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Sperm Motility Development in the Epididymis Is Associated with Decreased Glycogen Synthase Kinase-3 and Protein Phosphatase 1 Activity[1]

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ABSTRACT

Immotile bovine caput epididymal sperm contain levels of protein phosphatase activity twofold higher than do mature motile caudal sperm. Comparison of the inhibition profiles of endogenous phosphatase activities detected by okadaic acid (OA) and calyculin A (CA) revealed a pattern consistent with the predominance of a type 1 protein phosphatase (PP1). Immunoblot analysis identified $PP1\gamma_2$ (the testis-specific isoform of PP1) as the only PP1 isoform in sperm and showed little protein phosphatase 2A (PP2A). In addition, of the known PP1 inhibitors, i.e., DARPP-32, inhibitor 1 (I1), and inhibitor 2 (I2), only I2-like activity was detected in sperm. Inhibition of PP1 by the heat-stable I2-like activity purified from sperm could be reversed with purified glycogen synthase kinase-3 (GSK-3). Furthermore, sperm extracts contain an inactive complex of PP1 and I2 (termed PP1I) that could also be activated by purified GSK-3. The presence of GSK-3 in sperm was demonstrated by activation of purified PP1I, and quantitation revealed that immotile caput sperm contained sixfold higher GSK-3 activity than motile caudal sperm. Immunoblot analysis confirmed the expression of GSK-3 in sperm and revealed the occurrence of both the α and β isoforms.

Our findings suggest that the higher PP1 activity measured in immotile sperm, presumably due to higher GSK-3 activity, is responsible for holding motility in check. This conclusion was supported by the observation that the phosphatase inhibitors OA and CA, at micromolar and nanomolar levels, respectively, were able to induce motility in completely immotile bovine caput epididymal sperm and to stimulate the kinetic activity of mature caudal sperm. The intrasperm levels of cAMP, pH, and calcium were unaltered by treatment with these inhibitors. The results suggest a biochemical basis for the development and regulation of sperm motility and a possible physiological role for the PP1/I2/GSK-3 system.

INTRODUCTION

The biochemical mechanisms underlying sperm maturation and the development of motility remain largely undefined. Mammalian sperm acquire the capacity for motility and fertilization during epididymal transit. During this passage through the epididymis, changes are observed in the intrasperm levels of cAMP, pH, and calcium [1, 2], mediators known to regulate the kinetic activity of a variety of flagellated organisms, including prokaryotes, ciliated cells, and invertebrate and mammalian sperm. In addition, during epididymal maturation, sperm undergo changes in shape, metabolic patterns, enzyme activities, and chemical and physical properties of the plasma membrane [3–12]. The significance of these changes in relation to motility development is unclear. It is known that the potential for motility already exists in both immature testicular and epididymal sperm [13, 14], as evidenced by the ability of demembranated immature sperm, under appropriate conditions, to undergo motility activation.

The development of sperm motility in the epididymis is associated with an increase in intrasperm levels of cAMP in a number of species [1], and the motility of intact immature epididymal sperm can be induced in vitro by elevation of intracellular cAMP levels [15]. However, optimum intracellular calcium and pH are required in order for this increase in cAMP levels to be translated into motility. Changes in calcium and pH above or below their optimum ranges can stimulate or inhibit flagellar activity [16, 17]. The levels of these mediators are also associated with developmental changes in motile sperm in the female reproductive tract, where sperm undergo capacitation and hyperactivation before fertilization of the ovum [18]. Remarkably, these developmental changes that sperm undergo in the male epididymis and the female reproductive tract occur in the absence of transcriptional or translational activity, and they presumably involve reversible phosphorylation of key proteins regulating sperm kinetic activity and metabolism.

Sperm protein phosphorylation seems to be directly related to motility, and it is increased by agents that elevate sperm cAMP levels [19]. The importance of protein phosphorylation in sperm motility has also been inferred from studies with demembranated sperm model systems. Protein phosphatases isolated from other tissue sources inhibit the motility of demembranated sea urchin sperm when included in the reactivation media [20, 21]. It has been shown that bovine and sea urchin sperm extracts contain serine/

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threonine phosphatase activity, but the type of phosphatase expressed was not identified [22, 23]. More recently, calcineurin (PP2B) was identified in canine and sea urchin sperm and shown to modify cAMP-dependent protein phosphorylation and motility [24].

In somatic cells, the serine/threonine-specific protein phosphatases are involved in a wide range of cellular functions [25]. They can be classified into different types on the basis of their substrate specificity and responses to a defined set of inhibitors and activators. These criteria define four types of catalytic protein phosphatases: PP1, PP2A, PP2B, and PP2C [26, 27]. PP1 can be distinguished from the other types by its sensitivity to inhibition by two heat-stable inhibitors, I1 and I2. I1 activity is regulated by cAMP-dependent phosphorylation of a single threonine residue by protein kinase A (PKA) and by calcium/calmodulin-dependent dephosphorylation of the same residue by PP2B [25, 28]. PP1 can thus integrate and modify the actions of cAMP and calcium. I2 binds to the catalytic subunit of PP1 to form an inactive cytoplasmic form of the enzyme (PP1I) that can be converted to active PP1 by phosphorylation of the bound I2 by glycogen synthase kinase-3 (GSK-3) [28–31]. Dephosphorylated and dissociated I2 can bind and inactivate PP1 again. The physiological role of this unique activation/inactivation cycle of PP1 activity via I2 is not clear. Furthermore, several isoforms are known to exist for each type of protein phosphatase catalytic subunit. At least three different genes are known to encode mammalian PP1 isoforms; they are called PP1α, PP1β, and PP1γ [32–34]. Moreover, PP1γ can undergo alternative splicing to generate two proteins that differ at their extreme C-terminal ends. Whereas $PP1\gamma_1$ is expressed in most tissues, $PP1\gamma_2$ is expressed solely in the testis [32–34]. The present study was designed to investigate the role played by GSK-3 and PP1 in the control of sperm motility.

