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Role of Tyrosine Phosphorylation of Flagellar Proteins in Hamster Sperm Hyperactivation[1]

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ABSTRACT

Despite extensive study of sperm motility, little is known of the mechanism of mammalian sperm hyperactivation. Here we describe a novel method for preparation of rodent sperm flagella and use it to show a correlation between tyrosine phosphorylation of flagellar proteins and hyperactivation of hamster sperm. When hyperactivation was produced by a 3.5-h incubation in a medium supporting capacitation, four major tyrosine-phosphorylated peptides of 90-, 80-, 62-, and 48-kDa mass were detected in flagellar extracts. Incubation with calyculin A, an inhibitor of protein phosphatases 1 and 2A, produced hyperactivation within 40 min but only a single 80-kDa phosphotyrosine-containing flagellar component. Conversely, incubation with inhibitors of either protein kinase A (H8) or protein tyrosine kinase (tyrphostin 47) prevented both hyperactivation and the production of tyrosine-phosphorylated flagellar peptides. These results indicate a strong correlation of hyperactivation with the tyrosine phosphorylation of sperm flagellar peptides, and they strongly implicate an 80-kDa component as a major mediator of the mechanism that produces hyperactivated motility of hamster sperm.

INTRODUCTION

Freshly ejaculated mammalian sperm display vigorous motility but are unable to fertilize an oocyte. In order to become fertilization competent, sperm must spend a period of time in either the female reproductive tract or under an appropriate in vitro environment. This process of becoming fertilization competent is referred to as ''capacitation'' and involves a series of biochemical and functional changes in the sperm [1, 2].

Recent studies have shown that changes in protein tyrosine phosphorylation are correlated with mouse, human, and bovine sperm capacitation [3–7]. Moreover, both tyrosine phosphorylation and sperm capacitation are stimulated by cAMP analogues but inhibited by cAMP antagonist or protein kinase A (PKA) inhibitor, indicating that the cAMP/PKA signaling pathways are involved in the two processes [6, 8, 9]. Further support for the role of cAMP-dependent protein phosphorylation during sperm capacitation comes from the observation that protein phosphatase 1 and 2A inhibitor, calyculin A [10], enhances sperm capacitation and protein phosphorylation [9, 11].

One striking phenomenon occurring during capacitation is that the sperm flagellum displays a frantic movement called hyperactivation [12]. Hyperactivation was first described by Yanagimachi in golden hamster sperm [13]. Compared with activated (non-hyperactivated) sperm, hyperactivated sperm exhibit a high-amplitude flagellar waveform [14–16]. It has been proposed that hyperactivation occurs to enable the sperm to generate adequate force to penetrate the cumulus matrix, or zona pellucida, of the oocyte [14, 15].

Although sperm hyperactivation is a capacitation-associated phenomenon, the two processes do not appear to be coupled. In a capacitating medium containing a low concentration of bicarbonate, hamster sperm become capacitated, but not hyperactivated [17, 18]. Conversely, hyperactivation can proceed independently of capacitation in hamster and mouse sperm [19–21]. This has led a number of investigators to suggest that sperm capacitation and hyperactivation should be considered independent events [12, 15, 22].

Many physiological studies on sperm hyperactivation have been conducted; however we have a limited understanding of the cytoplasmic, biochemical mechanisms of this important event. This is due in part to the difficulty in obtaining pure sperm flagella, particularly from small laboratory animals, e.g., hamsters, mice, and rats, in which sperm hyperactivation is well defined [14]. While some methods, such as homogenization or sonication, for isolating sperm flagella have been reported [23, 24], these methods require a considerable quantity of materials and inevitably yield flagellar fragments. In the present study, we describe a novel method for obtaining uniform flagella from rodent sperm by the use of glass beads and centrifugation. We have used immunofluorescence to demonstrate the presence of tyrosine-phosphorylated proteins in the sperm flagellum and immunoblotting of extracts of isolated flagella to characterize flagellar peptides whose tyrosine-phosphorylation status correlates with hyperactivation.

