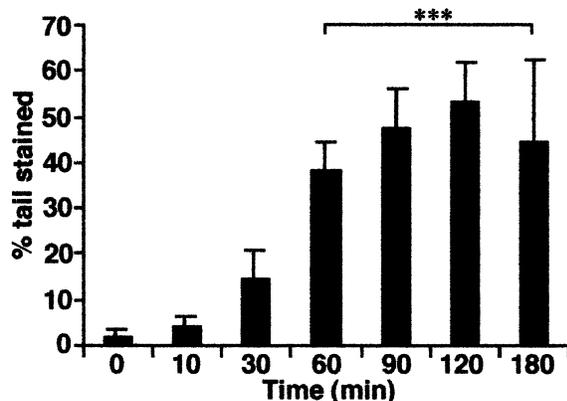


FIG. 2. The percentage of mouse sperm that displayed tail-associated tyrosine phosphorylation following incubation in BWW for the times indicated. Protein tyrosine phosphorylation was detected by indirect immunofluorescence using an anti-phosphotyrosine antibody. Values are the mean \pm SEM of four different experiments. ANOVA revealed a highly significant difference ($P < 0.001$) due to time. ***Significant ($P < 0.001$) difference in the mean compared with 0 min.

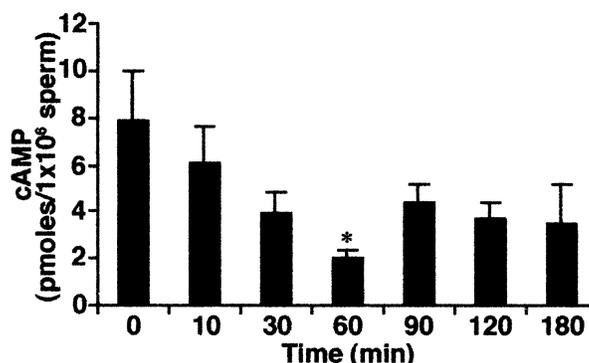


FIG. 3. Intracellular cAMP levels of mouse caudal spermatozoa incubated in BWW for the times indicated. Values are the mean \pm SEM of five different experiments. ANOVA revealed a highly significant ($P < 0.001$) difference due to time. *Significant ($P < 0.05$) difference in the mean compared with 0 min.

protein band corresponding to the leukocyte marker CD45 by immunoblotting with a commercially available antibody against this protein (data not shown). This spontaneous chemiluminescence increased dramatically after a lag time of approximately 10–40 min of incubation and then stayed high for the remainder of the 100-min incubation period. The lag time was longer for the highest concentration of spermatozoa tested than for the lower concentrations and