MATERIALS AND METHODS

Sperm Preparation

Bovine caput, corpus, and caudal epididymal sperm suspensions were prepared as previously described [35]. Twice-washed sperm were resuspended in caudal epididymal sperm diluent (CESD; 100 mM NaCl, 40 mM KCl, 20 mM Tris-HCl [pH 7.4]) containing 10 mM glucose and 5 mg/ml BSA for motility studies, or in an homogenization buffer for preparation of extracts for enzyme assays as described below.

Sperm Extracts and Phosphatase Assay

Sperm pellets were resuspended at 10^8 cells/ml of homogenization buffer (50 mM Tris-HCl [pH 7.0], 1 mM EDTA, 1 mM EGTA, supplemented with 10 mM benzamidine, 4 μg/ml leupeptin, 1 mM PMSF, and 0.1 mM *N*-tosyl-L-phenylalanine chloromethyl ketone; all ingredients from Sigma Chemical Co., St. Louis, MO) and sonicated with three 5-sec bursts of a Biosonik III (Bronwill Scientific, Rochester, NY) sonicator at maximum setting. After centrifugation at 16 000 × *g*, the supernatants (termed sperm extracts) were either stored at −20°C or further centrifuged at 100 000 × *g* for 30 min for preparation of soluble and membrane fractions. The 100 000 × *g* supernatants were used as a source of sperm phosphatase in all experiments except those represented in Figure 1, where the 16 000 × *g* supernatants were used.

Phosphatase activity was assayed by means of ^{32}P-labeled glycogen phosphorylase *a* (Gibco-BRL, Gaithersburg, MD). In a typical assay, 20 μl of diluted sperm extract was incubated for 10 min with 20 μl of ^{32}P-phosphorylase *a* (3 mg/ml) and 20 μl of assay buffer (20 mM imidazole-HCl [pH 7.6], 0.1 mM EDTA, 1 mg/ml BSA, 0.1% mercaptoethanol) at 30°C. The reaction was terminated with 180 μl of 20% trichloroacetic acid (TCA). The amount of radioactive phosphate released was quantitated in the TCA supernatants (200 μl) by scintillation counting. One unit of enzyme activity is defined as that amount that dephosphorylates 1 μmol phosphorylase *a* per minute in this standard assay. Okadiac acid and calyculin A (CA; LC Laboratories, Woburn, MA) were dissolved in dimethyl sulfoxide (DMSO) and stored at −20°C. Appropriate concentrations for the experiments were made by serial dilutions of the stock solutions. Control tubes without inhibitor received an equivalent amount of DMSO.

Assay for Heat-Stable Phosphatase Inhibitors

Sperm extracts were boiled for 30 min and centrifuged at 16 000 × *g* for 15 min. The supernatant was adjusted to 10% TCA and, after centrifugation, the precipitate was dispersed in 0.5 M Tris and dissolved by extensive dialysis against a buffer containing 5 mM Tris, 1 mM EDTA, and 1 mM EGTA. The dialysate was boiled for 10 min, and after centrifugation, the supernatant (termed the heat-stable sperm extract) was stored at −20°C and used as the source of endogenous PP1 inhibitory activity. Methods for assaying I1- and I2-type activities [36] were as follows. For the assay for I1-type activity, aliquots of the heat-stable extracts were preincubated for 15 min with Mg-ATP in the absence or presence of PKA and boiled, and their inhibitory activity against purified recombinant PP1γ was measured in the phosphatase assay described above. Heat-stable extract prepared from monkey brain striatum was used as a positive control, since it is known to be rich in I1 and the I1-like inhibitor DARPP-32 [37]. For the assay for I2-type activity, purified PP1 was inactivated by preincubation with heat-stable sperm extracts; purified GSK-3 was then added in the absence or presence of Mg-ATP for an additional 10 min, and phosphatase activity was assayed as described. In control experiments, the ability of Mg-ATP to antagonize the

inhibition of PP1 activity was not observed in the absence of added GSK-3.

GSK-3 Activity

Caput and caudal sperm extracts were prepared essentially as described above. The 16 000 × *g* supernatants were further centrifuged at 100 000 × *g*. The pellets (membrane fractions) were then resuspended in homogenization buffer by sonication, and the supernatants (cytosolic fractions) were further purified by Sephadex G-50 (Pharmacia, Piscataway, NJ) chromatography. GSK-3 was assayed enzymatically in both fractions by its ability to activate purified inactive PP1I [29]. Endogenous phosphatase activity present in the fractions was taken into account in the calculations of GSK-3 activity (1 unit of GSK-3 activity is defined as the amount of enzyme that increases the phosphorylase phosphatase activity of PP1I by 1 mU/ml). All experiments were performed with freshly prepared extracts adjusted to equal cell numbers. PP1I present in sperm extracts was assayed in an analogous fashion by means of GSK-3 purified from rabbit skeletal muscle [38]. The difference in phosphatase activity measured in the presence and absence of ATP was attributed to PP1I.

SDS-PAGE and Immunoblotting

SDS-PAGE was performed in 12% acrylamide slab gels using 50 μg of total protein per lane. Sperm tail fragments were prepared by sonication followed by centrifugation twice at 1200 × *g*. Head and midpiece fragments sedimented during this centrifugation, while the supernatant contained essentially pure tail fragments. After electrophoresis, proteins were electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH). Nonspecific sites on the filter were blocked with 3% nonfat dry milk in Tris buffered saline (TBS; 25 mM Tris-HCl [pH 7.4], 150 mM NaCl). The blots were washed twice for 15 min with TBS containing 0.1% Tween 20 (TTBS) and then incubated for 1–4 h with the antibodies (made in our laboratory) directed against PP1α, PP1γ, or PP1γ_2 (diluted 1:5000 in TTBS containing 0.5% BSA). The blots were washed twice with TTBS as above and incubated with the appropriate biotinylated secondary antibody for 1 h (ABC Kit, Vector Labs., Burlingame, CA). Subsequent detection procedures were performed according to the manufacturer's instructions. The production of PP1α- and PP1γ-specific antibodies has been described [34, 39]. Identical methods were used to generate specific antibodies against the unique C-terminal sequence of PP1γ_2 (VGSGLNPSIQKASNYRNNTVLYE).