MATERIALS AND METHODS

Reagents and Culture Medium

Dibutyryl cAMP (dbcAMP), protein tyrosine kinase (PTK) inhibitor, tyrphostin 47 (3,4-dihydroxy-a-cyanothiocinnamamide; RG-50864), PKA inhibitor, H-8(*N*-(2-[methylamino] ethyl)-5-isoquinolinesulfonamide-hydrochloride), and glass beads ($\leq$ 106 μm) were purchased from Sigma Chemical Company (St. Louis, MO). Anti-phosphotyrosine monoclonal antibody (Clone PY20) was purchased from Transduction Laboratories (Lexington, KY). Horseradish peroxidase-conjugated goat anti-mouse IgG was obtained from Bio Source International (Camarillo, CA). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG was from Cappel Research Products (Durham, NC). Fraction V BSA was from Armour Pharmaceutical Corporation (cat. #40125R; Tokyo, Japan) or Calbiochem Corporation (cat. #126591; La Jolla, CA). Other chemicals were of reagent grade from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Sodium vanadate was made up as 10 mM stock solution in 0.1 N NaOH. Stock solutions of calyculin A, okadaic acid, and tyrphostin 47 were prepared in dimethyl

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sulfoxide and stored at −20°C. Controls without inhibitors received an equivalent amount of dimethyl sulfoxide. Modified Tyrode's albumin lactate pyruvate (TALP) medium [25] was used throughout these experiments. The preparation of the medium was previously described [26].

Sperm Preparation

Male golden hamsters (*Mesocricetus auratus*) were raised and maintained in a light-controlled room (12L:12D) at constant temperature (22 ± 1°C). Sexually mature (10–12 wk of age) hamsters were killed by ethyl ether inhalation. Caudae epididymides were excised. After removal of blood from the epididymal surface with tissue paper, the distal portion of the epididymis was pierced with a 23-gauge syringe needle, and sperm were squeezed out with forceps. Approximately 50 μl of epididymal sperm mass was placed at the bottom of a 100-ml beaker, then gently covered with 100 ml of modified TALP medium and incubated at 37°C under 5% CO_2 in air. Quantitative analysis of activated sperm motility was performed within 5 min of sperm activation. For hyperactivated sperm, motility was quantified after 3.5 h of incubation.

Quantitative Analysis of Sperm Movement

Methods for examination and recording of sperm movement were described previously by Si [26]. The sperm motility parameters analyzed were straight-line velocity (VSL), curvilinear velocity (VCL), maximal principal bend (MPB) and maximal reverse bend (MRB) angles during one-beat cycles in the middle piece of the flagellum, and flagellar beat frequency. Sperm hyperactivation was evaluated by videotape analysis of asymmetric, circular flagellar movement with large principal bend and reverse bend.

Flagellar Preparation

Aliquots (10 ml) of the sperm ''swim-up fraction'' were pipetted out at various intervals of incubation and immediately demembranated by adding 10 μl of 10% (w:v) Triton X-100. The demembranated sperm were subjected to two washes by centrifugation at 2000 × *g* for 5 min in a Ca^{2+}- and Mg^{2+}-deficient PBS. The sperm pellet was resuspended in PBS, and the final sperm concentration was adjusted to ~1 × 10^7 cells/ml (determined with a hemocytometer). One milliliter of the sperm suspension was mixed approximately 3:1 (v:v) with ≤ 106-μm glass beads and vortexed in a 1.5-ml microcentrifuge tube for 1 min. The vortexed sperm were then transferred to a 10-ml centrifuge tube, and the glass beads were washed twice by pipetting with PBS to release the flagella, which were collected and also transferred to the 10-ml centrifuge tube. The sperm suspension was 20-fold diluted with PBS, distributed to two 10-ml centrifuge tubes, and centrifuged for 10 min at 100 × *g*. The supernatant containing flagella was pipetted out and centrifuged at 2000 × *g* for 10 min to recover sperm flagella.

To remove sperm mitochondrial sheaths (MS), the isolated sperm flagella were extracted for 3 min with a solution of 0.1% Triton X-100 and 2 mM dithiothreitol (DTT) in PBS at pH 9.0. The outer dense fibers (ODF) were isolated by resuspending the MS-free flagella in PBS at pH 7.4 plus 0.03% SDS, which dissolves the axoneme and fibrous sheath (FS) selectively. The disappearance of FS was checked by phase-contrast microscopy. The ODF were collected by centrifugation at 5000 × *g* for 10 min. Protein phosphatase inhibitor (100 μM sodium orthvanadate) and protease inhibitors (1 mM PMSF and 10 μg/ml leupeptin) were included in all steps of the procedure for sample preparation.

