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Primate Sperm Contain Protein Phosphatase 1, a Biochemical Mediator of Motility[1]

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ABSTRACT

Sperm motility initiation, capacitation, and hyperactivation are modulated by an interplay of intracellular Ca^{2+}, cAMP, and pH. Mechanisms by which these mediators alter sperm function have not been elucidated but may involve reversible alterations in regulatory protein phosphorylation. Studies were designed 1) to investigate the influence of the protein phosphatase (PP) inhibitor calyculin A (CA) on human sperm motility and 2) to characterize the CA-sensitive PP and its endogenous regulators in human and rhesus monkey sperm. CA (50 nM) treatment of human sperm resulted in an increase in percentage motility and an acceleration in mean path velocity. Inhibition of either protein phosphatase-1 (PP1) or protein phosphatase-2A (PP2A) could be responsible for this motility stimulation, since both of these phosphatases are sensitive to nanomolar quantities of CA. PP activity in human (n = 4) and rhesus monkey (n = 4) sperm sonicates was measured using [^{32}P]-phosphorylase-*a*, the preferred substrate for PP1 and PP2A, in the absence of divalent cations. Human ($6.2 \pm 4.5 \times 10^{-2}$ mU/10^6 sperm) and monkey ($4.3 \pm 0.8 \times 10^{-2}$ mU/10^6 sperm) sonicates contained activity tentatively identified as PP1 on the basis of inhibition profiles in okadaic acid (OA) and CA. Western blot analysis with antibodies against various isoforms of PP1 subsequently documented the presence of $PP1_{\gamma 2}$ in human and monkey sperm. PP1 activity in most tissues is regulated by the heat-stable inhibitors I1 or I2. Sperm sonicates contained inhibitor activity similar to I2 as well as glycogen synthase kinase-3 (GSK-3) activity, which is involved in the activation of the PP1-I2 complex. These results indicate, for the first time, that human and rhesus monkey sperm contain PP1 and regulators of PP1 and that inhibition of PP1 activity by CA can enhance motility.

INTRODUCTION

During the period following spermiation, yet preceding interaction with the cumulus-oocyte complex, morphologically mature sperm undergo motility initiation [1–3], capacitation [4–6], and, in many species, hyperactivation [7]. Intracellular Ca^{2+}, cAMP, and pH have all been identified as biochemical mediators of motility and fertility [7], yet the mechanisms by which these mediators influence sperm function have not been delineated. In addition, all the sperm alterations mentioned can be mimicked in vitro with membrane-permeable cAMP analogues and/or phosphodiesterase inhibitors [8–10]. Since motility initiation, capacitation, and hyperactivation occur when sperm are relatively void of transcription and translation [3], it is likely that these processes are controlled by an alteration in the phosphorylated state of latent proteins.

The reversible phosphorylation of proteins is now recognized as a major intracellular control mechanism involved in a wide range of eukaryotic cellular responses [11]. Serine/threonine protein phosphatases (PPs) stimulate dephosphorylation of phosphoproteins by phosphate group hydrolysis and thus oppose the actions of protein kinases. Classification of serine/threonine PPs is based on substrate specificity and response to a defined set of inhibitors and activators [12]. Type 1 PP (PP1) is sensitive to the heat- and acid-stable inhibitors 1 and 2 (I1 and I2), whereas type 2 PPs (PP2s) are insensitive to I1 and I2 [13]. Inactivation of PP1 by I1 (PP1-I1) is dependent on I1 phosphorylation by cAMP-dependent protein kinase [14], whereas I2 does not require phosphorylation for PP1 inhibition [15]. The inactive PP1-I2 complex is activated by dissociation of PP1 and I2, a reaction controlled by glycogen synthase kinase-3 (GSK-3) phosphorylation of I2 [16, 17]. Type 2 PPs can be subclassified into PP2A, PP2B, and PP2C on the basis of cation requirements. PP1 and PP2A do not require divalent cations for activity whereas PP2B and PP2C require Ca^{2+}/calmodulin and Mg^{+}, respectively, for activity [18]. Several isoforms are known to exist for each PP type. For example, the catalytic subunit of PP1 can be encoded by three different genes, termed PP1α, PP1β, and PP1γ [19, 20]. PP1γ can undergo alternative splicing generating two proteins that differ at their extreme C-terminal ends. $PP1\gamma_1$ is ubiquitously expressed in most tissues, whereas $PP1\gamma_2$ is expressed solely in the testis [19, 21].

Okadaic acid (OA) and calyculin A (CA) are potent, specific, cell-permeable, pharmacological phosphatase inhibitors [22, 23]. PP1 and PP2A are inhibited completely at approximately 1.0 nM CA. However, PP1 is 100-fold less sensitive to OA inhibition, while PP2A has a similar sensitivity to OA and CA. PP2B and PP2C are essentially insensitive to OA and CA [23, 24]. This distinguishing sensitivity

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of PP1 and PP2s to exogenous inhibitors is highly conserved in several systems, including plants and yeast [12, 24].

Sea urchin [25, 26], bovine [27], caprine [28], canine, porcine [26], and human [29] sperm extracts contain PP activity. Tash and colleagues [26, 29] further characterized the canine, porcine, and human sperm PP as PP2B and demonstrated its involvement with calcium-dependent regulation of flagellar motility (reviewed in [30]).

