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Extracellular Adenosine 5′-Triphosphate Alters Motility and Improves the Fertilizing Capability of Mouse Sperm[1]

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ABSTRACT

Extracellular adenosine 5′-triphosphate (ATPe) treatment of human sperm has been implicated in improving in vitro fertilization (IVF) results. We used the mouse model to investigate mechanisms of action of ATPe on sperm. ATPe treatment significantly enhanced IVF success as indicated by both rate of pronuclear formation and percentage cleavage to the 2-cell stage. However, ATPe did not increase the percentage of sperm undergoing spontaneous acrosomal exocytosis nor change the pattern of protein tyrosine phosphorylation normally observed in capacitated sperm. ATPe altered sperm motility parameters; in particular, both noncapacitated and capacitated sperm swam faster and straighter. The percentage of hyperactivated sperm did not increase in capacitated ATPe-treated sperm compared to control sperm. ATPe induced a rapid increase in the level of intracellular calcium that was inhibited by two distinct P2 purinergic receptor inhibitors, confirming that these receptors have an ionotropic role in sperm function. The observed motility changes likely explain, in part, the improved fertilizing capability when ATPe-treated sperm were used in IVF procedures and suggest a mechanism by which ATPe treatment may be beneficial for artificial reproductive techniques.

acrosomal exocytosis, acrosome reaction, assisted reproductive technology, extracellular ATP, fertilization, hyperactivation, intracellular calcium, purinergic receptor, sperm motility and transport

INTRODUCTION

Both intracellular and extracellular ATP molecules have critical roles in sperm function. Intracellular ATP is the main energy source in sperm, acts as a substrate for the generation of the second messenger cAMP by adenylyl cyclases, and serves as a phosphate donor for protein phosphorylation. Glycolysis is the principal metabolic pathway for ATP generation in sperm, at least in the mouse, although oxidative phosphorylation and the conversion of ADP to ATP and AMP by adenylate kinase can also contribute [1, 2]. Extracellular ATP (ATPe) is an important signaling molecule. Because ATP is a strongly charged anion, having four negative charges at physiologic pH, it does not readily cross the plasma membrane. Rather, the role of ATPe is presumably mediated by cell-surface P2 purinergic receptors [3]. The levels of ATPe can be quite variable and are dependent on a number of factors including the tissue type and the presence of ecto-ATPases that hydrolyze ATP to ADP and adenosine. In the epididymis where sperm acquire motility, the epithelial cells, sperm, and neurons innervating the epididymis are sources of ATPe [4]. ATPe is present in the female reproductive tract and is maximal at ovulation [5], but the cell types that produce ATP in these tissues have not been determined.

Extracellular ATP was reported to increase the percentage of human and bovine sperm that undergo acrosomal exocytosis (AE) [6, 7], but different mechanisms have been proposed to mediate this effect. Luria et al. [7] suggested that ATPe activates a P2 purinergic receptor that elevates intracellular calcium while Foresta et al. [6] proposed that an ATPe-gated sodium channel was involved. Regardless, the reported increase in AE in human sperm led to a study in which oocytes from 22 women were inseminated in vitro with ATPe-treated and -untreated sperm obtained from their partners with male factor infertility [8]. The fertilization percentage in the group of oocytes inseminated with ATPe-treated sperm was significantly increased compared to control sperm as indicated by the presence of pronuclei and embryo cleavage. Embryos from the treated and control groups were transferred together, and seven patients delivered nine healthy babies. Although it could not be determined whether the ATPe-treated sperm were responsible for any of the successful pregnancies, these results suggested that the use of ATPe for treatment of human sperm during IVF procedures could be warranted. However, the biological rationale for treating sperm with ATPe (assuming the major downstream effect is premature induction of AE) is not clear because induction of AE prior to sperm-zona pellucida binding would likely prevent and not promote fertilization.

Because previous reports suggested that ATPe is beneficial in IVF procedures, we investigated the effects of ATPe on various parameters of sperm function and examined potential mechanisms for this improvement using the well-characterized mouse model system. We found that ATPe dramatically improves fertilization success in the mouse model, most likely by initiating P2 purinergic receptor-mediated ion fluxes and causing alterations in sperm motility.

MATERIALS AND METHODS

Animals

CF1 and CD1 mice were purchased from Harlan (Indianapolis, IN) and B6SJLF1/J mice were purchased from Jackson Laboratories (Bar Harbor, ME). All experiments were conducted under the approval of the Institutional Animal

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Care and Use Committee of the University of Pennsylvania in accordance with the Guide for Care and Use of Laboratory Animals.

Assessment of Acrosomal Exocytosis

Caudal epididymal sperm were collected from transgenic mice expressing proacrosin/enhanced green fluorescent protein (EGFP) in the sperm acrosome [9]. Sperm were allowed to swim out from the epididymides into modified Krebs-Ringers solution (mKRS; 154 mM NaCl, 5 mM KCl, 1.7 mM $CaCl_2$, 1 mM KH_2PO_4, 1 mM $MgSO_4$, 5 mM $NaHCO_3$, 5 mM glucose, and 20 mM HEPES, pH 7.4). The sperm were counted and the volume adjusted so that the final concentration in each sample was 1×10^7 sperm/ml. The sperm were incubated with 2.5 mM ATPe or 10 μM ionomycin (Sigma, St. Louis, MO) or mKRS medium alone. Aliquots were removed at 60 and 120 min and fixed with 1.9% paraformaldehyde for 30 min at 4°C. For some experiments, the sperm were incubated in medium that supports capacitation (mKRS containing 15 mM $NaHCO_3$ and 3 mg/ml BSA) for 90 min prior to addition of 2.5 mM ATPe, 10 μM progesterone, or 1 μm ionomycin; the sperm were incubated for an additional 30 min prior to fixation. The sperm were placed on a glass slide and allowed to settle for 15 min. Slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA) and examined using both brightfield and epifluorescence illumination on an inverted epifluorescence microscope (Nikon TE-2000U, Nikon Instruments, Melville, NY) for the presence or absence of green fluorescence in the acrosomes. Sperm were scored as "acrosome-intact" if fluorescence was observed in the acrosome while "acrosome-reacted" sperm had no fluorescence or only a speckled pattern of fluorescence remaining in the acrosome region.