SDS-PAGE and Western Blotting

Isolated hamster sperm flagella (~6 × 10^5 cells) were solubilized in Laemmli sample buffer and subjected to electrophoresis on 8% SDS-PAGE gels [27]. The proteins were electroblotted to a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA) according to the method of Towbin et al. [28]. Membranes were blocked for 1 h with 5% BSA in Tris-buffered saline (TTBS; 150 mM NaCl, 20 mM Tris-HCl, 0.1% Tween 20, pH 7.6) followed by incubation for 1 h with monoclonal anti-phosphotyrosine antibody PY20 (1 μg/ml in TTBS). The blots were then washed (three times, 10 min each, with TTBS) and incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse IgG at 1:1000 dilution. After 1-h incubation, the membrane was washed (three times, 20 min each, with TTBS), and the peroxidase activity was detected using 4-chloro-1-naphtol as a substrate [29]. The reaction was stopped by washing in double-distilled water. Secondary antibody alone was used as a control.

Immunofluorescence Microscopy

Demembranated hamster sperm were extracted with Triton X-100 and DTT to remove MS as described above. The MS-free sperm were washed twice with PBS. One hundred microliters of sperm suspension (1 × 10^6 cells/ml) was settled on a coverslip, air dried, fixed with methanol for 10 min, and air dried. The coverslip was blocked with 5% BSA in PBS for 30 min. Monoclonal anti-phosphotyrosine antibody PY20 (10 μg/ml in PBS) was applied to the coverslip and incubated for 30 min. The coverslip was then washed with PBS and incubated for 30 min with FITC-labeled goat anti-mouse IgG secondary antibody (1:1000 dilution). Finally, the coverslip was mounted onto a glass slide and viewed using a Nikon (Garden City, NY) fluorescence microscope with a ×40 objective. Secondary antibody alone served as a control.

Data Analysis

Data are presented as the mean ± SD of multiple experiments. Significant differences between treatments were determined by the Newman-Keuls test after ANOVA. Results were considered significantly different when $p < 0.05$.

RESULTS

Comparison of Hamster Sperm Activation with Hyperactivation

Sperm activation and hyperactivation were evaluated by videotape analysis of swimming velocity, flagellar beat frequency, and bending angles. When the immotile caudal epididymal sperm were activated by being suspended in the modified TALP medium, they showed a low-amplitude flagellar waveform (Fig. 1A) and a progressive movement with an average beat frequency of 11.9 ± 0.9 Hz, a VSL of 223.5 ± 25.2 μm/sec, and a VCL of 348.2 ± 47.4 μm/sec. The MPB and MRB angles in the middle piece of the flagellum were on average 0.8 ± 0.1 rad and 0.8 ± 0.1 rad, respectively. In order to induce hyperactivation, the activated sperm were further incubated for 3.5 h at 37°C under

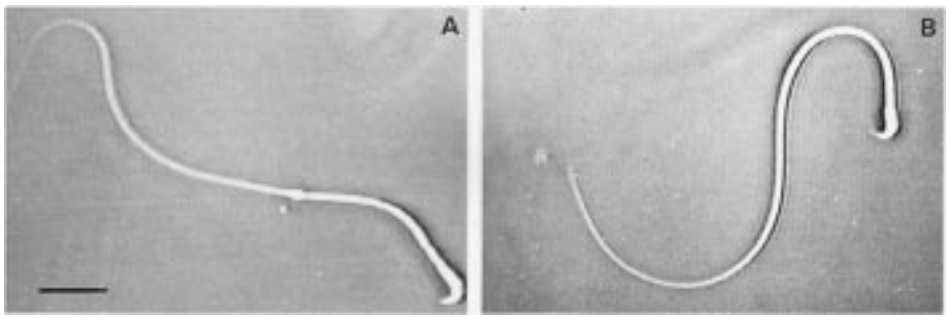

FIG. 1. A comparison of flagellar waveforms of activated (**A**) and hyperactivated (**B**) hamster sperm. Pictures from video records. Bar = 25 μm. Temperature, 37°C.

5% CO_2 in air. Hyperactivated sperm showed a high-amplitude flagellar waveform (Fig. 1B) and a circular movement with an average beat frequency of 7.9 ± 1.1 Hz, a VSL of 189.8 ± 20.4 μm/sec, and a high VCL of 747.8 ± 85.2 μm/sec (Table 1). The MPB and MRB angles in the middle piece were on average 1.6 ± 0.2 rad and 1.7 ± 0.2 rad, respectively (Table 1).