The objectives of the present study were to investigate the influence of the PP inhibitor CA on human sperm motility and to characterize the CA-sensitive PP and its endogenous regulators in human and rhesus monkey sperm.

MATERIALS AND METHODS

Semen Collection and Evaluation

Human semen samples utilized in these studies were collected by healthy adult men, after at least 48 h of abstinence, under evaluation at the Oregon Health Sciences University Andrology Laboratory. These samples had an average sperm concentration of 56.6×10^6 cells per milliliter (range: $33.8–72.0 \times 10^6$), average percentage motility of 53.7% (range: 45–65), and an average volume of 1.5 ml (range: 0.7–2.5). All samples were allowed to liquify at 37°C, washed, and processed for sperm sonicates, Western blot extracts, or motility assessment (described below) within 3 h of collection.

Rhesus monkey semen samples were collected via penile electroejaculation [31] from a population of males of proven fertility housed at the Oregon Regional Primate Research Center. These samples had an average sperm concentration of 62.6×10^7 cells per milliliter (range: $33.7–121.3 \times 10^7$), average percentage motility of 79.5% (range: 71–87), and an average volume of 0.4 ml (range: 0.2–0.9 ml). All samples were allowed to liquify at room temperature for approximately 10 min; this was followed by washing and sperm processing (described below).

After liquification, human and monkey semen samples were washed twice with 3.0 ml of either PBS, pH = 7.4 (for sperm sonicate and Western blot extract preparation), or human tubal fluid medium supplemented with HEPES [32] (HTF-HEPES; for motility assessment) by centrifugation at $350 \times g$ for 7 min at room temperature.

CA and Motility

To determine the optimal concentration of CA to be used in subsequent experiments, washed human sperm were resuspended in HTF-HEPES to 6×10^7 cells per milliliter, and 500 μl was placed into individual tubes containing no CA (control) or containing added CA to a final concentration of 10, 20, 50, or 100 nM. Four additional human sperm samples were treated with or without CA (final concentration 50 nM). Tubes were incubated at 37°C for 10 min before head motion parameters were analyzed with MacCASMA, a computer-assisted sperm motility analysis system (modified to run on a Macintosh computer), as previously described [33]. After treatment and incubation, 2 μl of sperm suspension was placed on a 20-μm-deep counting chamber (Petroff-Hausser, Horsham, PA), which had been pretreated with polyglutamate-lysine to prevent sperm adhesion, and was maintained at 37°C. After bulk fluid movement had subsided (30–60 sec), six video recordings of 10 sec each were taken at 6 different random locations on the slide (×875 magnification). Nonoverlapping video segments from each of the six recordings were analyzed to yield 6 replicate determinations for each treatment. The head motion parameters measured were percentage motility (%M; determined with path and progressive velocity thresholds at 14 μm/sec) and average path velocity (Va; determined as an eight-point moving geometric average of head centroid points).

Sperm Sonicates and Extracts

For evaluation of PP, inhibitor, and GSK-3 activity, sperm pellets were diluted with 0.5 ml of ice-cold homogenization solution [33] composed of 20 mM Tris, 2 mM EDTA, 2 mM EGTA (pH = 7.0), 0.1% 2-β-mercaptoethanol, 10 mM benzamidine, 1.0 mM PMSF, 0.1 mM *N*-tosyl-L-phenylalanine chloromethyl ketone, and 4.0 μg/ml leupeptin (all ingredients from Sigma Chemical Co., St. Louis, MO). The suspensions were then sonicated (four times, 5 sec each; 1-min intervals) at maximum setting with a Biosonik III (Bronwill Scientific, Rochester, NY) sonicator on ice. Sonicated suspensions were then centrifuged at $10\,000 \times g$ for 15 min at 4°C, and individual fresh supernatants were subjected to PP and GSK-3 assays. Pooled human and pooled monkey sperm sonicates were stored at −20°C, for no longer than 2 mo, until PP or GSK-3 assays were conducted. For evaluation of PP inhibitor, sonicated supernatants were placed in a 100°C water bath for 15 min, followed by cooling on ice and centrifugation at $10\,000 \times g$ for 5 min at room temperature. Heat-stable supernatants were stored at −20°C until assayed for PP inhibitor activity.

For Western blot analysis, washed human or monkey sperm pellets were resuspended in 0.5 ml of 2% SDS, 5% 2-β-mercaptoethanol, placed in a boiling water bath for 10 min, and subsequently centrifuged at $12\,000 \times g$ for 10 min at room temperature. The resulting supernatant was stored at −20°C until electrophoresis was performed.

Enzyme Assays

The PP assay system (Gibco/BRL, Gaithersburg, MD) employed radiolabeled phosphorylase-*a* as the substrate [34], which was prepared by incubation of phosphorylase-*b* (10 mg/ml), phosphorylase kinase, and 0.2 mM [γ^{32}P]-ATP (10^6 cpm/nmol) for 60 min at 30°C in buffer containing 100 nM

Tris-HCl, 100 mM sodium glycerol 1-phosphate (pH 8.2), 0.1 mM $CaCl_2$, and 10 mM magnesium acetate. [^{32}P]-Phosphorylase-*a* was precipitated by ammonium sulfate, dialyzed, and centrifuged as previously outlined [34, 35] before being resuspended at 50 mg/ml in 0.05 M Tris-HCl (pH 7.0) containing 0.1 mM EGTA, 0.1% 2-β-mercaptoethanol, and 15 mM caffeine and stored in aliquots at 4°C.