In Vitro Fertilization

Female CF1 mice were superovulated by intraperitoneal injections of 5 IU eCG (EMSCO, Philadelphia, PA), followed 48 h later by 5 IU hCG (Sigma). Eggs were collected from the oviducts 13–15 h following hCG administration into HEPES-buffered Whitten medium [10]. When necessary, cumulus masses were removed by a brief incubation in 0.1% hyaluronidase. The eggs were washed and then placed into 250 μl fertilization drops of TYH medium (120 mM NaCl, 4.7 mM KCl, 1.7 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 5.5 mM glucose, 0.5 mM sodium pyruvate, 10 mg/ml gentamicin, 0.0006% phenol red, and 4 mg/ml BSA) [11] under light mineral oil. All gamete and embryo culture was performed at 37°C in a humidified atmosphere of 5% CO_2 in air.

Mouse sperm were collected from either adult CD1 or B6SJLF1/J males. Sperm from one epididymis was released into BSA-free TYH medium and the other epididymis into BSA-free TYH medium containing 2.5 mM ATPe. After 1 h, the sperm were capacitated for 90 min in complete TYH medium containing 4 mg/ml BSA. The sperm concentration was determined after capacitation and was always approximately equivalent in each group. Eggs were then inseminated at a final concentration of $1–8 \times 10^5$ sperm/ml, washed free of unbound sperm 3 h after insemination, and cultured in KSOM + AA medium (Specialty Media, Phillipsburg, NJ). The success of fertilization was determined by morphological assessment of pronucleus formation and cleavage to the 2-cell stage. In some cases, embryos were cultured in KSOM + AA to the blastocyst stage.

Assessment of Sperm Motility

Caudal epididymal sperm from adult CD1 mice were collected into TYH medium containing 0.01% polyvinyl alcohol (TYH/PVA) ± 2.5 mM ATPe; the PVA was added to prevent the sperm from sticking to glass surfaces. After 15 min, adequate dispersion of the sperm was determined visually, and an aliquot was diluted 1:10 in the same medium. Sperm were evaluated immediately after dilution (noncapacitated, 15 min) or incubated for a total of 60 min prior to evaluation (noncapacitated, 60 min). For some experiments, the sperm were capacitated for 90 min after the initial 60 min incubation in ATPe by adding BSA to the culture medium to achieve a final concentration of 4 mg/ml; these sperm were evaluated at the end of the 150 min incubation time (capacitated). A sperm aliquot was placed on a prewarmed 100 μm-depth counting chamber slide (CellVision, Hopedale, MA) and analyzed using computer-assisted semen analysis (CASA) (Hamilton-Thorne IVOS V12.2L; Hamilton Thorne Research, Danvers, MA). For some experiments, sperm were collected and incubated for 60 min in TYH/PVA ± 0.25–2.5 mM β, γ-methyleneadenosine 5′-triphosphate (AMP-PCP) (Sigma), an ATP analogue that is relatively resistant to hydrolysis, prior to analysis by CASA.

At least 300 motile sperm and 5 fields were assessed by CASA for each treatment group. Parameters assessed in this study were: motility (%), curvilinear velocity (VCL, μm/sec), straight-line velocity (VSL, μm/sec), average path velocity (VAP, μm/sec), amplitude of lateral head displacement (ALH, μm), beat-cross frequency (BCF, Hz), linearity (LIN = VSL/VCL, %), straightness (STR = VSL/VAP, %), and percentage of rapid sperm (VAP > 25 μm/sec, %). The CASA motility parameters used were those recommended by the manufacturer for mouse sperm. Hyperactivated motility was defined as motility with high amplitude thrashing patterns and short trajectory distances [12]. The criteria for detecting hyperactivated spermatozoa were VCL > 180 μm/sec, ALH > 9.5 μm, and LIN < 38%.

Gel Electrophoresis and Immunoblotting

Mouse sperm were collected into TYH/PVA ± 2.5 mM ATPe. After 1 h, the sperm were capacitated for 90 min in TYH medium containing 4 mg/ml BSA. Aliquots of 10^6 sperm were removed immediately after collection (15 min), after incubation for 60 min, and after capacitation (150 min). Sperm were washed, resuspended in SDS sample buffer, and evaluated for tyrosine phosphorylation by immunoblotting as described previously [13]. To confirm equal loading of samples, each blot was reprobed using a monoclonal anti-α-tubulin antibody (Invitrogen, Carlsbad, CA) at a final concentration of 1 μg/ml in PBS containing 0.2% Tween 20.

Determination of Sperm Intracellular Calcium

Mouse sperm were collected into modified Whitten medium (25 mM HEPES, 1.2 mM $MgSO_4$, 109 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1 mM sodium pyruvate, 4.8 mM lactic acid, 1.7 mM $CaCl_2$, 25 mM $NaHCO_3$, 5.5 mM glucose, 3 mg/ml BSA, pH 7.2). The sperm were loaded with the fluorescent probe fluo-4 acetoxymethyl ester (fluo-4 AM) (Invitrogen Inc., Carlsbad, CA) by incubation for 20 min in 3 μM fluo-4 AM and 0.015% Pluronic F-125 (Invitrogen) in modified Whitten medium. Coverslips were washed overnight with 85% ethanol, rinsed with water, and coated with laminin by adding a drop of 20 μg/ml laminin (Sigma) and allowing it to air dry. The laminin-coated coverslips were placed into a Leiden chamber and fluo-4 AM-loaded sperm (30–100 μl) were added to the coverslip and incubated for 30 sec at 37°C.