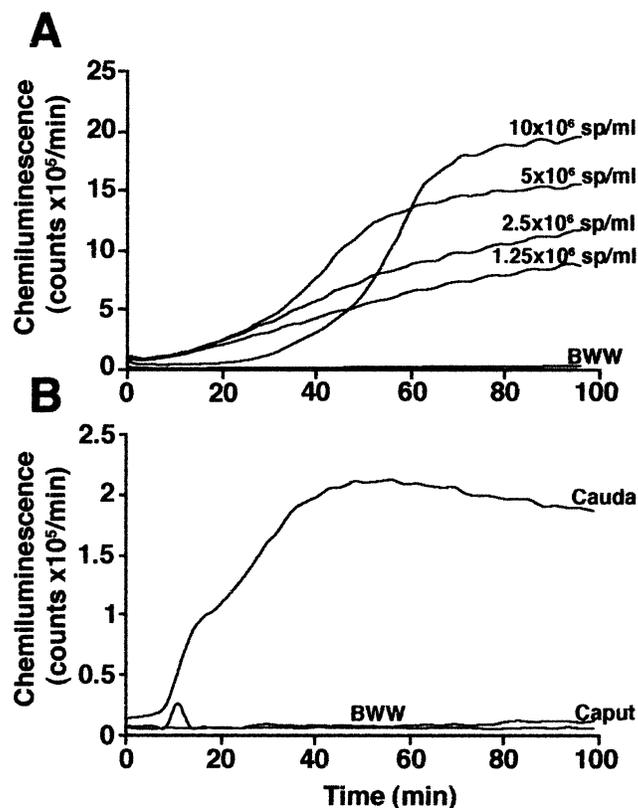


FIG. 4. Spontaneous generation of luminol-peroxidase-dependent chemiluminescence by mouse epididymal spermatozoa. A) Spermatozoa ($1.25\text{--}10 \times 10^6$ sperm/ml) from the cauda epididymis were incubated in medium containing luminol ($25 \mu\text{M}$) and horseradish peroxidase (4.4 U), and spontaneous chemiluminescence was monitored over 100 min. B) Caudal (10^6 sperm/ml) and caput (10^7 sperm/ml) spermatozoa were incubated in the same medium, and spontaneous chemiluminescence was monitored over 100 min. The results are representative of four independent experiments.

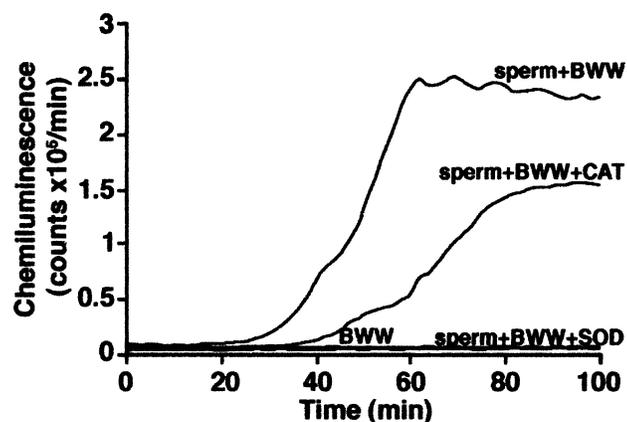


FIG. 5. Inhibition of the spontaneous luminol-peroxidase chemiluminescent signal produced by caudal mouse spermatozoa by SOD and catalase (CAT). Caudal spermatozoa were added to medium containing luminol-peroxidase and either SOD (300 U/ml) or CAT (3000 U/ml), and their ability to generate chemiluminescence was monitored for 100 min. The results are representative of three independent experiments.

differed among independent experiments. However, after 60 min of incubation, the signal was directly cell concentration dependent for all four replicates. In contrast to caudal cells, caput spermatozoa, which do not engage in tyrosine phosphorylation when released into medium that supports capacitation, did not produce a spontaneous chemiluminescent signal even when incubated at the highest concentration (Fig. 5B). This finding indicates that the capacity of the spermatozoa to display this redox activity coincides with their ability to activate the signaling pathway leading to tyrosine phosphorylation, which develops during epididymal transit.

Studies to characterize the free radical species respon-

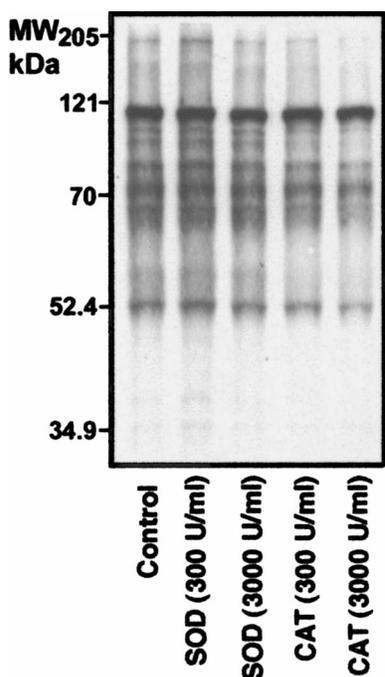


FIG. 6. Effect of SOD and CAT on protein tyrosine phosphorylation in mouse spermatozoa. Caudal mouse spermatozoa were incubated in BWW for 90 min with or without SOD (300, 3000 U/ml) or CAT (300, 3000 U/ml), and sperm proteins were extracted with 2% SDS. The Western blot is representative of four independent experiments.