In the standard PP assay, duplicate 10-μl individual human sperm sonicates (n = 4) diluted 1:25 in assay buffer (20 mM imidazole-HCl, 0.1 mM EDTA, 0.1% 2-mercaptoethanol, and 1 mg/ml BSA; pH 7.6), 10-μl individual rhesus sperm sonicates (n = 4) diluted 1:50, or increasing volumes of similarly diluted pooled human and pooled monkey sperm sonicates were analyzed for PP activity. [^{32}P]-Phosphorylase-*a* (20 μl; ~1.0 × 10^6 cpm) and assay buffer (to bring final volume to 60 μl) were added, and the mixture was incubated at 30°C for 10 min. The reaction was terminated with the addition of 180 μl of ice-cold 20% trichloroacetic acid (TCA) on ice for 10 min; centrifugation at 10 000 × *g* was then performed for 3 min at 4°C. Radioactivity was determined in the TCA supernatants (200 μl) with a beta counter (Packard Instrument Co. Inc., Downers Grove, IL).

CA and OA (LC Labs, Woburn, MA) inhibition profiles were obtained by incubating pooled human or monkey sperm sonicates with either CA (final concentration 0.34–6.25 nM), OA (final concentration 0.5–1000 nM), or no inhibitor (control) for 10 min at 30°C. [^{32}P]-Phosphorylase-*a* was then added, and PP activity was assayed as described above.

To determine the presence of a PP inhibitor, samples (8 μl) of individual, boiled sperm sonicates were incubated in duplicate with 20 μl of diluted (1:25) pooled human sperm sonicate, as the PP source, for 10 min at 30°C before a standard PP assay was carried out. For the assessment of a concentration dependency, increasing volumes of undiluted (2–16 μl) heat-stable sonicates were incubated in duplicate with a PP source under the conditions specified above before measurement of PP activity. To characterize the PP inhibitor (I1 or I2) present in pooled human or monkey sperm, heat-stable sonicates (5 μl in duplicate) were incubated with 10 μM ATP (Sigma) with or without 1–2 mU protein kinase A (PKA; prepared in our laboratory) for 10 min at 30°C. Reactions were terminated in a 100°C water bath for 10 min and cooled to 30°C; a PP source was added before the PP assay was conducted.

GSK-3 activity was assessed indirectly by the extent of dissociation of PP1-I2, resulting in increased PP activity. Samples containing 10 μl of PP1-I2, generated in vitro by combining equimolar amounts of purified rabbit PP1 and I2 (prepared in our laboratory), were incubated in duplicate for 10 min at 30°C with either 10 μl purified GSK-3 (10 mU/ml) or diluted (1:25) pooled human or diluted (1:50) pooled monkey sperm sonicates as the GSK-3 source. Since GSK-3 activity is Mg-ATP-dependent, samples were coincubated with and without Mg-ATP (5 mM $MgCl_2$; 0.5 mM ATP). After incubation, 20 μl [^{32}P]-phosphorylase-*a* was added and a standard PP assay was conducted.

Electrophoresis and Western Blot Analysis

Twenty-five microliters of undiluted pooled human and monkey sperm extract proteins was separated by one-dimensional SDS-PAGE [36]. Resolving gels were cast using 12% acrylamide; stacking gels contained 4% acrylamide. Gels were equilibrated in 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol for 15 min before protein electrophoretic transfer to nitrocellulose in the same buffer. Blots were blocked with 5% nonfat dry milk in 20 mM Tris-HCl (pH = 7.5), 0.1% Tween 20, 0.5 M NaCl, and 2% NaN_3 (TTBS; ingredients from Sigma) for 2 h and washed two times for 10 min in TTBS. Blots were incubated with either anti-PP1α (RU36; diluted 1:2000), anti-PP1γ_1 (RU31; diluted 1:25 000), anti-PP1γ_2 (G503; diluted 1:10 000), (all prepared in our laboratory) or anti-GSK-3 (diluted 1:1000; provided by Dr. Woodgett, Ontario Cancer Institute/Princess Margaret Hospital, Toronto, ON, Canada) antibodies in TTBS plus 0.1% BSA overnight. The production of PP1α- and PP1γ_1-specific antibodies has been described [20]. Identical methods were used to generate PP1γ_2-specific antibodies against the unique C-terminal peptide (VGSGLNPSIQKASNYRNNTLVYE). Blots were washed twice in TTBS, incubated for 30 min with biotinylated anti-rabbit IgG (Vector Laboratories Inc., Burlingame, CA), washed twice in TTBS, and developed with the avidin-biotin (peroxidase) ABC kit (Vector).

Statistical Analysis

Data collected from motility experiments in which human sperm were treated with or without 50 nM CA were subjected to square-root transformation (because of heterogeneity of variance) before analysis by way of Student's paired *t*-test. Sperm PP and inhibitor dose-dependent activity were analyzed with linear regression. Differences were considered to be significant when $p < 0.05$.