The cover slips were washed twice with modified Whitten medium to remove unattached sperm and extracellular fluo-4 AM and then covered with 400 μl of medium. When inhibitors were used to block the effect of ATPe on P2 purinergic receptors, this final 400-μl medium contained 10 μM Reactive Blue 2 (RB-2; Sigma) or 10 μM pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate (PPADS; Sigma). The chamber was placed onto the stage of an inverted epifluorescence microscope in a temperature-controlled environment at 37°C and imaging was begun immediately. Epifluorescence images were collected with a 100X 0.5–1.3 NA S Fluor oil objective and MetaFluor software (Universal Imaging, West Chester, PA) as previously described [14]. Basal sperm fluorescence levels were recorded at time 0, and without interruption of the imaging, either ATPe or NaCl was added to reach a final concentration of 2.5 mM or 5 mM, respectively. The fluorescence signal was captured every 500 msec for 2–3 min; exposure time was 100 msec.

The recorded epifluorescence images were analyzed quantitatively using MetaFluor software. The total fluorescence signal in each sperm head was measured by drawing a region of interest around the sperm head and then calculating the median fluorescence intensity in the region. This was done for each sperm head that was not overlapping with other sperm, and only sperm with moving tails were included. The fluorescence was expressed as a ratio of F/F_0 where F_0 was the median sperm head fluorescence before ATP or NaCl addition and F was the median fluorescence intensity in the same sperm head measured every 500 msec. For the inhibitor experiments, at least 25 sperm per group were analyzed qualitatively for the presence or absence of a calcium response by visual examination of the recorded images.

Statistical Analysis

The percentage of acrosome-reacted sperm, IVF experiments, and calcium responses were analyzed by Fisher exact test using Prism 4.0 Software (GraphPad Software, San Diego, CA). The sperm motility parameters were analyzed by the nonparametric test, Wilcoxon signed-rank test for paired data. In the particular case of sperm motility parameters when a single control group was used for comparison to each of the test groups, ATP or AMP-PCP, *P* values were Bonferroni-adjusted to account for multiple comparisons. Otherwise, two-tailed *P* values of <0.05 determined statistical significance.

RESULTS

ATPe-Treated Sperm Showed Increased Fertilizing Capacity in IVF Procedures

To determine whether treatment of mouse sperm with ATPe affected the success of IVF, sperm from hybrid B6SJLF1/J

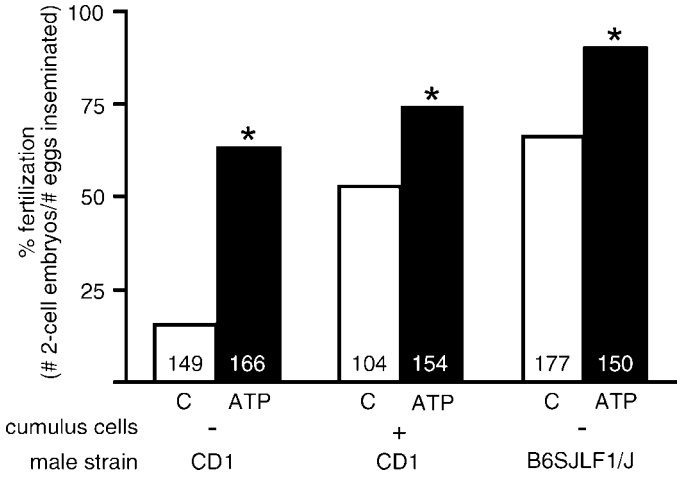

FIG. 1. Effect of ATPe treatment of sperm on in vitro fertilization. Either cumulus cell-intact or cumulus cell-free eggs were inseminated with capacitated sperm from CD1 or B6SJLF1/J mice, as indicated. Sperm had been incubated with or without 2.5 mM ATPe prior to capacitation. Successful fertilization was defined by visualizing a 2-cell embryo after 27 h. The data represent cumulative results of five experiments for the cumulus cell-free egg/CD1 male condition and four experiments for the other two conditions. Fertilization percentages for control (C) and ATPe-treated sperm (ATP) are indicated by open bars and solid bars, respectively. Asterisks indicate significant differences between control and ATPe-treated groups (Fisher exact test, $P < 0.01$).

males, a strain with very high in vitro fertilization success, were treated with ATPe or control medium before use for insemination. The percentage of eggs fertilized as indicated by cleavage to the 2-cell stage was 68.8% (122/177) for control sperm and 92.0% (138/150) for ATPe-treated sperm ($P < 0.0001$) (Fig. 1). We then examined whether similar results would be obtained using a mouse strain whose performance in IVF (at least in our hands) is less reliable than that from B6SJLF1/J males. Using sperm from CD1 males, the percentage of eggs fertilized was 16.1% (24/140) for control sperm and 63.3% (105/166) for ATPe-treated sperm ($P < 0.0001$). Finally, when IVF was performed with cumulus-intact eggs and sperm from CD1 males, the percentage of eggs fertilized was 52.9% (55/104) in the control group and 73.4% (113/154) in the ATPe-treated group ($P < 0.001$). In spite of the variation in control fertilization rates under these different conditions, ATPe treatment of sperm consistently increased the percentage of eggs fertilized.

Treatment of sperm with ATPe also increased the fertilization rate, i.e., how quickly the inseminated eggs were fertilized, as indicated by the timing of pronuclear formation. Within 7 h after insemination, 70.4% (328/466) of the eggs that were fertilized with ATPe-treated sperm had visible pronuclei vs. 55.6% (200/360) of the eggs fertilized with control sperm ($P < 0.0001$). The difference in IVF rate was even greater when eggs were inseminated with the CD1 sperm. Using these sperm with cumulus-intact eggs, 81.3% (208/256) of the eggs reached the 2-pronuclei stage within 7 h when ATPe-treated sperm were used vs. 60.3% (114/189) for control sperm ($P < 0.0001$). These results suggest that the effect of ATPe was more pronounced in sperm with suboptimal performance in IVF procedures.