Motility Analysis

Sperm motility was monitored with a computerized image analysis system (CASMA) whose performance characteristics have been previously described [40, 41]. Sperm were resuspended in CESD with 10 mM glucose and 5 mg/ml BSA at 5 × 10^7 cells/ml, and 4 μl of the suspension was placed on a bacterial counting chamber under a coverslip on a microscope stage maintained at 37°C. In some experiments, mature motile caudal sperm were first rendered submotile by incubation at room temperature. The coverslip and slide were precoated with polyglutamate-lysine to prevent sperm from sticking to glass [35]. Six fields of view were chosen at random for each slide and recorded on a videotape for subsequent analysis via the image analysis system. Thirty frames or 1/2 sec of the video sequence for each sample was analyzed. A wide range of parameters was studied, including flagellar beat parameters [40, 41]. We report here the results observed on percentage motility (defined as the number of sperm moving at velocity greater than 30 μm/sec) and average velocity (Va). Average path velocity expressed in μm/sec is the moving 5-point average of the coordinates of the center of the head [40]. Details of the flagellar analysis have been previously described [41]; here we report only the flagellar tracings of sperm movement through 30 frames. For head motion analysis, at least 200 sperm were examined per sample. For statistical comparisons, Student's *t*-test was used since all parameters selected for study were normally distributed. Differences were considered significant when $p < 0.05$.

RESULTS

Phosphorylase Phosphatase Activity in Epididymal Sperm Extracts

In preliminary experiments, we determined that approximately 60% of the total phosphorylase phosphatase activity in caput and caudal sperm extracts was present in the 100 000 × *g* supernatant fraction of sperm sonicates, and that caput sperm extracts consistently showed higher enzyme activity than caudal sperm. However, the phosphatase activity measured was found to increase with increasing dilution of the extracts in the assay even though the extracts were first passed through Sephadex G-50 to remove interfering molecules of small molecular weight. A similar nonlinear relationship of phosphatase-specific activity with respect to dilution has been documented for extracts from other tissues [42]. Therefore, all subsequent experiments were conducted with extracts derived from stock cell suspensions adjusted to equal sperm numbers. Figure 1 shows the comparison of phosphorylase phosphatase activity of caput and caudal soluble sperm extracts measured at two dilutions. Caput sperm extracts reproducibly contained about twofold higher enzyme activity than caudal sperm extracts.

PP1 and PP2A can be differentiated on the basis of their differing sensitivities to inhibition by okadaic acid (OA) and

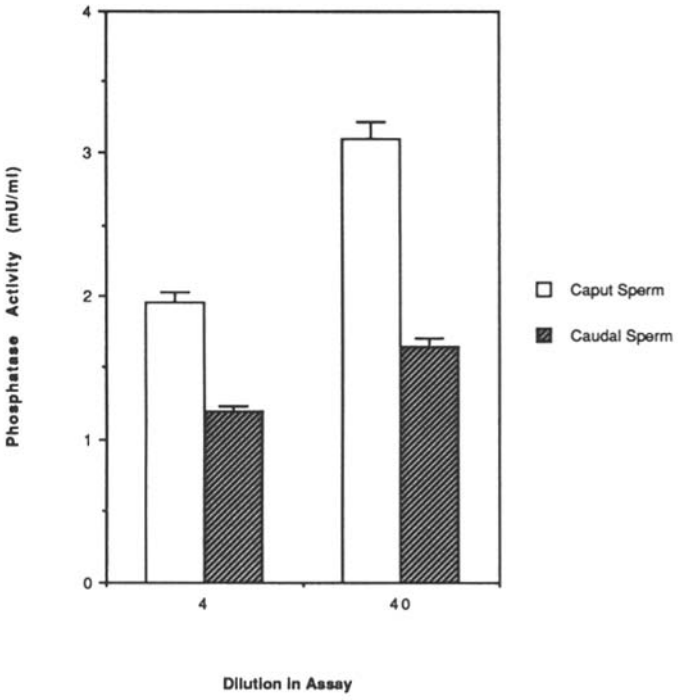

FIG. 1. Protein phosphatase activity in caput and caudal epididymal sperm. Caput and caudal sperm extracts (100 000 × *g* supernatant) were analyzed for phosphorylase phosphatase activity at the indicated dilutions, as described in *Materials and Methods*. The values are the means ± SEM of six experiments. Each experiment was conducted in duplicate with sperm extracts pooled from three different testes (the stock sperm suspension was adjusted to 1 × 10^9 cells before preparation of extracts). Data represent phosphatase activity in mU/ml of undiluted extract.

calyculin A (CA). The OA and CA inhibition profiles of endogenous sperm phosphorylase phosphatase activity are shown in Figure 2. The curves are essentially identical for caput and caudal epididymal sperm extracts and are also similar to the inhibition profiles determined for PP1 from a variety of other tissues [43, 44]. Negligible inhibition of endogenous phosphatase activity was observed at 1 nM OA, but virtually complete inhibition was obtained with 1 μM OA. In contrast, 1 nM CA completely inhibited sperm phosphatase activity in the assay. The 30–100-fold difference in the observed IC_{50} for OA and CA (Fig. 2) is consistent with the predominance of type 1 phosphatase activity in both caput and caudal sperm. This conclusion was strengthened by immunoblot analysis of sperm using antibodies specific for PP1α, PP1γ, PP1γ_2, and PP2A. Testis extracts were previously shown to contain PP1α, PP1γ_1, and PP1γ_2 [32–34]. However, sperm were found to express solely the PP1γ_2 isoform of PP1 (Fig. 3), whereas the other more commonly expressed PP1 isoforms, PP1α (not shown) and PP1γ_1 (Fig. 3), could not be detected. Weak immunoreactivity was detected with PP2A antibodies, indicating that this phosphatase is perhaps expressed at relatively low levels in sperm (Fig. 3). The immunoblot data are consistent with the OA and CA inhibition profile data (Fig. 2). Immunoblot (Fig. 3) and immunocytochemical (not shown) analysis of sperm