RESULTS

CA and Motility

The addition of increasing concentrations of CA to human sperm resulted in enhanced percentage motility and path velocity. Incubation of sperm in the presence of 20 nM CA for 10 min yielded a 54% increase in the number of motile sperm in comparison to the control value. Further increases in CA did not result in the recovery of additional motile sperm. CA exposure also stimulated mean sperm path velocity in a dose-dependent manner. Treatment of sperm with CA, at a final concentration of 50 nM, resulted in a 30% increase in path velocity compared to that in the

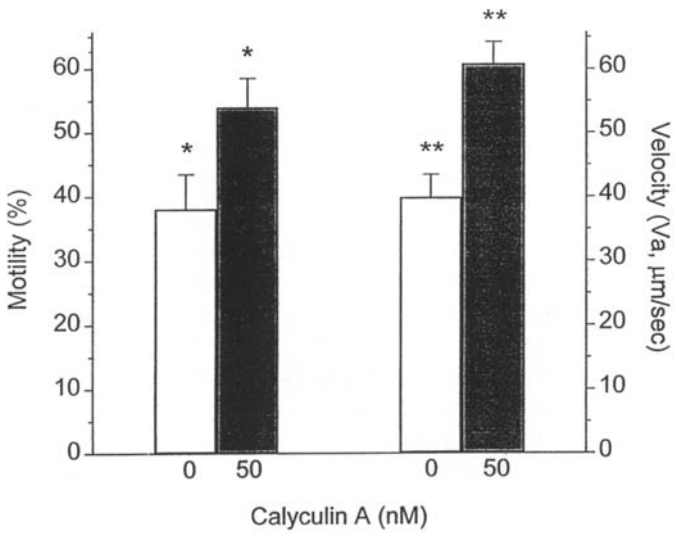

FIG. 1. Influence of the PP inhibitor, CA (50 nM), on human sperm motility. Washed human sperm samples (n = 4) were resuspended to 6 × 10^7 cells per milliliter and treated with or without 50 nM CA for 10 min before analysis of sperm percentage motility and path velocity with CASMA. This sperm concentration has previously been shown to be optimal in minimizing errors in tracking [32]. Motility values for each sperm sample were derived from a minimum of 200 sperm analyzed in 6 random fields. Columns represent means ± SEM. Columns with similar symbols are significantly different (* $p < 0.05$; ** $p < 0.01$).

control. Therefore, subsequent experiments were conducted with individual sperm samples treated with or without CA, at a final concentration of 50 nM.

All four human sperm samples treated with 50 nM CA showed an enhanced percentage motility and path velocity (Fig. 1). Treatment of human sperm samples with 50 nM CA caused a significant increase ($p < 0.05$) in percentage motile sperm in comparison to the value obtained with 0 nM CA (range 42–63, mean ± SE = 54.0 ± 4.5 and range 31–54, mean ± SE = 38.1 ± 5.4%, respectively). Even more noticeable was the significant enhancement ($p < 0.01$) in path velocity caused by 10-min incubation with 50 nM CA in comparison to the control value (range 52–67, mean ± SE = 60.8 ± 3.4 and range 30–45.5, mean ± SE = 39.9 ± 3.5 μm/sec, respectively).

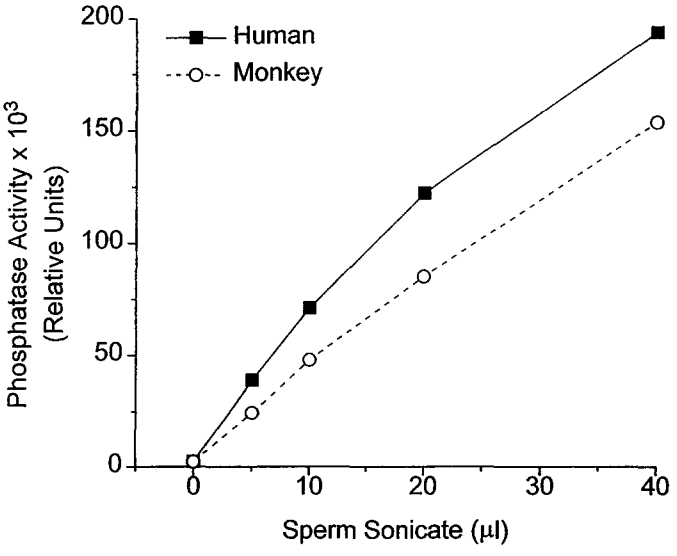

FIG. 2. Dose-dependent increase of PP activity in human and monkey sperm sonicates. PP activity in pooled human or monkey (n = 4 per pool) sperm sonicates was evaluated at increasing volumes with phosphorylase-*a* used as the substrate. Points represent means of duplicate samples. All values were determined in a single assay. Phosphatase activity is reported as uncorrected cpm for ease in visualizing a dose-dependent response.

Primate Sperm PP

Initially, four samples of human and monkey sperm were evaluated individually for PP activity. All sonicates tested contained PP activity, with an average of 6.2 × 10^{-2} ± 4.5 × 10^{-2} and 4.3 × 10^{-2} ± 0.8 × 10^{-2} mU/10^6 sperm for human and rhesus monkey sperm, respectively. All further PP characterizations were conducted on pooled sperm sonicates. PP activity increased in a dose-dependent manner with sperm sonicate volume resulting in R values = 0.989 ($p < 0.001$) and 0.998 ($p < 0.001$) for human and monkey sperm, respectively (Fig. 2). The phosphatase activities reported in Figure 2 are expressed in uncorrected cpm for ease of visualizing the dose-dependent response. When these values, based on pooled samples, were converted to mU/10^6 sperm and averaged within species (human: 14.7 × 10^{-2} ± 1.4 × 10^{-2}; monkey: 3.3 × 10^{-2} ± 0.2 × 10^{-2} mU/10^6 sperm), they were consistent with the averages for individual semen samples reported above.