ATPe Does Not Affect Acrosomal Exocytosis in Mouse Sperm

The increase in fertilizing capability when ATPe-treated mouse sperm were used in IVF procedures (Fig. 1) indicated that ATPe affects sperm function in some manner. Foresta et al. [6] showed that ATPe, at concentrations ranging from 50 μM to 5 mM (2.5 mM showed the maximal effect), induces AE of noncapacitated human sperm, and this finding was used to explain the increase in IVF success for male factor infertility [8]. To determine if ATPe affected AE in mouse sperm, experiments were performed using a transgenic line expressing an acrosin-EGFP fusion protein. Because this protein localizes to the acrosome and is freely diffusible following AE, acrosome-intact sperm will fluoresce whereas acrosome-reacted sperm will not; thus, this mouse line provides an objective assessment of AE [9]. When noncapacitated sperm were incubated for 60 or 120 min with ATPe, the percentage of sperm that underwent AE did not increase over controls (Fig. 2, A and B). The calcium ionophore, ionomycin, was used as a positive control and resulted in AE in more than 90% of sperm at 60 and 120 min. Incubation of mouse sperm under capacitating conditions increased the percentage of AE in control sperm from 11% to 23%, and progesterone increased this percentage to ~43% (Fig. 2C). ATPe had no effect on the percentage of capacitated sperm that were acrosome reacted. These results indicated that under the incubation conditions used here, ATPe does not induce AE in either noncapacitated or capacitated sperm.

Motility Is Altered in ATPe-Treated Sperm

Although ATPe did not affect AE, our visual assessment of ATPe-treated mouse sperm suggested that they were swimming differently than control sperm. To quantitate motility changes, CASA was performed after exposing noncapacitated sperm to ATPe. After 15 min, there were no significant differences in motility parameters (Supplemental Table S1 available online at www.biolreprod.org). After 60 min continuous exposure to ATPe, VCL, VSL, and VAP were significantly increased when compared to controls, and LIN (linearity) and STR (straightness) were also increased (Fig. 3 and Supplemental Table S1). When ATPe-treated sperm were capacitated by incubation in BSA prior to analysis, there were significant increases over controls in VCL, VSL, VAP, BCF, LIN, and STR (Fig. 4 and Supplemental Table S1).

Because adenosine has effects on sperm motility and ciliary movement [15, 16], we examined whether the observed changes in sperm motility in response to ATPe could be due to the degradation of ATP to adenosine by ecto-ATPases. Mouse sperm were incubated with 0.25–2.5 mM AMP-PCP, an ATP analogue and purinergic receptor agonist that is relatively resistant to hydrolysis, and then analyzed by CASA. There were no significant differences in motility parameters between sperm treated with 2.5 mM AMP-PCP and those treated with 2.5 mM ATPe (Fig. 5 and Supplemental Table S2). These results indicate that the motility changes are not likely to be mediated by adenosine.

ATPe Does Not Affect Protein Tyrosine Phosphorylation of Capacitated Sperm

When sperm are incubated under capacitating conditions, a subset of proteins are typically tyrosine phosphorylated, and there is a tight link between protein tyrosine phosphorylation in a sperm population and motility alterations and fertilization competence reported for sperm of several mammalian species [13, 17, 18]. To determine if the effects of ATPe to improve IVF success could be explained by altered tyrosine phosphorylation, we sampled aliquots of sperm during ATPe treatment for the IVF experiments and evaluated the tyrosine phosphor-