Time-Dependent Changes in Tyrosine Phosphorylation of Sperm Flagellar Proteins during Hyperactivation

When sperm motility is initiated, cAMP-dependent protein phosphorylation occurs [30, 31]. Using an anti-phosphotyrosine monoclonal antibody we asked whether protein phosphorylation is also associated with hyperactivation of sperm motility. The basic mechanochemical component of sperm hyperactivation is the flagellum (the tail). Therefore, before we could perform biochemical studies on flagellar movement, we needed to fractionate the sperm tail from the head. Figure 2 shows the uniform flagella isolated from hamster sperm according to the novel method described in *Materials and Methods.* Nearly all flagella appeared to be intact rather than fragmentary and were ~180 μm long, suggesting that the glass beads disassociated the sperm tail from the head at the neck.

Western blot analysis revealed that the anti-phosphotyrosine monoclonal antibody did not detect any proteins in the sperm flagellar extracts taken at 5 or 30 min of incubation (Fig. 3, lanes B and C). In contrast, after 1-h incubation, the antibody recognized two proteins of 90 and 80 kDa (Fig. 3, lane D). Furthermore, after a 3.5-h incubation, sperm were hyperactivated, and four proteins of 90, 80, 62, and 48 kDa cross-reactive to the antibody were observed (Fig. 3, lane F). The proteins were specifically phosphorylated on tyrosine residues, since preimmunoabsorption of the antibody to *O*-phospho-DL-tyrosine completely abolished immunoreactivity (data not shown). These results indicate that tyrosine phosphorylation of hamster flagellar proteins occurred in a time-dependent manner during hyperactivation.

Effects of dbcAMP, Calyculin A, and Okadaic Acid on Sperm Hyperactivation and Protein Tyrosine Phosphorylation

As demonstrated above, tyrosine phosphorylation of a number of flagellar proteins was associated with sperm hyperactivation. To determine which phosphoproteins might be important for hyperactivation, our first aim was to decrease the incubation time required for hyperactivation by treating sperm with cell-permeant cAMP analogue, dbcAMP, or with the protein phosphatase inhibitors, calyculin A or okadaic acid. As shown in Figure 4, under the modified TALP medium alone, 3–4 h of incubation was required to produce hyperactivated motility. However, in the same medium supplemented with 5 mM dbcAMP, the movement characteristics typical of hyperactivated sperm motion, as determined by videotape analysis, were observed in the majority of the sperm (≥ 80%) within 90 min (Table 1). Alternatively, in the presence of 2 μM calyculin A or 5 μM okadaic acid, over 80% of sperm had become fully hyperactivated within 40 min (Fig. 4 and Table 1). Apparently, protein phosphatase inhibitors, supposed to inhibit protein dephosphorylation, had a stronger effect on accelerating sperm hyperactivation than had dbcAMP.

When the calyculin A- or okadaic acid-treated sperm flagella were subjected to Western blot analysis with anti-phosphotyrosine antibody, a single 80-kDa protein was tyrosine phosphorylated (Fig. 5, lane B). In contrast, in the control flagella without calyculin A or okadaic acid treatment, no proteins cross-reactive to the antibody were observed (Fig. 5, lane A). These results indicate that preferential phosphorylation of an 80-kDa protein occurred when hyperactivation was promoted by calyculin A.

Effect of PKA and PTK Inhibitors on Sperm Hyperactivation and Protein Tyrosine Phosphorylation

On the other hand, under conditions conducive to hyperactivation, when sperm were coincubated with 1 mM PKA inhibitor, H8, or 1 mM PTK inhibitor, tyrphostin 47,

TABLE 1. Comparison of motility parameters of hyperactivated sperm treated with dbcAMP, calyculin A, or okadaic acid with that of control sperm (mean ± SD, n ≥ 8).*

	Treatment			
Parameter	Control	dbcAMP	Calyculin A	Okadaic acid
Beat frequency (Hz)	7.9 ± 1.1	7.8 ± 1.2	8.2 ± 1.2	8.1 ± 1.2
VSL (μm/sec)	189.8 ± 20.4	177.6 ± 18.3	210.1 ± 22.2	198.2 ± 21.0
VCL (μm/sec)	747.8 ± 85.2	735.6 ± 70.3	767.2 ± 90.2	753.4 ± 81.2
MPB (rad)	1.6 ± 0.1	1.5 ± 0.1	1.6 ± 0.2	1.5 ± 0.2
MRB (rad)	1.7 ± 0.2	1.6 ± 0.3	1.7 ± 0.2	1.6 ± 0.2

* Control sperm were incubated for 3.5 h in modified TALP medium; dbcAMP, calyculin A, and okadaic acid-treated sperm were incubated for 90 or 40 min, respectively (temperature, 37°C).