In individual as well as pooled sperm sonicates, PP assay conditions did not include Ca^{2+} or Mg^{+}, which are required for PP2B and PP2C activity, respectively. Thus we concluded that the PP activity reported above was attributable to either PP1 or PP2A. To ascertain which PP (PP1 or PP2A) was present, experiments were conducted to assess the sensitivity of sperm PP to OA as well as to CA. Both PP1 and PP2A are very sensitive to the inhibitor CA. PP2A sensitivity to OA approximates its sensitivity to CA, whereas PP1 is much less sensitive to OA. The inhibitory patterns observed with OA or CA were similar for the human and rhesus monkey (Fig. 3). The concentration of OA required for 50% inhibition (IC_{50}-OA) of human and rhesus sperm PP activity was 38.8 and 37.2 nM, respectively. In contrast, the concentration of CA required for 50% inhibition (IC_{50}-CA) of human sperm PP (0.75 nM) and monkey sperm PP (0.59 nM) was approximately 1/60th the IC_{50}-OA. This heterogeneous response of primate sperm PP to the inhibitors OA and CA is consistent with the presence of PP1.

To further confirm and characterize the activity as PP1, samples were subjected to one-dimensional SDS-PAGE; Western blots were then analyzed with affinity-purified antibodies to the PP1 isoforms PP1α, PP1γ_1, and PP1γ_2 (Fig. 4). Neither human nor monkey sperm extracts reacted with PP1α antibody (data not shown); however, both human and

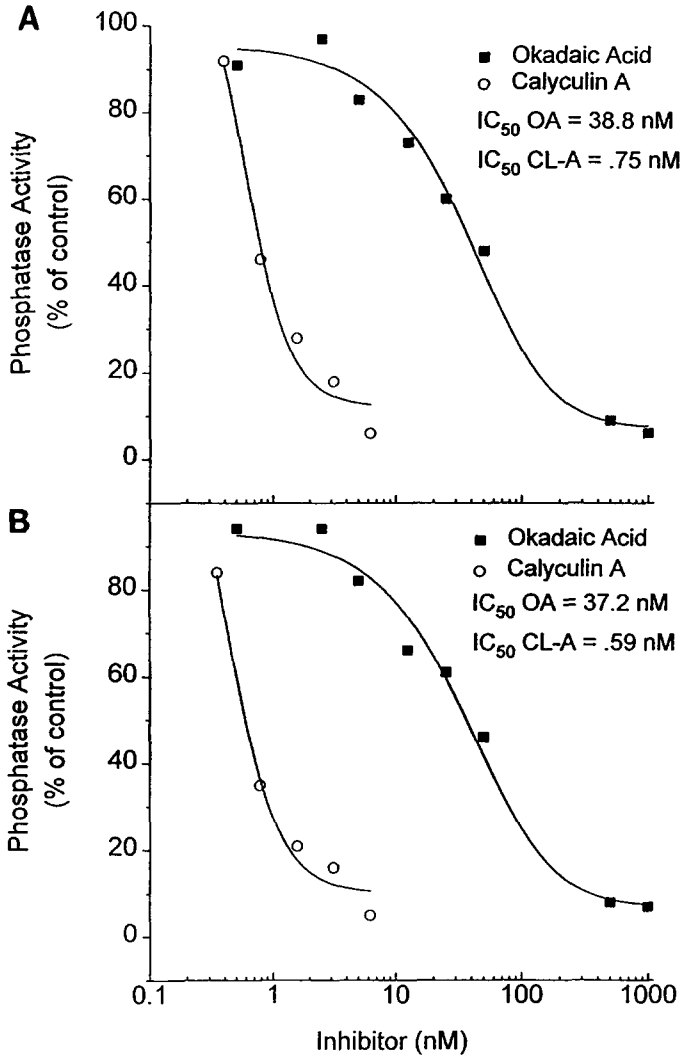

FIG. 3. Inhibition profiles of human (A) and monkey (B) sperm PP activity with OA and CA. Pooled sperm extracts were incubated with increasing concentrations of either OA or CA for 10 min. Individual points represent the means of duplicate samples with all values determined in a single assay. The IC_{50} concentration of OA or CA, which would yield 50% inhibition of PP activity in relation to the control value (no inhibitor), was determined from the best-fit line.