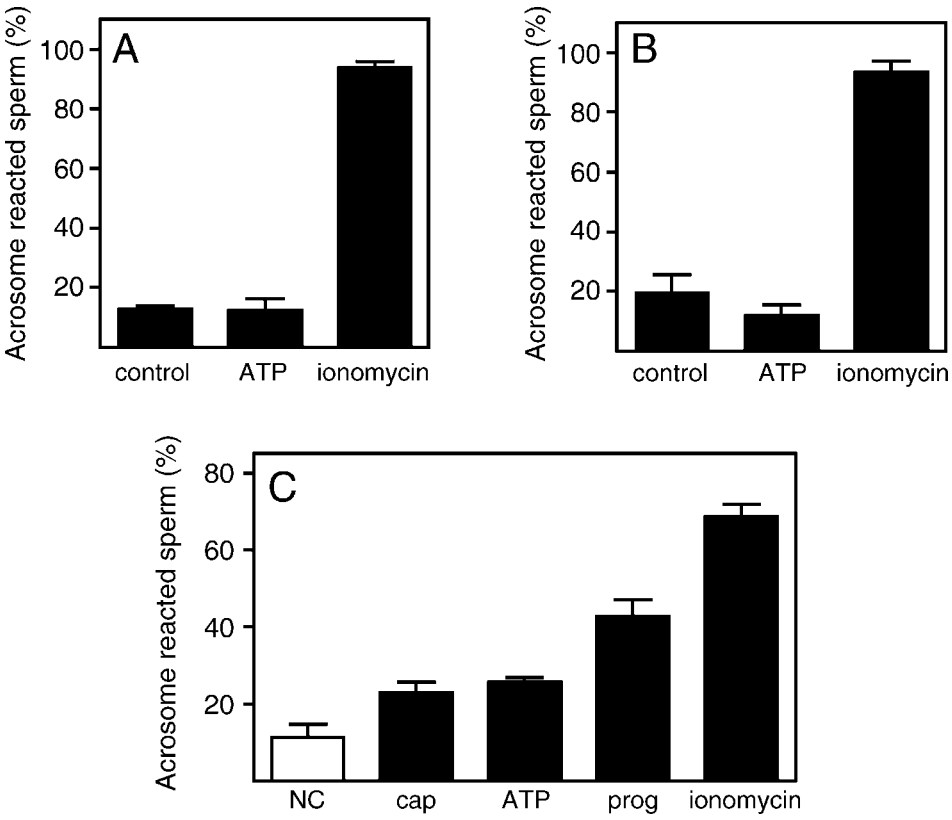

FIG. 2. Effect of ATPe on mouse sperm acrosomal exocytosis. Mouse sperm containing EGFP in their acrosomes were incubated for 1 h (**A**) or 2 h (**B**) in mKRS alone (control), in mKRS containing 2.5 mM ATPe (ATP), or in mKRS containing 1 μm ionomycin (ionomycin). In **C**, the sperm were incubated for 120 min in medium that does not support capacitation (NC; white bar) or for 90 min in medium that supports capacitation followed by 30 min in the same medium containing either no additive (cap; capacitated), 2.5 mM ATPe (ATP), 10 μM progesterone (prog), or 1 μm ionomycin (ionomycin). Acrosome-reacted sperm were identified by the disappearance of green fluorescence. All graphs represent the mean ± SEM of three independent experiments.

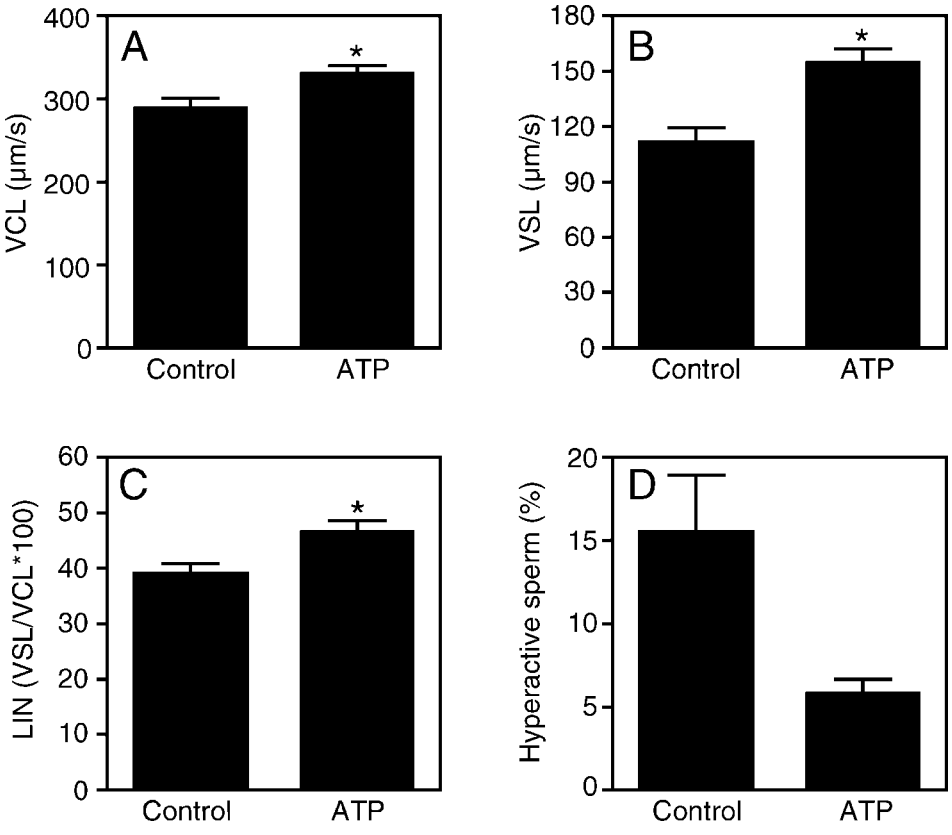

FIG. 3. Effect of ATPe on motility parameters of noncapacitated mouse sperm. Sperm were collected and incubated in TYH/PVA (Control) or TYH/PVA + 2.5 mM ATPe (ATP). CASA was performed after incubation for 60 min. Graphs represent the mean ± SEM of six independent experiments. **A**) VCL. **B**) VSL. **C**) Linearity. **D**) Hyperactivation. Asterisks indicate significant differences between control and ATPe-treated groups ($P < 0.05$, Wilcoxon signed rank test).