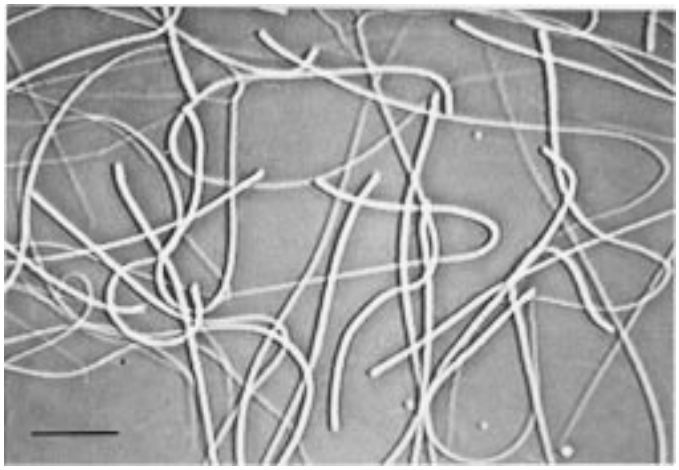

FIG. 2. Isolated hamster sperm flagella using glass bead and centrifugation method. Picture from video records. Bar = 20 μm.

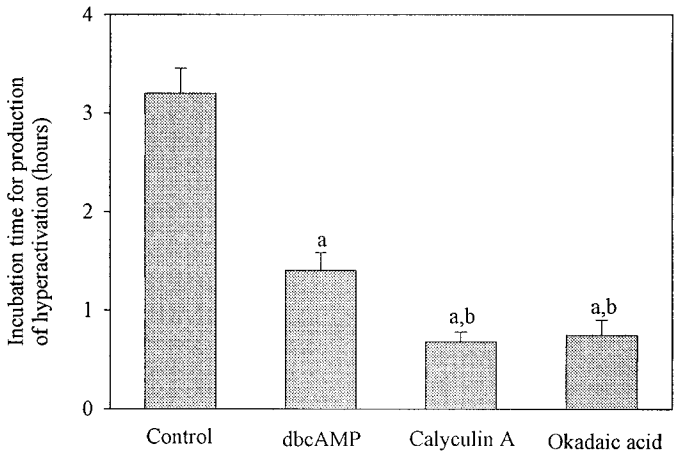

FIG. 4. Effects of dbcAMP, calyculin A, and okadaic acid on decrease of hyperactivation time in the hamster (mean ± SD, n = 6). Sperm were incubated in modified TALP medium alone (control) or supplemented with 5 mM dbcAMP, 2 μM calyculin A, or 5 μM okadaic acid. In all treatments, the percentage hyperactivated sperm was over 80%. [a]Significantly different from control sperm. [b]Significantly different from sperm treated with dbcAMP.

they displayed motility similar to that of the control for the first hour of incubation. However, after 3.5 h of incubation, the majority of the inhibitor-treated sperm lost their vigorous motility and were almost immotile as shown in Table 2. On Western blots of the H8- and tryphostin 47-treated sperm, no proteins cross-reactive to phosphotyrosine antibody were observed (Fig. 5, lanes C and D), indicating that both H8 and tryphostin 47 markedly prevented protein tyrosine phosphorylation. Thus, in contrast to the enhancing effect of calyculin A on sperm hyperactivation and tyrosine phosphorylation, PKA and PTK inhibitors had the opposite effects.

Localization of the Tyrosine-Phosphorylated Proteins in Sperm Flagella

In addition to the axoneme, the flagellum of mammalian sperm contains some accessory components such as outer dense fibers (ODF) extending from the connecting piece of the flagellum, the mitochondrial sheaths (MS) in the middle piece, and the fibrous sheath (FS) in the principal piece [32, 33]. To localize the proteins phosphorylated during hyperactivation, we examined MS-free flagella [34] and ODF (Fig. 6). Western blots of extracts from MS-free flagella (lane B) showed the same tyrosine-phosphorylated protein band as found in the extracts of demembranated flagella (lane A). On the other hand, no proteins cross-reactive to the antibody were observed on the Western blot of ODF (lane C). These results indicate that the prominent tyrosine-phosphorylated proteins of the flagellum are not components of the MS or ODF.