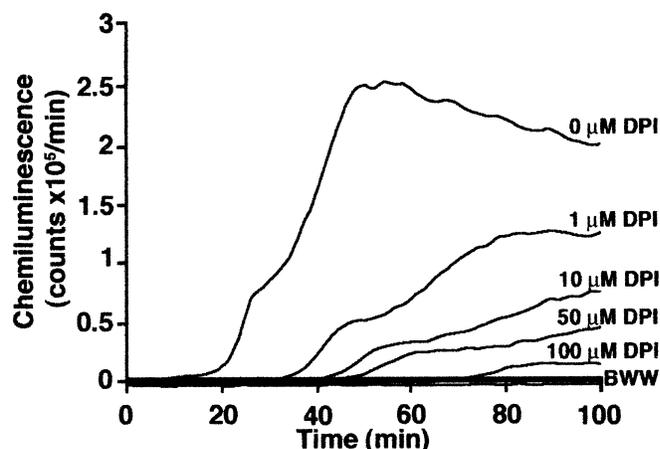


FIG. 7. Concentration-dependent inhibition of spontaneous luminol-peroxidase-dependent chemiluminescence of mouse spermatozoa by DPI. Caudal spermatozoa were added to medium containing luminol-peroxidase and 0–100 μ M DPI, and their ability to generate chemiluminescence was monitored for 100 min. This result is representative of three independent experiments.

sible for this chemiluminescent signal indicated that hydrogen peroxide was involved because when catalase, an enzymatic scavenger of this oxidant, was added to the medium at the start of the incubation, the chemiluminescence was suppressed by approximately 30% (Fig. 5). The inability of catalase to abolish the signal completely may reflect the limited access of this extracellular enzyme to the intracellular sites where luminol-dependent chemiluminescence was generated. Alternatively, redox active species other than hydrogen peroxide, such as peroxynitrite, may have been involved in generating the chemiluminescent signal. The fact that the chemiluminescent signal was completely abolished by addition of SOD to the incubation medium (Fig. 5) suggests the concomitant generation of superoxide anion [48]. However, because the luminol-peroxidase system requires the formation of superoxide to oxygenate luminol and thus generate a chemiluminescent signal, this effect of SOD may reflect its ability to disrupt the chemistry of the probe. Thus, the generation of superoxide anion by mouse spermatozoa cannot be established using this chemiluminescent technique.

Redox Regulation of Tyrosine Phosphorylation

To determine whether tyrosine phosphorylation is dependent on the presence of either superoxide anion or hydrogen peroxide produced endogenously, spermatozoa were incubated in the presence of SOD or catalase. SOD had no effect on tyrosine phosphorylation during 90 min of incubation (Fig. 6). In contrast, catalase caused a dose-dependent inhibition of tyrosine phosphorylation. However, even high concentrations of catalase did not completely suppress tyrosine phosphorylation, suggesting that hydrogen peroxide is not the only factor regulating this signal transduction system or that the limited access of catalase to intracellular sites prevented it from completely suppressing tyrosine phosphorylation.

The effect of DPI (1–100 μ M) on spontaneous luminol-peroxidase-dependent chemiluminescence was assessed, because it acts by targeting flavin groups in redox enzymes. Addition of DPI to the incubation medium resulted in a dose-dependent decrease in the signal generated by spermatozoa, such that approximately 80% of the redox activity

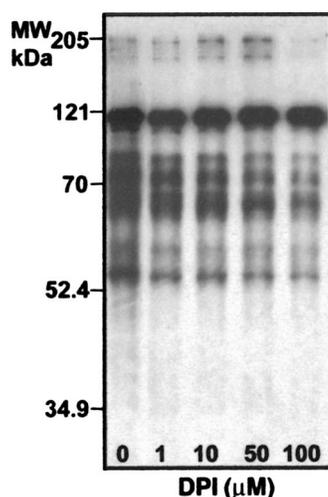


FIG. 8. Concentration-dependent inhibition of protein tyrosine phosphorylation in mouse spermatozoa by DPI. Caudal mouse spermatozoa were incubated in BWB containing DPI (0–100 μ M) for 90 min, and then proteins were extracted with 2% SDS. The result shown is representative of three independent experiments.

was inhibited at 100 μ M DPI (Fig. 7). Addition of DPI also resulted in a dose-dependent inhibition of tyrosine phosphorylation in these cells over the same concentrations used to inhibit the chemiluminescent signal (Fig. 8). However, as with catalase, DPI did not completely inhibit tyrosine phosphorylation.

To determine whether the redox regulation of sperm capacitation was through an effect on cAMP metabolism, intracellular cAMP levels were measured in spermatozoa following incubation for 90 min in the presence of DPI, SOD, or catalase (Fig. 9). There was no significant change in cAMP content following incubation with any of these factors over the concentrations ranges tested.