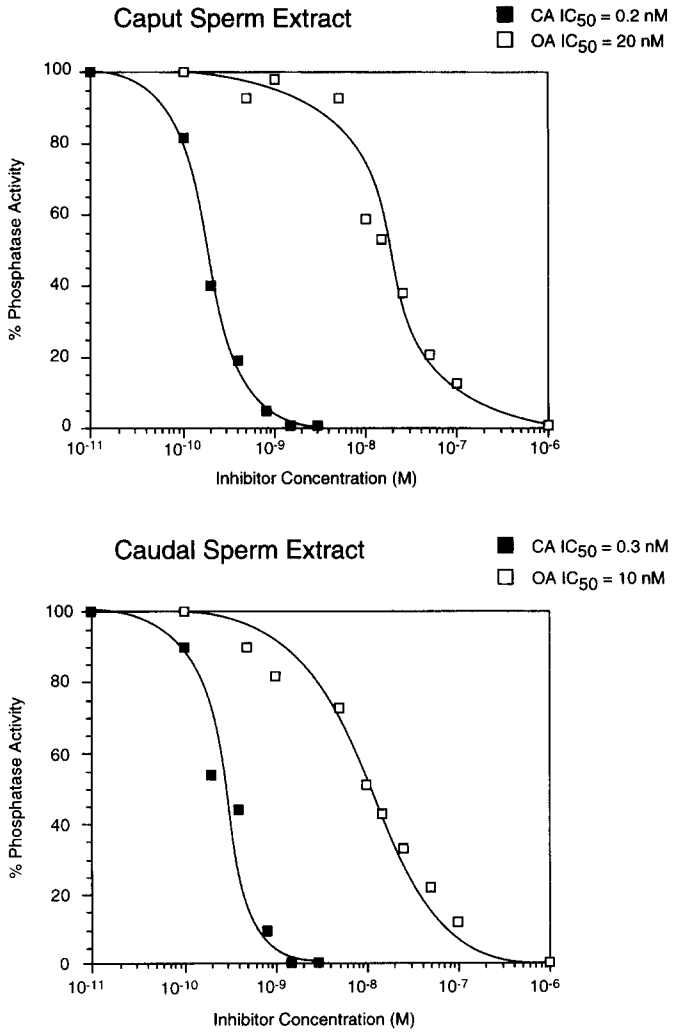

FIG. 2. Characterization of protein phosphatase activity present in bovine epididymal sperm extracts. Caput and caudal epididymal sperm extracts (16 000 × *g*) were prepared as described in *Materials and Methods* and diluted to give 0.1 mU of phosphorylase phosphatase activity in the assay. Phosphorylase phosphatase activity was measured in the presence of the indicated doses of OA and CA. The results (expressed as percentage of control) are the means of four separate determinations in two independent experiments.

with PP1γ_2-specific antibodies showed immunoreaction product in the tail and in the equatorial and acrosomal head regions of sperm.

Heat-Stable Inhibitors of PP1 in Sperm

The total amount of immunoreactive PP1 did not appear to differ significantly between caput and caudal sperm (Fig. 3), suggesting that the observed difference in phosphatase activity between caput and caudal sperm might be due to different PP1 activity states. Therefore, heat-stable extracts were prepared from caput and caudal sperm and assayed for I1/DARPP-32- and I2-type activity. These extracts were capable of inhibiting purified PP1 in a dose-dependent manner, and their inhibitory potency was unaffected by

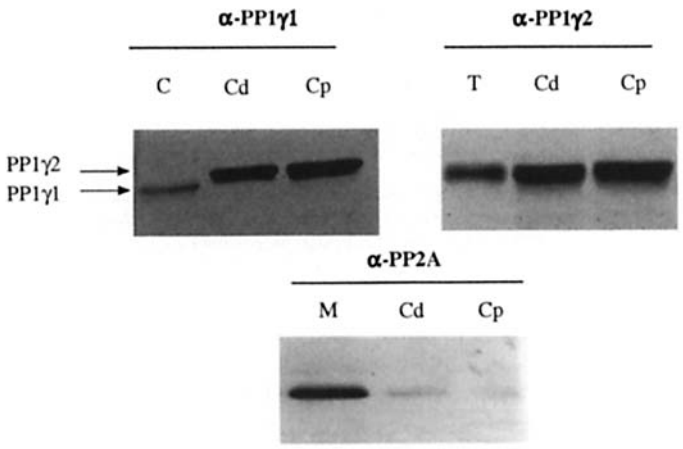

FIG. 3. Immunoblot analysis of sperm extracts probed with antibodies against PP1. Protein extracts (50 μg) were subjected to SDS-PAGE followed by immunoblotting. Blots were probed with a PP1γ-specific antibody capable of detecting both $PP1\gamma_1$ and $PP1\gamma_2$ (α-PP1γ), or with a $PP1\gamma_2$-specific antibody (α-PP1γ2), or with a PP2A-specific antibody (α-PP2A). C, control rhesus monkey striatal extract; Cd, bovine caudal sperm; Cp, bovine caput sperm; T, tail fragments from bovine caudal sperm; M, rhesus monkey myometrium. Molecular weights of PP1γ1, PP1γ2, and PP2A were 37, 39, and 34 kDa, respectively, determined by means of prestained protein standards.