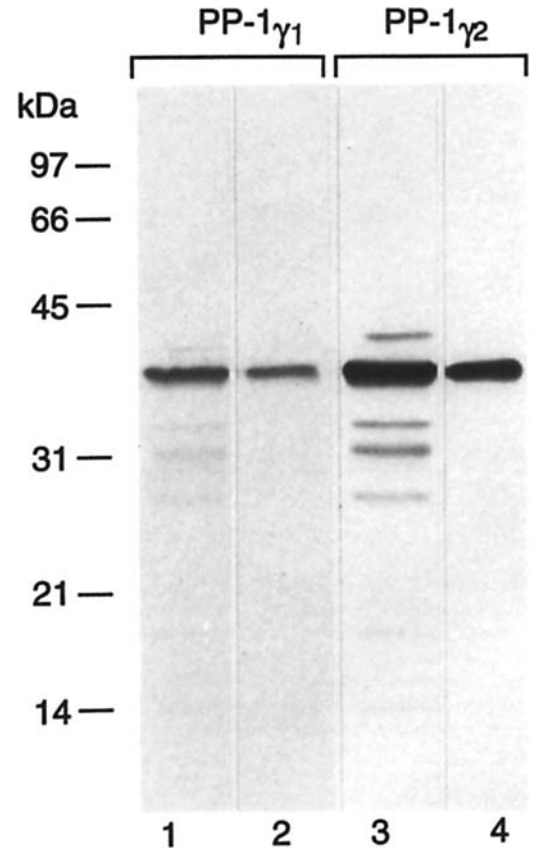

FIG. 4. Anti-PP1γ_1 and anti-PP1γ_2 immunoblotting of human and monkey sperm extracts after SDS-PAGE. Approximately 50 µg of human (lanes 1 and 3) or monkey (lanes 2 and 4) total sperm protein was added per lane; this was followed by electrophoresis under denaturing conditions, electrophoretic transfer to nitrocellulose, and immunoblotting with either PP1γ_1 or PP1γ_2 antibodies.

monkey sperm extracts reacted with PP1γ_1 and PP1γ_2 antibodies, yielding a predominant band at approximately 39 kDa.

Primate Sperm PP Inhibitor

One mechanism by which PP1 is modulated involves endogenous heat-stable inhibitors, referred to as I1 and I2. All human and rhesus monkey sperm sonicates tested contained PP inhibitory activity. For example, when 8 µl of individual human (n = 4) or rhesus monkey (n = 4) boiled sonicates was coincubated with human sperm sonicates as a PP source, the average inhibition of PP activity compared to that in controls (no heat-stable sperm extract) was 77.0 ± 3.7% for human and 66.8 ± 3.7% for rhesus monkey. Furthermore, inhibition was dose-dependent; increasing volumes of either human or monkey heat-stable sperm sonicates resulted in decreasing PP activity (Fig. 5), yielding R values of 0.96 ($p < 0.02$) for both.

I1 and I2 are distinguishable by their response to phosphorylation; the inhibitory activity of I1 is stimulated after phosphorylation by PKA, whereas I2 is PKA-independent. Coincubation of either boiled human or monkey sperm extracts with PKA did not alter their inhibitory potential (Table 1). When PKA was coincubated with monkey brain striatum extract (known to contain both I1 and I2), a threefold increase in the inhibitory potential of this extract was observed. Thus, the biochemical properties of the human and rhesus monkey sperm PP inhibitor resemble those of I2.

Primate Sperm GSK-3

GSK-3 in the presence of Mg-ATP causes the phosphorylation of I2, the liberation of PP1 from PP1-I2, and increased phosphatase activity [37]. To verify the existence of this relationship in primate sperm, in vitro-generated PP1-I2 was incubated with either purified GSK-3 or human or monkey

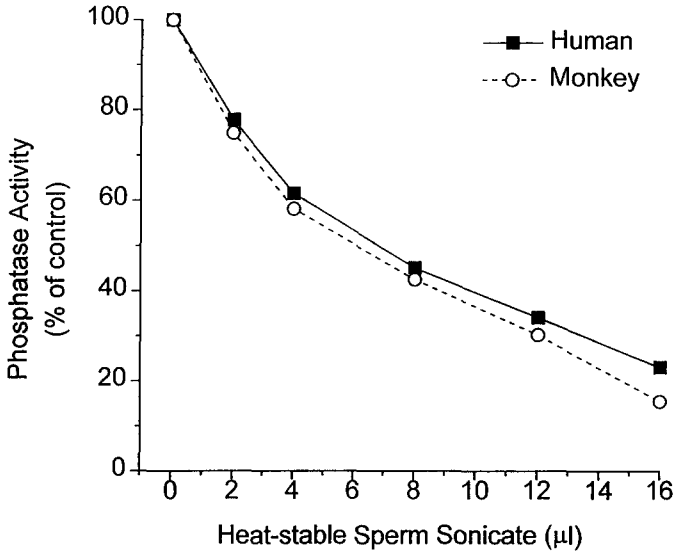

FIG. 5. Dose-dependent increase of inhibitor activity in human and monkey sperm sonicates. Inhibitor activity in boiled pooled human (n = 4) and pooled monkey (n = 4) sperm extracts was evaluated at increasing doses with human sperm extract used as the PP source and phosphorylase-*a* as the substrate. Points represent means of duplicate samples. All values were determined in a single assay. Phosphatase activity is reported as percentage of control (no boiled sperm extract added) for ease in visualizing a dose-dependent response.