Furthermore, indirect immunofluorescence was used to examine the distribution of these proteins in methanol-fixed hyperactivated sperm; this revealed strong labeling just in the principal piece where the FS is exclusively located (Fig. 7, C and D). In contrast, no labeling was seen in non-

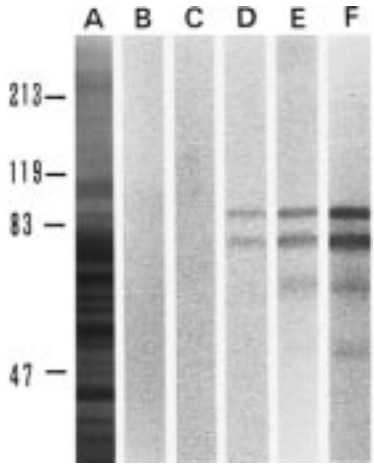

FIG. 3. Time course of protein tyrosine phosphorylation in caudal epididymal hamster sperm. **A**) Coomassie blue-stained gel of demembranated flagella. Sperm were incubated in modified TALP medium supporting capacitation. At 5 min (lane B), 30 min (lane C), 1 h (lane D), 2 h (lane E), and 3.5 h (lane F) of incubation, a 10-ml aliquot was taken, and sperm were demembranated and washed with PBS as described in *Materials and Methods.* Equal numbers of detergent-insoluble sperm flagella were fractionated by SDS-PAGE and probed with anti-phosphotyrosine antibody. Molecular weight standards (× 10^{-3}) are indicated to the left.

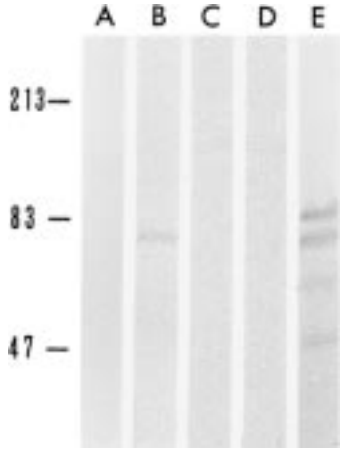

FIG. 5. Effects of calyculin A, H8, and tyrphostin 47 on the appearance of phosphotyrosine-containing proteins in caudal epididymal hamster sperm. Sperm were incubated for 40 min in the absence (**A**) and presence (**B**) of 2 μM calyculin A. Sperm were incubated for 3.5 h in modified TALP medium alone (**E**) or in the presence of 1 mM H8 (**C**) or 1 mM tyrphostin 47 (**D**). Equal numbers of detergent-insoluble sperm flagella were solubilized for SDS-PAGE and immunoblotting with anti-phosphotyrosine antibody. Molecular weight standards (× 10^{-3}) are indicated to the left.

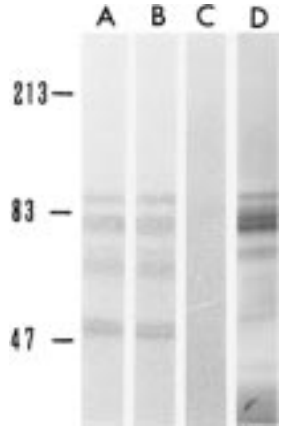

FIG. 6. Immunoblot analysis of phosphotyrosine-containing proteins in hamster sperm. **A**) Whole sperm flagellar extracts. **B**) MS-free flagellar extracts. **C**) Isolated ODF. **D**) Coomassie blue-stained gel of isolated ODF. Molecular weight standards ($\times 10^{-3}$) are indicated to the left.

hyperactivated (Fig. 7B) or control sperm incubated with secondary antibody alone (Fig. 7A). Together with the immunoblot results, these data suggest that the tyrosine-phosphorylated proteins are FS components.

TABLE 2. Effects of PKA and PTK inhibitors on sperm motility parameters (mean ± SD, n ≥ 8).*

	Treatment		
Parameter	Control	H8	Tyrphostin 47
Beat frequency (Hz)	7.9 ± 1.1	2.7 ± 0.6	3.1 ± 0.5
VSL (μm/sec)	189.8 ± 20.4	40.7 ± 5.2	36.2 ± 4.7
VCL (μm/sec)	747.8 ± 85.2	71.5 ± 9.1	67.2 ± 7.4

* Sperm were incubated for 3.5 h in modified TALP medium alone as a control or supplemented with 1 mM H8 or tyrphostin 47 (temperature, 37°C).