PKA phosphorylation, indicating the absence of I1/DARPP-32-like activity from sperm (Table 1). In contrast, the inhibitory activity of control monkey brain striatal extracts was greatly enhanced by PKA phosphorylation (22% inhibition before phosphorylation compared to greater than 95% inhibition after phosphorylation), consistent with the high levels of I1 and DARPP-32 known to be present in this tissue. Moreover, whereas the inhibitory activity of the phosphorylated striatal extract could be reversed by incubation with PP2A, the inhibitory activity present in sperm extracts was unaffected by PP2A pretreatment (data not shown). These results further indicated that the PP1 inhibitory activity present in sperm is not of the I1/DARPP-32 type, suggesting rather that it might be of the I2 type. In support of this possibility, the in vitro inactivation of purified PP1 by the heat-stable sperm extracts could be antagonized by purified GSK-3 (Table 2), confirming the I2-type nature of sperm endogenous PP1 inhibitory activity.

TABLE 1. Absence of I1-like activity from sperm.*

Extract		PP1 activity (mU × 10) − PKA	+ PKA
None (Control)		0.86	0.89
+ Caudal Sperm	1 μl	0.99	0.86
	2 μl	0.73	0.75
	3 μl	0.64	0.67
	5 μl	0.38	0.36
+ Caput Sperm	1 μl	0.86	0.86
	2 μl	0.75	0.70
	3 μl	0.67	0.63
	5 μl	0.55	0.57
+ Striatum	2 μl	0.72	0.04

*Phosphatase activity of recombinant $PP1\gamma$ was measured in the presence and absence of inhibitors as described in *Materials and Methods*. Values are means of two determinations. Data shown here are representative of 2 to 5 similar experiments.

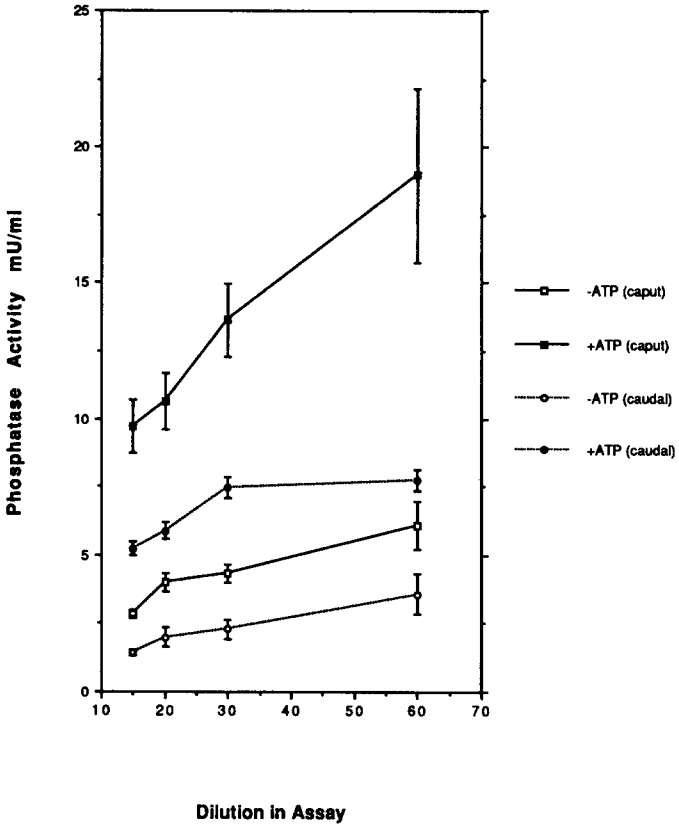

FIG. 4. ATP-dependent protein phosphatase activity in sperm extracts. Caput and caudal sperm extracts (100 000 × *g*; prepared from 1×10^9 cells/ml) were preincubated at the indicated dilutions for 10 min at 30°C with or without 50 μM Mg-ATP. Phosphorylase phosphatase activity was measured at the end of this preincubation. Values are means ± SEM from three different experiments, each conducted in duplicate.

GSK-3 Activity in Sperm Extracts

The detection of I2-like inhibitory activity in sperm prompted us to investigate the expression of a GSK-3-like kinase activity (the only known physiological regulator of I2). The inactive PP1-I2 complex is activated by ATP-dependent phosphorylation of I2 catalyzed by GSK-3. Preincubation of either caput or caudal sperm extracts in the

TABLE 2. I2-like activity in sperm.*

Extract	Phosphatase activity (% of control) − GSK-3	− GSK-3 (+ ATP)	+ GSK-3	+ GSK-3 (+ ATP)
None	100	100	100	100
2 μl	65	65	63	88
4 μl	49	49	44	79
6 μl	37	37	31	67
8 μl	30	30	27	63
10 μl	27	27	25	58

*Heat-stable sperm extracts were incubated with recombinant $PP1\gamma$ in the presence and absence of ATP and purified GSK-3. Values are the means of results from two experiments, each carried out in duplicate.

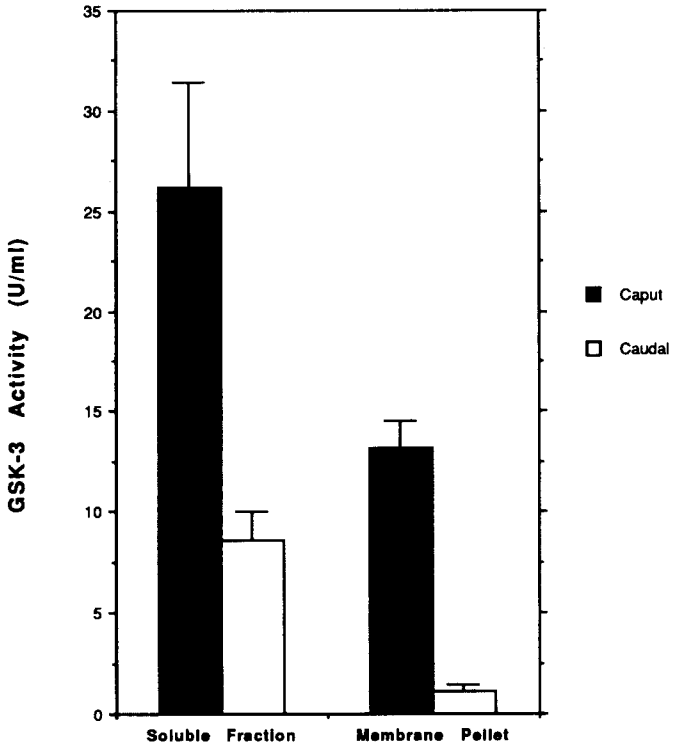

FIG. 5. Comparison of GSK-3 activity in caput and caudal sperm extracts. Caput and caudal sperm extracts (100 000 × *g*) were prepared and assayed for GSK-3 with purified PP1I as described in *Materials and Methods*. The values are the means of three different experiments, each conducted in duplicate.