sperm sonicates in the presence and absence of Mg-ATP. Incubation of PP1-I2 without a GSK-3 source, but in the presence of Mg-ATP, did not result in phosphatase activity. A Mg-ATP-dependent increase in PP activity was observed in all cases in which a GSK-3 source was included. One unit of GSK-3 activity (U) is the amount of enzyme that increases the phosphorylase phosphatase activity of PP1-I2 by 1.0 mU/ml in the standard assay. Incubation of in vitro-generated PP1-I2 with purified GSK-3, in the presence of Mg-ATP, resulted in an increase in phosphatase activity from 0.03 (no Mg-ATP) to 1.4 mU. Phosphatase activity in the absence of Mg-ATP was 0.06 and 0.04 mU when human and rhesus sperm sonicates, respectively, were incubated with PP1-I2. With the addition of Mg-ATP, phosphatase activity was 0.62 and 0.43 U for human and rhesus sperm sonicates incubated with PP1-I2. This 10-fold, Mg-ATP-dependent increase in PP1-I2-liberated PP activity suggests that both human and

TABLE 1. Characterization of primate sperm PP inhibitor.

Phosphatase source	Inhibitor source**	Protein phosphatase activity × 10^{-2} (mU/10^6 sperm)*	
		− Protein kinase A	+ Protein kinase A
Human sperm	None	8.4	8.1
Human sperm	Human sperm	4.5	4.6
Human sperm	Monkey sperm	5.5	5.3
Human sperm	Monkey brain	2.2	0.8

* PP activity measured as the amount of [^{32}P] released from [^{32}P]-phosphorylase-*a* due to the activity of PP in pooled human sperm sonicates. Boiled monkey brain striatum was used as a positive control since it is known to contain both I1 (PKA-dependent) and I2 (PKA-independent) activity.
** Boiled sonicates.

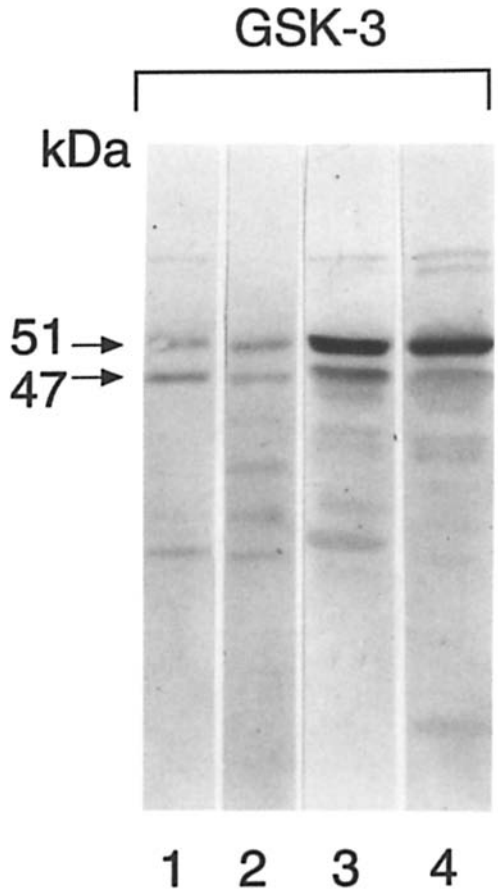

FIG. 6. Anti-GSK-3 immunoblotting of human sperm (lane 1), monkey sperm (lane 2), monkey testis (lane 3), and monkey myometrium (lane 4) extracts after SDS-PAGE. Extract proteins (50 µg) were added, followed by electrophoresis under denaturing conditions, electrophoretic transfer to nitrocellulose, and immunoblotting with a GSK-3 antibody that recognizes both isoforms, GSK-3α and GSK-3β.

monkey sperm contain GSK-3 at approximately 0.56 and 0.39 U, respectively.

To confirm and characterize the presence of GSK-3 in primate sperm, pooled human and pooled monkey sperm extracts were subjected to one-dimensional SDS-PAGE and Western blot analysis with a GSK-3 antibody that recognizes both GSK-3α and GSK-3β (Fig. 6). This antibody reacted to protein bands at approximately 51 and 47 kDa, the reported molecular masses of GSK-3α and GSK-3β, respectively [38]. Therefore it is concluded that the GSK-3 activity measured in primate sperm extracts is attributable to the presence of both GSK-3α and GSK-3β.

DISCUSSION

The results from this study demonstrate that a functional PP1 system is present in primate sperm, since human and monkey sperm contain not only PP1 but also a known endogenous PP1 stimulator (GSK-3) and inhibitor (I2). In addition, an association of the PP system with motility was demonstrated. Treatment of human sperm with the specific,

cell-permeable PP1 inhibitor, CA, resulted in a stimulation of both the percentage of motile sperm in a population and mean sperm path velocity. These experiments represent a significant step in addressing the question of the biochemical mechanisms involved in controlling sperm function.

PPs are involved in the control of processes as widespread as metabolism, contractility, membrane transport and secretion, gene expression, and cell division [11, 12] and have been identified in all eukaryotes examined [39]. The presence of both PP1 and PP2B (calcineurin) has been reported in testicular tissue [40–42]. Furthermore, sperm PP activity has been described in cattle [27], goats [28], pigs, dogs [26], and, most recently, humans and roosters [29, 43]. This PP activity has been characterized as PP2B in the dog, pig, and human [26, 29] and as PP1 in the rooster [43].