DISCUSSION

Physiological studies on sperm hyperactivation have been carried out in a variety of mammals including livestock, rodents, and primates. Extracellular factors such as Ca^{2+}, HCO_3^-, BSA, and metabolic substrates have been found to be essential for the initiation and maintenance of normal hyperactivated motility [14, 16]. However, very little is known of the intracellular biochemical pathways that regulate sperm hyperactivation.

The results presented in this study demonstrate that 1) hamster sperm hyperactivation was significantly accelerated by dbcAMP, phosphatase inhibitors, calyculin A, and oka-

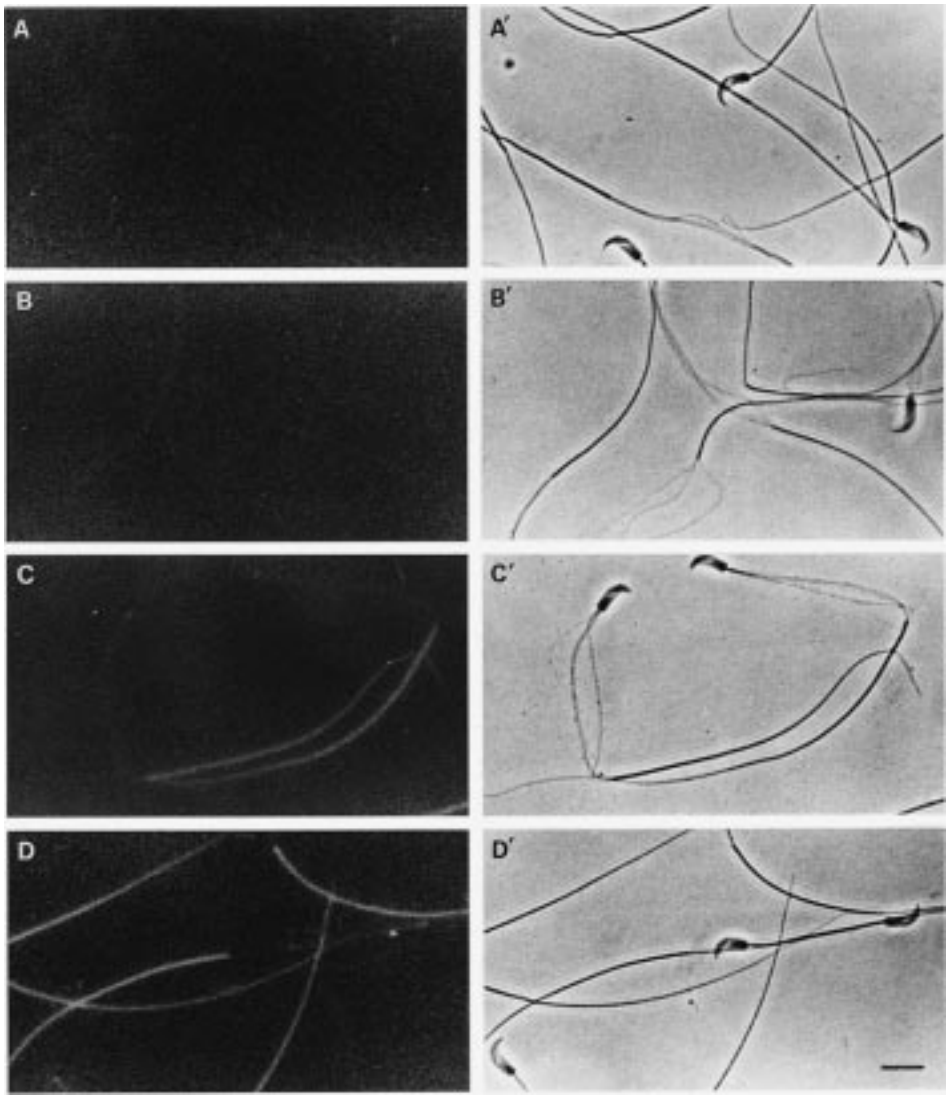

FIG. 7. Immunofluorescent localization of phosphotyrosine-containing proteins in hamster sperm. **A**) Hyperactivated sperm were reacted with secondary antibody alone as a control. At 40 min of incubation in the absence (**B**) and presence of calyculin A (**C**), and 3.5 h of incubation in the modified TALP medium alone (**D**), sperm were reacted with anti-phosphotyrosine monoclonal antibody. Panels on the right are phase-contrast images that correspond to panels on the left. Bar = 20 μm.

daic acid, but was inhibited by PKA inhibitor H8 and PTK inhibitor tyrphostin 47; 2) protein tyrosine phosphorylation was enhanced by calyculin A, but blocked by H8 or tyrphostin 47. These results suggest that increased protein tyrosine phosphorylation is associated with sperm motility hyperactivation.