presence of ATP resulted in an ATP-dependent activation of endogenous phosphatase activity (Fig. 4). In addition, quantitation of GSK-3 activity, measured by the ability of extracts

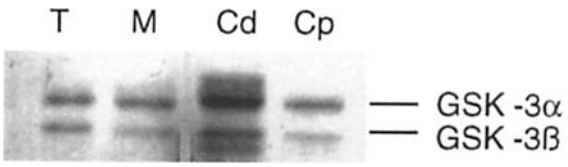

FIG. 6. Immunoblot analysis of sperm extracts probed with antibodies against GSK-3. The immunoblot was probed with a monoclonal antibody recognizing both GSK-3α and GSK-3β isoforms (49 and 51 kDa, respectively). T, bovine testis; M, myometrium; Cd, bovine caudal sperm; Cp, bovine caput sperm.

to activate purified PP1I, revealed considerably higher enzymatic activity in both the cytosolic and membrane fractions of caput sperm compared to caudal sperm (Fig. 5). Immunoblot analysis of sperm extracts probed with monoclonal antibodies recognizing the α and β isoforms of GSK-3 confirmed the expression of both isoforms in caput and caudal sperm (Fig. 6).

Effects of OA and CA on the Motility of Epididymal Sperm

Our findings suggested that the higher phosphatase activity observed in immotile caput sperm, presumably due to higher GSK-3 activity, might be responsible for holding their motility in check. To test this possibility, we investigated the effect of the known PP1 and PP2A inhibitors OA and CA on the motion parameters of bovine epididymal sperm. In preliminary experiments, immotile caput epididymal sperm were incubated with 1, 5, and 25 μM OA for 10 min and head motion analysis was performed. In an analysis of more than 200 sperm for each concentration point, maximal activation occurred at 5 μM OA, with lower values obtained at lower or higher concentrations of OA. When caput sperm

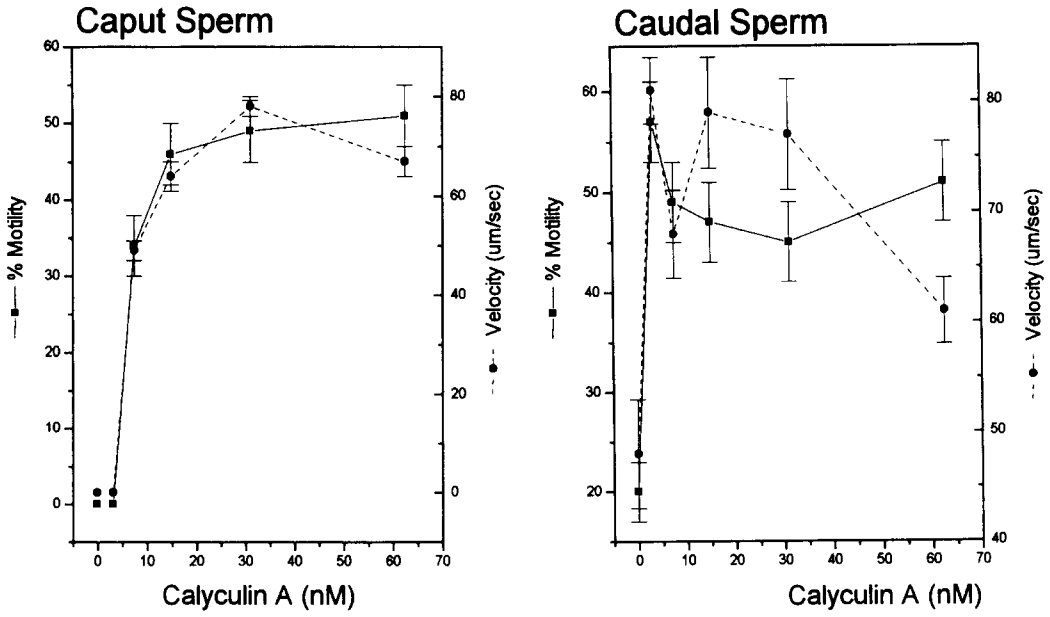

FIG. 7. Effect of CA on motility of caput and caudal sperm. Caput and caudal epididymal sperm (5×10^7 cells/ml) were prepared as described in *Materials and Methods*. Caudal epididymal sperm were rendered submotile [49, 50] by preincubation at room temperature for 60 min before treatment with CA. Motility was video-recorded 10 min later and analyzed by CASMA [39 40].