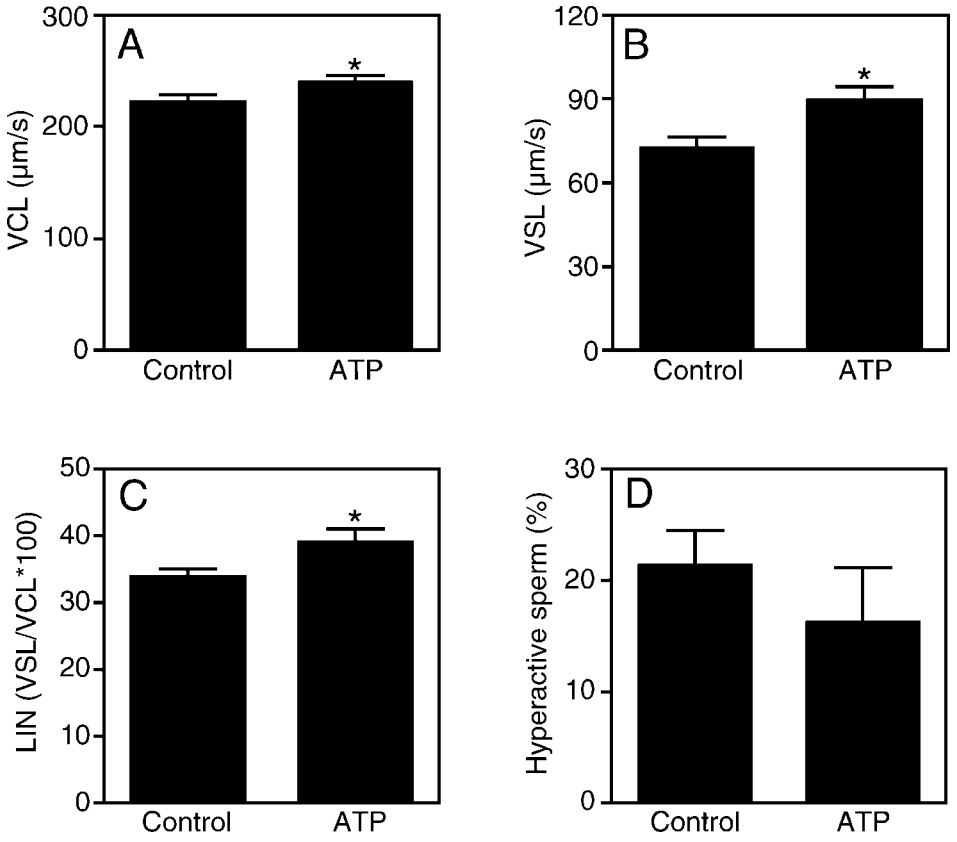

FIG. 4. Effect of ATPe on motility parameters of capacitated mouse sperm. Sperm were collected and incubated for 60 min in TYH/PVA alone (Control) or TYH/PVA + 2.5 mM ATPe (ATP). BSA was then added to a final concentration of 4 mg/ml, and after an additional 90 min incubation, CASA was performed. Graphs represent the mean ± SEM of eight independent experiments. **A**) VCL. **B**) VSL. **C**) Linearity. **D**) Hyperactivation. Asterisks indicates significant differences between control and ATPe-treated groups ($P < 0.05$, Wilcoxon signed rank test).

ylation status of the sperm proteins. Sperm were analyzed at three time points: immediately after collection into medium ± ATPe, after 60 min incubation in medium ± ATPe, and after capacitation for 90 min, i.e., just prior to their use for insemination. The presence of 2.5 mM ATPe did not increase the global pattern of tyrosine phosphorylation as compared to controls at any of these time points (Fig. 6).

ATPe Causes a Calcium Flux in Sperm

To examine the mechanism underlying the change in sperm motility observed after ATPe treatment, we measured intracellular calcium levels using the fluorescent probe, fluo-4. Prior to the addition of ATPe, most sperm showed fluorescence predominantly in the acrosomal region (Fig. 7A). When sperm were stimulated with ATPe, a rapid increase in intracellular calcium was seen in the whole head (Fig. 7, B and C). This increase was followed by a relatively slower decline (Fig. 7C). Because of rapid flagellar movement, we were not able to detect alterations in flagellar intracellular calcium in most sperm. However, occasional sperm were seen to have a calcium influx in the midpiece coincident with the calcium changes in the sperm head.

In any given experiment, ~75% of the sperm showed the calcium response with only a few sperm showing no calcium increase after ATPe treatment (e.g., sperm number 7 in Fig. 7, A and B). In most cases, the sperm that did not respond had a higher baseline level of fluorescence in the midpiece. Because ATPe is supplied as a disodium salt, an equivalent molar amount (5 mM) of sodium ion was used as a control and had no effect on sperm calcium, indicating that the effect was due directly to ATPe (Fig. 7, D–F).

To determine whether the calcium response was mediated by ATPe action on P2 purinergic receptors, two inhibitors were tested for their ability to block this response. PPADS and RB-2 are nonselective P2 purinergic receptor antagonists that block both ionotropic P2X and metabotropic (G protein-coupled) P2Y receptors but do not interact with adenosine A1 receptors [3, 19–21]. Treatment of sperm with PPADS almost completely prevented sperm from generating a calcium response to ATPe, i.e., 4/48 (8%) PPADS-exposed sperm vs. 56/78 (72%) control sperm displayed a calcium response ($P < 0.0001$, Fisher exact test). RB-2 prevented a calcium response in about half of the sperm, i.e., 13/28 (46%) RB-2-exposed sperm vs. 35/48 (73%) control sperm ($P = 0.028$, Fisher exact test).

DISCUSSION

The present study was undertaken to determine the effects of ATPe on various parameters of mouse sperm function. We found that ATPe did not affect AE nor did it alter the pattern of protein tyrosine phosphorylation in capacitated sperm. However, ATPe altered sperm motility parameters; in particular, both noncapacitated and capacitated sperm swam faster and straighter after treatment with ATPe. These motility changes likely explain, at least in part, the improvement in fertilizing capability when ATPe-treated sperm were used in IVF procedures.

The effects of treating mammalian sperm with ATPe has been examined previously by several groups. Foresta et al. [6] and Luria et al. [7] showed that ATPe increases AE in human and bovine sperm, respectively. Our results do not support their conclusion for both mouse (this report) and human [22] sperm. These discrepancies could be explained in some cases by the specific methods used for measuring AE. In the mouse studies, we used a highly objective assay, i.e., release of EGFP from the sperm acrosome. In contrast, assessment of acrosomal status using Pisum sativum agglutinin, which was used by several groups (including ours when we examined human sperm), is more subjective because the staining patterns are more difficult