Role of Bicarbonate

The role of bicarbonate in the redox regulation of capacitation was assessed during 100 min of incubation (Fig.

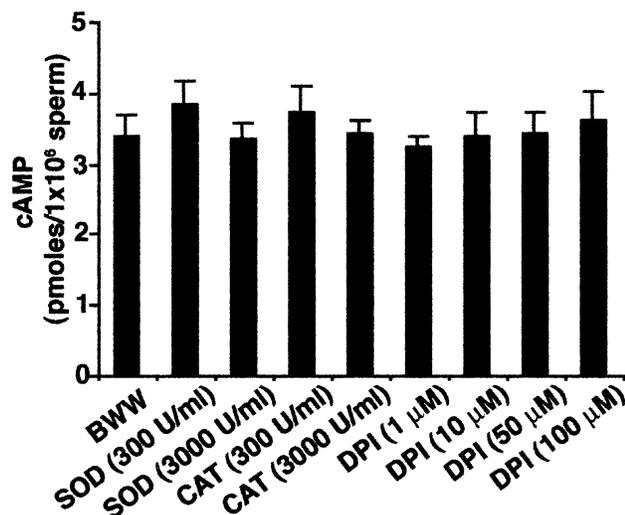


FIG. 9. Effect of SOD, catalase, and DPI on intracellular cAMP levels in mouse spermatozoa. Caudal spermatozoa were incubated in BWB for 90 min alone or in the presence of SOD (300, 3000 U/ml), CAT (300, 3000 U/ml), or DPI (1–100 μ M). Values are the mean \pm SEM of five independent experiments.

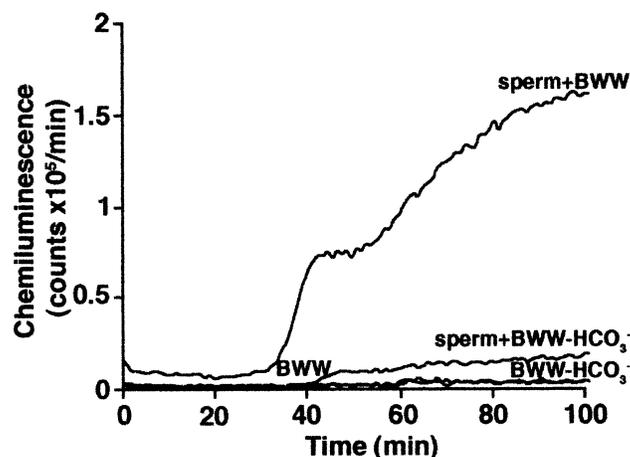


FIG. 10. Effect of bicarbonate on spontaneous luminol-peroxidase-dependent chemiluminescence by mouse spermatozoa. Caudal spermatozoa were incubated with and without bicarbonate for 100 min. The results are representative of four independent experiments.

10). When spermatozoa were incubated in medium with no added bicarbonate their spontaneous luminol-peroxidase-dependent chemiluminescence was reduced by 80–90% compared with that of spermatozoa incubated in complete medium. This effect was not prevented by the addition of pentoxifylline (Ptx) and dibutyl cAMP to increase intracellular cAMP levels (data not shown). Omission of bicarbonate from the incubation medium also significantly reduced tyrosine phosphorylation within the cells (Fig. 11A), but this effect was prevented by including dibutyl cAMP and Ptx in the incubation medium. This finding was confirmed by immunocytochemical analyses of the spermatozoa. Thus, there was a significant ($P < 0.001$) inhibition of tail-associated tyrosine phosphorylation in medium devoid of bicarbonate following 90 min of incubation (Fig. 11B). However, this deficiency could be overcome by including dibutyl cAMP and Ptx in bicarbonate-deficient medium. Under these conditions, levels of tyrosine phosphorylation were similar to those of the controls.

DISCUSSION

The results of this study provide the first evidence that the tyrosine phosphorylation events associated with the capacitation of mouse caudal spermatozoa are redox regulated. This redox activity could be detected using the luminol-peroxidase system and appeared to be generated via a flavoprotein-dependent process involving mediation by hydrogen peroxide. In contrast to caudal cells, caput spermatozoa did not produce a chemiluminescent signal, indicating that the capacity of the spermatozoa to display this redox activity coincides with their ability to activate the signaling pathway leading to tyrosine phosphorylation.