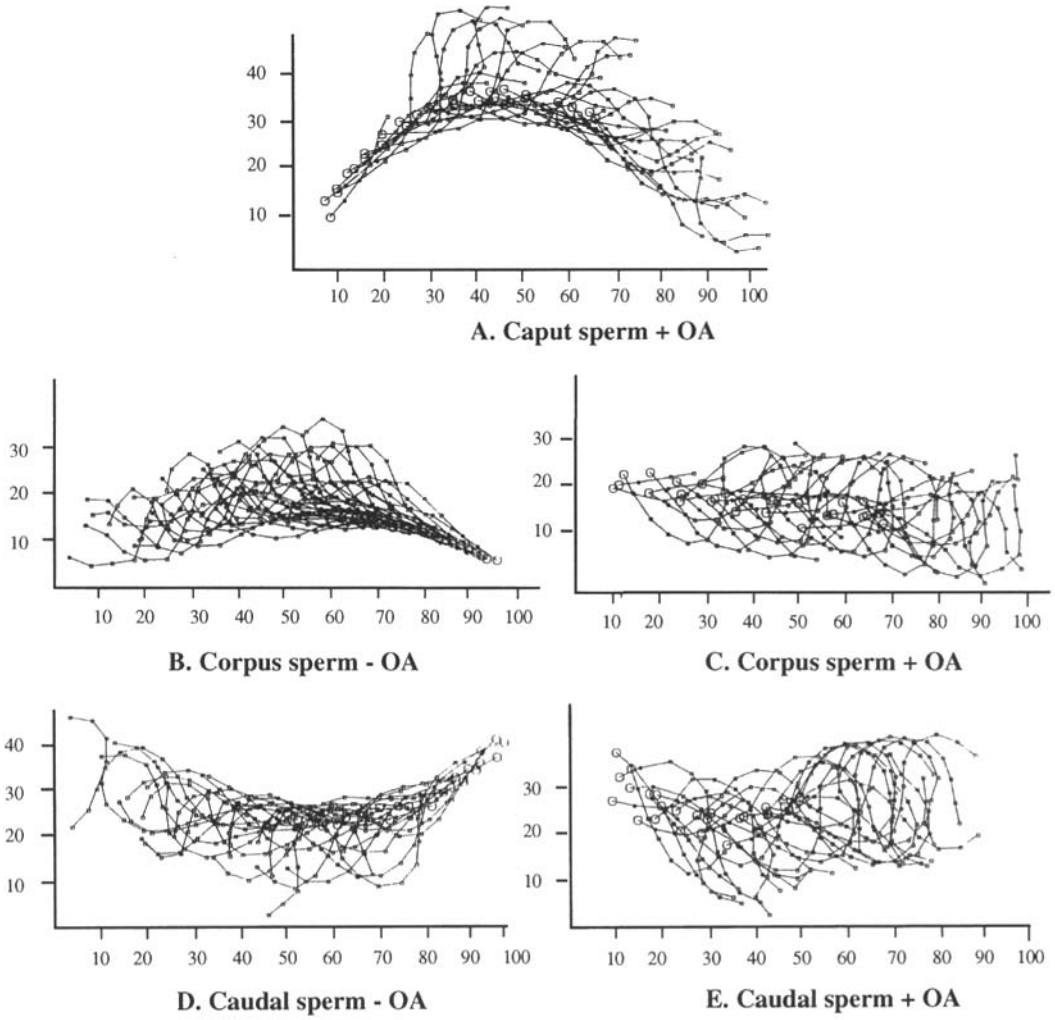

FIG. 8. Flagellar tracings of epididymal sperm treated with OA. Sperm were prepared from each epididymal section, and flagellar motility was determined as described in *Materials and Methods*; 5 μM OA in ethanol was added and the cells were incubated for 10 min before CASMA flagellar analysis. The tracings of representative sperm through 30 successive frames are shown here. The units of the axes are in micrometers. Data derived from analysis of 10–12 sperm showed that the beat amplitudes (A40 in μm) of caput (13.5 ± 0.8), corpus (15.8 ± 0.5), and caudal sperm (17.7 ± 0.7) were significantly different from control values (0, 12.5 ± 1.1, and 11.0 ± 0.8, respectively). Wavelength of beat was 29–31 μm and was not different in control and treated sperm.

were incubated with 5 μM OA for 0, 10, 20, or 30 min and similarly analyzed, maximal stimulation of motility was observed with the 10-min treatment.

Quantitation of the effects of 5 μM OA treatment for 10 min on the motion parameters of caput, corpus, and caudal epididymal sperm is shown in Table 3. OA induced motility in 53% of the cells of completely immotile caput sperm, resulting in an average velocity of 79 μm/sec. Although less dramatic, owing to higher basal levels of motility and velocity, significant stimulation ($p < 0.05$) was also observed with caudal sperm. The effect on corpus epididymal sperm was intermediate. The motility effects of a 10-min treatment with varying concentrations of CA on caput and caudal epididymal sperm are shown in Figure 7. Remarkably, this phosphatase inhibitor induced motility in caput sperm at concentrations as low as 7 nM and stimulated the motility of submotile caudal sperm at 3.5 nM (maximal effects were observed with 20 nM CA). No significant changes in cAMP, pH, or intracellular calcium were associated with OA or CA treatment of sperm. One of the significant features of motility induction in caput and corpus sperm treated with OA was that their flagellar motion characteristics were similar to those of mature caudal sperm (Fig. 8).

DISCUSSION

The known dependence of sperm motility initiation on intracellular mediators such as Ca^{2+} and cAMP [1, 2] raised the possibility that such effects might be mediated via changes in the phosphorylation state of a target protein,

TABLE 3. Motility induction by OA in bovine epididymal sperm.*

Section	OA	% Motility	Velocity (Va)
Caput	−	0	0
Caput	+	53 ± 4	79 ± 3
Corpus	−	16 ± 3	56 ± 5
Corpus	+	74 ± 4	101 ± 3
Caudal	−	78 ± 3	81 ± 2
Caudal	+	90 ± 2	122 ± 4

* Sperm were prepared from each epididymal section indicated and flagellar motility was analyzed by CASMA. OA was added at a final concentration of 5 μM and the cells were incubated for 10 min at 37°C before analysis. Data are expressed as mean ± SEM. Both motility parameters in treated sperm were statistically significant compared to the respective values in control sperm.

since these second messengers are known modulators of kinase and phosphatase activities. Recent work on the serine/threonine-specific phosphatases has led to an increasing awareness of their importance in the control of a variety of cellular pathways [28] and prompted us to assess their possible role in the control of sperm motility. Thus, we assayed and compared the phosphorylase phosphatase activity levels present in bovine immotile caput and motile caudal epididymal sperm. Immotile caput sperm consistently demonstrated 2–3-fold higher basal and ATP-dependent phosphatase activity (Figs. 1 and 4). OA and CA were used to probe the nature of the endogenous sperm phosphatase, and the data indicated the possible involvement of a type 1 phosphatase (Fig. 2). Since immunoreactive PP1 was equivalent in caput and caudal sperm (Fig. 3), the observed difference in activities was presumably due to different PP1 activity states. The activity of GSK-3 was also found to be higher in caput compared to caudal sperm (Fig. 5). Although the basis for the observed differences in the activity of GSK-3, and therefore of PP1, in immotile and motile sperm remains to be fully characterized, it is possible that GSK-3 could be regulated by phosphorylation. Tyrosine phosphorylation of GSK-3 is required for its activity in somatic cells, and changes in GSK-3 phosphorylation have been reported in response to insulin [45–47].