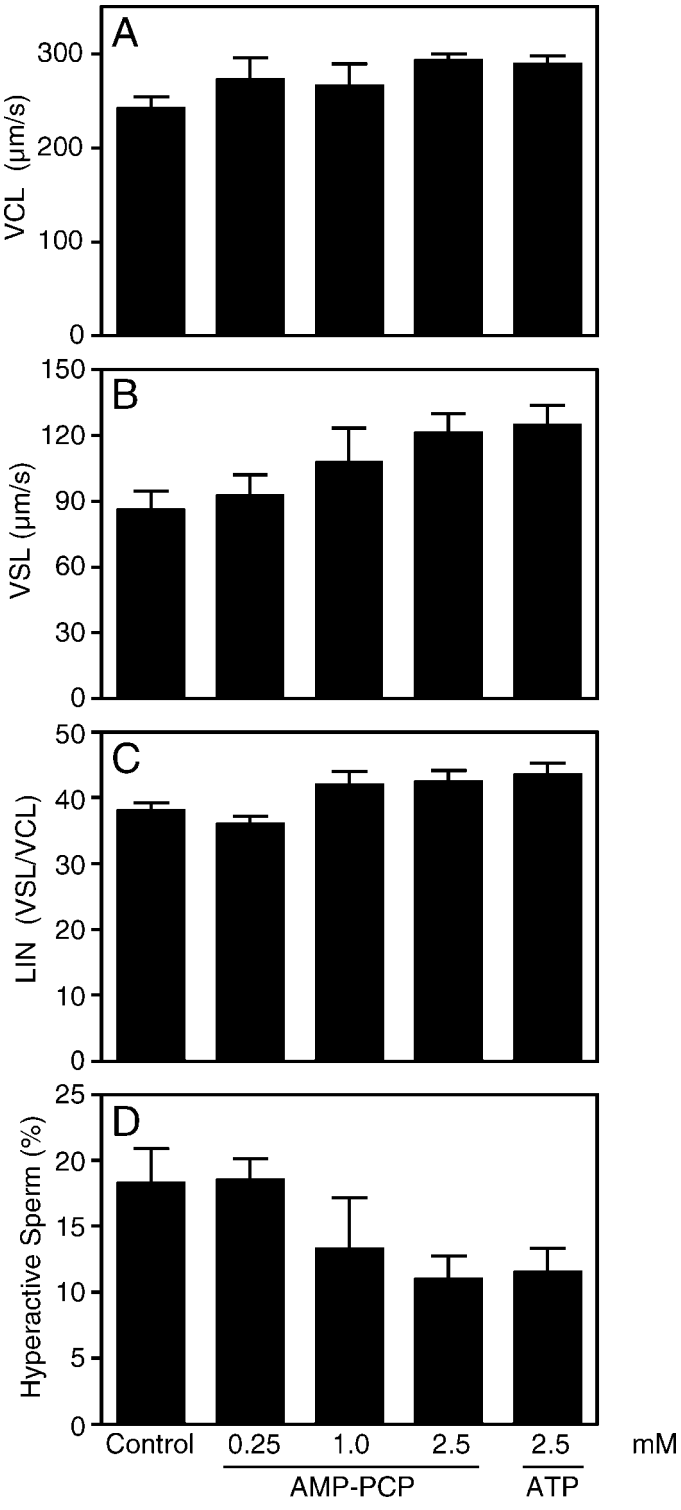

FIG. 5. Effect of β, γ-methyleneadenosine 5′-triphosphate (AMP-PCP) on mouse sperm motility. Sperm were collected into TYH/PVA (Control), TYH/PVA + AMP-PCP (AMP-PCP), or TYH/PVA + ATPe (ATP); the additive concentrations are indicated. After 60 min, sperm were analyzed by CASA. Graphs represent the mean ± SEM of four independent experiments. **A**) VCL. **B**) VSL. **C**) Linearity. **D**) Hyperactivation.

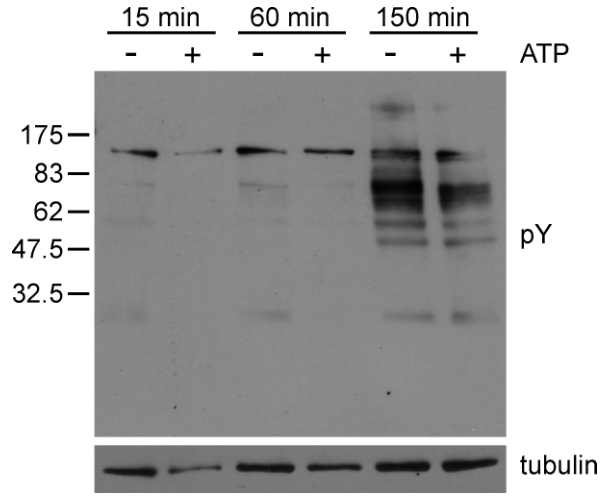

FIG. 6. Effects of ATP on protein tyrosine phosphorylation during mouse sperm capacitation. Sperm were collected into TYH/PVA + 2.5 mM ATPe (ATP) and then incubated in the same medium for 60 min. BSA was then added to the medium and incubation continued an additional 90 min. Aliquots of sperm were collected and analyzed for protein tyrosine phosphorylation (upper panel) at three time points: immediately after release into the medium ± ATP (15 min), after 60 min in the same medium (60 min), or after an additional 90 min incubation in the same medium + BSA (150 min). Molecular weight markers are indicated on the left (kDa). The blot was reprobed for α-tubulin as a loading control (lower panel). This immunoblot is a representative example of four experiments showing similar results. pY, phosphorylated tyrosine.

to interpret. Our finding that ATPe does not increase AE is consistent with the view that ATPe has the potential to enhance fertilization in mouse and human IVF [8]. Acrosome-reacted human sperm do not bind to the zona pellucida of human oocytes [23], suggesting that any procedure that stimulates precocious AE should inhibit, and not improve, the capacity of the sperm to fertilize intact eggs.

Incubation of noncapacitated mouse sperm with ATPe visually changed sperm motility, i.e., the sperm moved faster and more in a straight line. Consistent with this subjective visual observation, objective measurements showed that both straight-line velocity and linearity were significantly increased. Likewise, ATPe-treated capacitated sperm showed higher values for VSL and LIN. The lack of an increase in hyperactive sperm suggests that the increase in fertilizing capacity is not due to a greater proportion of the sperm population becoming hyperactive. Rather, it is possible that faster and straighter swimming sperm will increase IVF success. Whether similar results would be observed in vivo, when sperm must transit the tortuous female reproductive tract to reach the ovulated egg, is unknown.