The chemiluminescent activity observed under these conditions undoubtedly involves amplification of a primary oxidative signal through redox cycling of the probe. With the chemiluminescence protocol employed in this study, the initiating step involves the oxidation of luminol by hydrogen peroxide and horseradish peroxidase to create a luminol radical. This radical then reacts with ground state oxygen in the immediate vicinity to generate superoxide anion and concomitantly regenerate the probe. The superoxide artificially generated in this way is then responsible for the oxygenation of luminol to create an unstable endoperoxide that subsequently breaks down, with the emission of light.

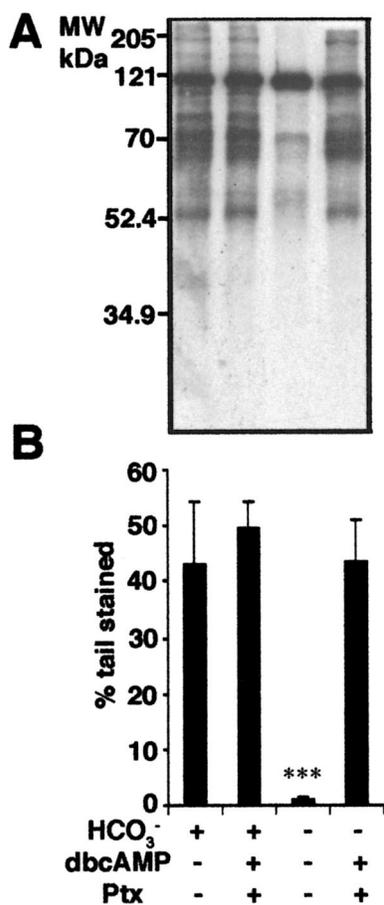


FIG. 11. Effect of bicarbonate on tyrosine phosphorylation in mouse spermatozoa as assessed by Western blot analysis (A) and immunocytochemistry (B). Spermatozoa were incubated in BWV with or without bicarbonate either alone or in the presence of dibutyl cAMP (5 mM) and Ptx (3 mM) for 90 min. Proteins were extracted with 2% SDS. The Western blot result shown is representative of five independent experiments. The graph shows the percentage of sperm that displayed fluorescence associated with the tail following indirect immunofluorescence using the anti-phosphotyrosine antibody. Values are the mean \pm SEM of three different experiments. ANOVA revealed a highly significant difference ($P < 0.001$) due to treatment. ***Significant ($P < 0.001$) difference in the mean compared with other time points.

The importance of superoxide in luminol endoperoxide formation accounts for the highly efficient inhibitory action of SOD on the spontaneous chemiluminescent signals generated by capacitating mouse spermatozoa. The fact that catalase inhibited this signal by only 30% suggests that a significant portion of the redox activity is generated at intracellular sites that could not be accessed by the extracellular presence of this enzyme. Alternatively, the creation of luminol radicals may be achieved by oxidizing species other than hydrogen peroxide, such as peroxynitrite.

Tyrosine phosphorylation of a broad range of sperm proteins in mouse caudal spermatozoa increases during incubation in vitro [13, 24]. In the present study, maximum levels of tyrosine phosphorylation were found following 90–120 min of incubation, corresponding with the time needed for mouse spermatozoa to capacitate in medium that supports this process [5]. The initial high cAMP levels may reflect an increase in cAMP associated with the activation of motility during ejaculation [49]; cAMP levels then fall before rising again over the time during which these cells engage in the capacitation process.

The findings described here suggest that production of

hydrogen peroxide by capacitating sperm populations during incubation in vitro modulates the tyrosine phosphorylation status of these cells, a process of collective autoregulation. Although the redox activity of mouse spermatozoa was cell concentration dependent, the initiation of this activity was delayed when spermatozoa were incubated at the highest concentration tested. Whether this inhibition is due to the presence of factor(s) in epididymal fluid that act to inhibit motility (reviewed in [50]) and/or decapacitation factors remains to be established. Hydrogen peroxide regulates phosphotyrosine-mediated signaling events in other cells through the induction of tyrosine kinase activity or the inhibition of the corresponding phosphatase [39, 51, 52]. A role for hydrogen peroxide in capacitation has been previously suggested for rat [15], hamster [34], and human [14] spermatozoa. These previous studies have shown that addition of catalase suppresses capacitation and/or that addition of hydrogen peroxide promotes the process by regulating the tyrosine phosphorylation status of these cells [36, 37]. The present results suggest a similar role for hydrogen peroxide in the mouse spermatozoon, in which this oxidant appears capable of controlling tyrosine phosphorylation at points downstream of cAMP metabolism, possibly via the regulation of phosphatase activity.