Our demonstration of the expression of PP1γ_2, the heat-stable PP1 inhibitor I2, and its modulating GSK-3 activity in sperm raised the possibility that regulation of the endogenous PP1 activity via I2 and GSK-3 might represent a mechanism for regulation of sperm motility. Sperm kinetic activity can be reversibly stimulated or inhibited by alterations in calcium and cAMP levels [1]. Calcium and cAMP can affect PP1 activity by modulating the phosphorylation or dephosphorylation of I1 and DARPP-32 [25, 28]. However, since sperm do not express either of these proteins, this pathway is unlikely to play a role in the regulation of sperm motility. A possible alternative is that such intracellular mediators might regulate sperm GSK-3 activity, which in turn can regulate the phosphorylation state of I2 and therefore control the level of active PP1 in sperm. It seems likely that the interconversion between inactive PP1I and active PP1 is physiologically relevant in the regulation of sperm function. It will be of considerable importance to establish the mechanisms regulating GSK-3 and PP1 activity during transit through the male epididymis and the female reproductive tract. Efforts are now underway to purify the endogenous GSK-3 activity in sperm and characterize its regulation. Sperm are a relatively simple model system in which to study the regulation of cytosolic PP1I in relation to motility and metabolism. These terminally differentiated cells are essentially devoid of transcriptional and translational activity. Thus, endogenous regulation of protein phosphorylation and motility by PP1 could represent an important mechanism for physiological regulation of a cell that encounters drastically different environments as it journeys through the seminiferous tubules and the female reproductive tract.

Given the different levels of PP1 activity in caput and caudal sperm, we postulated that if the activity of this enzyme was responsible for the control of sperm motility, then treatment of immotile caput sperm with known inhibitors of PP1 should result in stimulation of motility. Indeed, potent stimulation of motility (Table 3 and Fig. 7) was obtained with OA and CA at concentrations comparable to those required for inhibition of sperm extract phosphatase activity (Fig. 2). The most dramatic effect of these inhibitors was seen with caput immotile sperm (Table 3 and Fig. 7). Virtually motionless caput sperm treated with these inhibitors acquired movement characteristics similar to those of mature sperm. The effect of the inhibitors on mature sperm that were already motile was most apparent on the velocity parameters of motion (Table 3), although the inhibitors potently stimulated both the percentage motility and the velocity of submotile caudal sperm (Fig. 7). Results similar to those presented here for bovine sperm were also obtained with motile ejaculated monkey and human sperm [48]. Significantly, stimulation of motility by this means completely bypasses the requirement for elevation of cAMP and pH, conditions previously thought to be essential for induction of motility in caput sperm [49, 50]. These data suggest that high phosphatase activity could be responsible for limiting motility in immature sperm. These suggestions are consistent with our observations that immotile sperm extracts have at least twofold higher PP1 activity than motile sperm. In motile sperm, endogenous phosphatase activity is presumably already partially inhibited by endogenous mechanisms, and further pharmacologic inhibition of activity leads to the observed hyperactivity in these cells. Our conclusions are further supported by a recent report documenting that the avian-specific high temperature-dependent inhibition of motility of demembranated fowl spermatozoa could be reversed by inhibitors of PP1 [51]. OA and CA are also capable of significantly enhancing capacitation and agonist-induced acrosome reaction in ejaculated human sperm [52], processes associated with the onset of hyperactivity of sperm flagellar motion in several species [18].

We have demonstrated the presence of the serine/threonine-specific phosphatase PP1γ_2 in sperm. The OA and CA inhibition profiles of endogenous phosphatase activity are virtually identical to those observed for PP1 isolated from other tissue sources [43, 44]. Further, the observed differences in potencies of OA and CA as inducers of sperm motility (Table 3 and Fig. 7) are also consistent with the target phosphatase being type 1. However, we cannot definitively rule out the participation of other phosphatase types, such as PP2A, in the motility effects observed in the presence of the phosphatase inhibitors. The absence of an I1-like inhibitory activity but presence of an I2-like inhibitory activity in sperm extracts contrasts with the situation in most tissues, which express both I1 and I2 [28]. The physiological basis for the control of PP1 activity via I2 and GSK-3 remains to be elucidated [28, 31]. The well-established roles of PP1 and GSK-3 in the control of glycogen metabolism [28] are unlikely to be relevant in sperm, since mammalian sperm do not contain glycogen or glycogen-metabolizing enzymes [1]. Instead, sperm depend on exogenous glucose, fructose, lactate, or pyruvate as their energy source [28].

In summary, this report provides a possible biochemical basis for the regulation of sperm motility during epididymal sperm maturation, through the operation of the novel PP1/I2/GSK-3 system. We have found that this system also operates in human and rhesus monkey sperm [48]. Preliminary studies have shown that PP1α, I2, and GSK-3 activity are present in sea urchin sperm, suggesting that this phosphatase system could be a universal mechanism regulating protein phosphorylation and sperm function. Studies are in progress that are directed toward establishing the chemical basis for the regulation of the enzymes PPγ2 and GSK-3 in sperm during development in the testis, in mature sperm, and in sperm during maturation in the female reproductive tract.

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