The PP in primate sperm was identified as PP1γ_2 on the basis of substrate specificity, inhibition profiles, and Western blot analysis. CA, originally derived from the marine sponge *Discodermia calyx*, is a potent inhibitor of PP1 and PP2A [23, 44]. The influence of CA on human sperm motility suggests that a CA-sensitive PP is present in primate spermatozoa. All human and monkey sperm samples tested contained PP1 and/or PP2A activity, as concluded from phosphorylase-*a* substrate usage, and this activity increased in a dose-dependent manner as increasing amounts of primate sperm extracts were tested. The inhibitory profiles of primate PP activity for OA and CA are identical to those reported for PP1 isolated from other tissue sources [23], suggesting that the PP activity measured in primate sperm is PP1. However, this does not preclude the possibility that PP2A, PP2B, or PP2C is also present in primate sperm.

The existence of four isoforms of the active PP1 enzyme (PP1α, PP1β, PP1γ_1, and PP1γ_2) has been substantiated by cDNA cloning and immunoreactivities of specific antibodies against each isoform [19]. Immunoblot analysis of human and monkey sperm extracts identified an immunoreactive protein at 39 kDa with both PP1γ_1 and PP1γ_2 antibodies. PP1γ_2 has previously been reported to be a 39-kDa protein [42] that was found to be encoded by an alternatively spliced form of PP1γ_1 mRNA [19] with an extended carboxyl terminus [21]. This explains the protein band patterns recognized in primate sperm extracts with PP1γ_1 and PP1γ_2 antibodies. These data further confirm the presence of PP1, specifically PP1γ_2, in primate sperm.

Both human and monkey sperm extracts contained I2 activity, but did not contain any I1 activity. This observation was unexpected, since most tissues contain both I1 and I2 [45]. The presence of I2 in primate sperm, besides acting as a control mechanism to regulate phosphatase activity, is confirmative of the existence of PP1. One delineation point in the characterization of serine/threonine PPs into two groups is that PP1 (but not PP2s) is inhibited by heat- and acid-stable proteins, I1 and I2, as was found to be the case in primate spermatozoa.

The inactive cytosolic form of PP1 (PP1-I) was first identified in the bovine adrenal cortex [46] and was designated the Mg-ATP-dependent PP because preincubation with Mg-ATP was a prerequisite for activity. It was later reported that, in addition to Mg-ATP, another protein, Fa, was required to activate PP1-I [47]. Factor Fa contained kinase activity toward glycogen synthase [48] and was purified and reported to be identical to the enzyme GSK-3 [49]. GSK-3 was first identified as a protein kinase involved in regulating the activity of glycogen synthase, the rate-limiting enzyme in glycogen synthesis [50]. Our results suggest that human and monkey sperm contain an enzyme capable of causing dissociation of PP1 from in vitro-generated PP1-I2, and that this uncoupling is dependent on Mg-ATP. This in vitro-generated PP1-I2 presumably consists of a 1:1 complex of PP1 and I2, and its activation by GSK-3 and Mg-ATP is triggered by the phosphorylation of a threonine residue on I2 [16, 17]. It is important to recognize that mammalian sperm do not contain glycogen or enzymes responsible for glycogen metabolism [51] and that they utilize exogenous glucose, fructose, lactate, and pyruvate as their energy sources [52]. Therefore, the important roles that PP1 and GSK-3 play in regulating glycogen metabolism in somatic cells [12] would not apply in primate sperm. Molecular cloning experiments have identified two highly related isoforms of GSK-3 termed GSK-3α and GSK-3β. The enzyme GSK-3α is a 51-kDa protein whereas GSK-3β is a 46-kDa polypeptide with catalytic properties similar to those of GSK-3α [53]. Using an antibody that recognizes both GSK-3α and GSK-3β, we were able to demonstrate protein bands in human and monkey sperm extracts with molecular masses of approximately 51 and 47 kDa.

The influence of CA on human sperm motility strongly suggests that PP1 activity is involved in the regulation of primate sperm kinetic activity. This is in agreement with results obtained in the bovine [54] and fowl [43] systems. The effects of the PP1 inhibitor CA on the percentage of motile primate sperm are most noticeable in low-motility samples or in nonmotile caput epididymal sperm (unpublished results); these observations are similar to results obtained in the bovine model. If the initial percentage of motile sperm was high, then the influence of CA on motility parameters was most evident in sperm velocity. Thus it appears that PP1 activity is inversely correlated with primate sperm percentage motility and path velocity. Experiments are presently underway to determine PP1 activity with respect to a variety of sperm motility states in both the human and monkey models.

With respect to the sequence of events involved in controlling sperm motility, the data presented allow a wide range of novel speculations. One is that known biochemical mediators of motility, intracellular calcium, and cAMP [7] may alter the phosphorylated state and activity of GSK-3, which would ultimately regulate the PP1 activity in sper-

matozoa. In somatic cells, GSK-3 activity has been reported to be controlled by tyrosine phosphorylation [51] and phosphorylation catalyzed by protein kinase C [55]. The possibility that these two control pathways work through GSK-3, and thus mediate PP1 activity, represents a possible mechanism in the control of sperm kinetics that merits further investigation.

In conclusion, we have reported that human and monkey sperm contain PP1 and its endogenous regulators GSK-3 and I2. Furthermore, treatment of human sperm with the PP inhibitor CA was shown to stimulate motility parameters, presumably by altering the PP1 activity present in primate sperm.

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