It is well established that cAMP-dependent phosphorylation via PKA is critical for initiation of sperm motility [35]. The PKA, consisting of two regulatory subunits and two catalytic subunits, has been identified in mammalian sperm [36]. Demembranated, ATP-reactivated sperm motility was activated by the exogenous catalytic subunits of PKA in the absence of cAMP [37] but inhibited by the PKA inhibitor H8 [38]. On the other hand, parallel with the studies on PKA, serine/threonine protein phosphatases have been also identified in mammalian sperm flagella [39–41]. The addition of exogenous protein phosphatase inhibited demembranated, ATP-reactivated sperm movement and protein phosphorylation [42, 43]. However, treatment of live sperm with protein phosphatase 1 and 2A inhibitors, calyculin A or okadaic acid, initiated motility of hamster epididymal sperm (unpublished results) and promoted both capacitation and motility of primate sperm [9, 11, 41]. Thus, a balance of competition between protein phosphorylation by PKA and dephosphorylation by protein phosphatases may regulate sperm functions.

Besides PKA/protein phosphatase, PTK/protein tyrosine phosphatase also exists in sperm cells [44, 45]. Moreover, there is increasing evidence of cross-talk between intracellular PKA and PTK pathways. In rainbow trout sperm, the initiation of sperm motility is concomitant with cAMP-dependent phosphorylation of a 15-kDa protein on tyrosine residues [46]. In *Ciona*, sperm motility is stimulated by cAMP but inhibited by a PTK inhibitor [47]. With regard to mammalian sperm, protein tyrosine phosphorylation and capacitation are regulated by cAMP/PKA signaling pathway [3–9]. In the simplest explanation, cAMP-dependent phosphorylation of PTK would increase its activity. This hypothesis can explain how the serine/threonine phosphatase inhibitor calyculin A enhances both protein tyrosine phosphorylation and sperm hyperactivation.

Our data indicate that when calyculin A promotes hyperactivation, an 80-kDa peptide of the hamster sperm flagellum is preferentially phosphorylated by PTK. However, in the absence of calyculin A, both 80- and 90-kDa peptides are phosphorylated long before hyperactivation is observed (Fig. 3, lane D). One possible explanation for this observation is that the 90-kDa phosphopeptide prevents the hyperactivating action of the 80-kDa phosphopeptide.

Immunofluorescent staining revealed that tyrosine-phosphorylated proteins are localized only to the principal piece of the flagellum. Together with the results from immunoblots, these findings suggest that these proteins are FS components. The FS is a major cytoskeletal structure in the principal piece of mammalian sperm flagella consisting of two longitudinal columns and numerous circumferential ribs [32]. There is evidence that the FS not only acts as a passive elastic element but may also be an active regulator of sperm flagellar movement. In demembranated mouse sperm, the FS slides proximally from the principal piece to the connecting piece as microtubules are extruded from the axoneme; both cAMP and ATP are indispensable for FS sliding [31, 33, 34]. The ATP could be supplied in the principal piece by glycolysis, since the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase was shown to be covalently linked to the sperm FS from a number of mammalian species [48, 49]. Furthermore, a major FS polypeptide of 82 kDa in mouse sperm has been identified as a novel A-kinase anchoring protein (AKAP) [50, 51], and two research groups reported that sperm type II PKA was localized to the FS by association with an AKAP [4, 50–52]. Recently, the interaction of sperm AKAP with PKA II regulatory subunits has been shown to be critical for bovine sperm motility [53]. When these observations are taken into consideration, along with the present experimental results, it is apparent that there is a potential relationship between FS and sperm flagellar motility.

In summary, a novel method was developed to fractionate the rodent sperm flagellum from the head. For the first time, tyrosine-phosphorylated proteins were identified in hyperactivated sperm flagella. Anti-phosphotyrosine immunoblotting and immunofluorescence data suggest that the phosphotyrosyl proteins are FS components. Tyrosine phosphorylation of flagellar proteins is highly correlated with sperm hyperactivation. It is likely that tyrosine phosphorylation of FS components leads to a decrease in the stiffness of FS and subsequently enables the flagella to generate greater bends leading to sperm hyperactivation.

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