The results of this study also suggest that hydrogen peroxide is not the only redox active constituent that may be involved in the control of capacitation, because it was only partly responsible for the chemiluminescent signal produced by capacitating cells. Residual activity detected in the presence of extracellular catalase may represent an intracellular source of luminol-dependent chemiluminescence generated in conjunction with sperm peroxidase [44] that could not be accessed by extracellular catalase. The presence of a free radical-generating system within mouse spermatozoa was supported through the use of the flavin protein inhibitor DPI. A number of free radical-generating enzymes have been proposed to be present in sperm, including nitric oxide synthase [53, 54] and a NAD(P)H oxidoreductase [15, 41, 55], both of which are inhibited by DPI. Significantly, both superoxide anion and nitric oxide have been implicated in regulating capacitation, including inducing tyrosine phosphorylation in human and hamster sperm [36, 37, 56, 57]. However, the present results suggest that the redox control of tyrosine phosphorylation in the mouse is of limited significance compared with that in other species. Neither of the reagents found to modulate the redox status of these cells (catalase and DPI) affected intracellular cAMP levels. Moreover, the addition of SOD to the incubation medium did not influence tyrosine phosphorylation or intracellular cAMP levels of mouse spermatozoa. Thus, although redox activity may be a ubiquitous feature of mammalian spermatozoa [15, 37, 58], differences exist among species in the manner by which this activity is regulated and in its functional significance.

This study also shows for the first time that bicarbonate, a key medium constituent required to support tyrosine phosphorylation [25], also regulates the redox activity of mouse spermatozoa. This support may contribute to the diminished tyrosine phosphorylation status of cells incubated in bicarbonate-deficient medium. Identification of a bicarbonate-sensitive adenylyl cyclase in sperm [32] has led to the proposal that regulation of tyrosine phosphorylation occurs directly through this enzyme [25, 31]. However, the present results suggest that bicarbonate may also act to regulate tyrosine phosphorylation through influencing the redox activity of the spermatozoa, because a decrease in the

redox activity of these cells was correlated with a decrease in tyrosine phosphorylation. Bicarbonate regulation of both tyrosine phosphorylation and free radical generation probably is upstream of cAMP generation. The evidence for this assumption is that increases in the cAMP content of the cell bypassed the negative effects of bicarbonate omission on tyrosine phosphorylation but did not restore the chemiluminescent signal.

The effects of bicarbonate on capacitation are not limited to tyrosine phosphorylation [59, 60] but also have been associated with rapid changes to the sperm plasma membrane, including an increase in phospholipid disorder [61]. Therefore, bicarbonate may regulate the redox activity of the cell via a direct action on the plasma membrane oxidoreductase activity or indirectly through alterations to the plasma membrane, which subsequently enables this redox activity to be initiated. Thus, bicarbonate may increase tyrosine phosphorylation in mouse spermatozoa by acting directly on the adenylyl cyclase and by regulating the redox activity of the sperm, which then promotes phosphorylation at points downstream of cAMP metabolism.

The results presented here indicate that mouse caudal spermatozoa exhibit endogenous redox activity when incubated under conditions that support capacitation and that the redox status of these cells modulates the tyrosine phosphorylation events associated with this process. This flavin-containing redox system appears able to generate hydrogen peroxide within the cell, and this oxidant then regulates tyrosine phosphorylation, possibly via an inhibitory action on protein tyrosine phosphatases. The redox activity of mouse spermatozoa is regulated by extracellular bicarbonate, and thus the promotion of cellular redox activity might be one of the mechanisms by which this anion regulates the capacitation status of mouse spermatozoa. These findings add to a wealth of data obtained in a variety of different cell types in emphasizing the fundamental importance of redox activity in controlling tyrosine phosphorylation-dependent signal transduction pathways [62].

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