A number of studies demonstrate that the release of calcium from intracellular stores is involved in hyperactivated motility. Using a detergent-modeled system in which sperm are permeabilized with Triton X-100 and then motility is reactivated by the addition of various nucleotides and other components, Ho et al. [24] showed that calcium is required for hyperactivated motility. Furthermore, using sperm from the *Catsper1*- and *Catsper2*-null animals, *Catsper1*^{tm1Clph} and *Catsper2*^{tm1Clph}, Marquez et al. [25] demonstrated that extracellular calcium is required to supplement store-released calcium to produce a hyperactivated pattern of motility. Although we showed that ATPe caused a rapid transient elevation in intracellular calcium in mouse sperm as determined by calcium imaging of fluo-4-loaded sperm, no increase in the percentage of hyperactivated sperm was observed when these sperm were analyzed at later time points. It is possible that the location or amount of intracellular calcium released during treatment with 2.5 mM ATPe leads to an enhanced progressive, but not hyperactivated, pattern of motility.

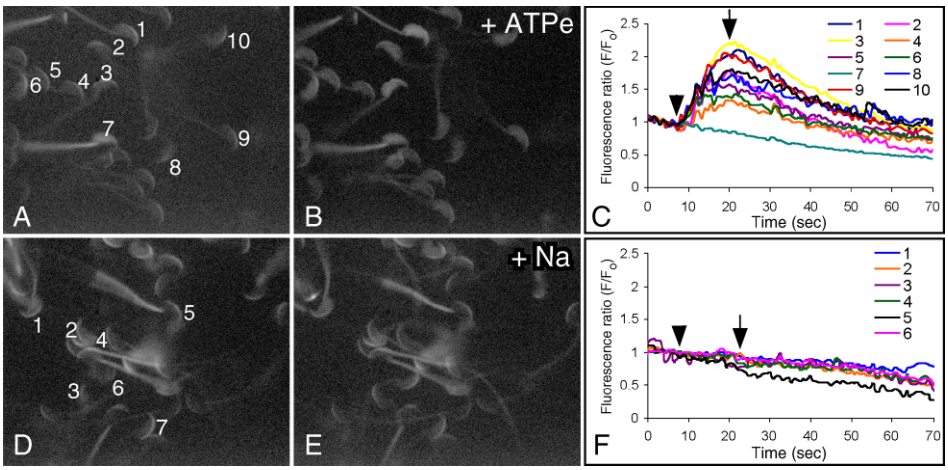

FIG. 7. Effect of ATPe on intracellular calcium in mouse sperm. Sperm fluorescence was recorded to determine the basal levels of intracellular calcium (**A** and **D**) before the addition of 2.5 mM ATPe (**B**) or 5 mM NaCl (+ Na, **E**). The intensity of fluorescence over time was tracked for each sperm (numbered in **A** and **D**) treated with ATPe (**C**) or NaCl (**F**). Original magnification for sperm micrographs was 1000×. Arrowheads indicate the time micrographs were recorded for **A** and **D**; arrows indicate the time micrographs were recorded for **B** and **E**.

Extracellular ATP increases intracellular calcium levels by activating P2 purinergic receptors. The ionotropic P2X receptors consist of ligand-gated cation channels permeable to sodium and calcium; sodium influx via these channels can stimulate subsequent calcium influx via voltage-gated calcium channels. The metabotropic P2Y receptors consist of G protein-coupled cell surface receptors that can increase intracellular calcium via phospholipase C activity [3]. In oviductal cells, ATPe affects ciliary beat frequency by acting through P2Y receptors, resulting in an influx of calcium [26]. Using P2X and P2Y receptor agonists and the same P2 receptor antagonists that we used in this study (RB-2 and PPADS), Luria et al. [7] concluded that ATPe causes an elevation of intracellular calcium in bovine sperm via P2Y receptors. However, both RB-2 and PPADS at the concentrations used in that study can inhibit both P2X and P2Y receptors [3]. In contrast, Foresta et al. [6] suggested that ATPe is not able to trigger a rise in intracellular calcium in human sperm. Additional studies by this group led to the conclusion ATPe causes activation of a monovalent cation channel, presumably a P2X receptor, that causes sodium influx into the sperm [27]. Interestingly, AMP-PCP, the relatively stable ATP analog that we used to address the possibility that the effects in sperm are due to degradation of ATP, is a P2X receptor agonist [28], suggesting that a P2X-mediated pathway is involved in these sperm events. P2X receptors are involved in other aspects of male fertility. When the *P2RX1* gene is ablated, fewer sperm are present in an ejaculate and male fertility is reduced by ~90%, presumably due to a reduction in contraction of the vas deferens to sympathetic nerve stimulation [29].

Our results strongly suggest that at least some of the effects of ATPe are mediated by purinergic receptor-mediated intracellular calcium increases. A rise in calcium could have several downstream effects leading to altered motility. Calcium increases would presumably activate the soluble adenylyl cyclase (ADCY10), leading to an increase in cAMP in the sperm [30]. Cyclic AMP activates protein kinase A and exchange proteins (EPACs), both of which are involved in the stimulation of sperm motility. EPACs regulate the conversion of microtubule sliding into flagellar bending [31], whereas protein kinase A is critical for hyperactivation [32]. Sodium may also be involved in sperm motility as it has been proposed that a putative sperm Na^+/H^+ exchanger (sNHE) is linked with ADCY10 in a signaling pathway [33]. In this regard, sNHE-null males are infertile, and their sperm have extremely poor motility [34]. However, sperm motility and infertility can be rescued with cyclic AMP analogues, suggesting that the role of the sodium is upstream of ADCY10. Last, there is evidence that CATSPER-mediated calcium influx into the midpiece may regulate sperm ATP homeostasis and NADH levels, either of which could affect motility [35]. ATPe-mediated calcium influx, which likely occurs in the midpiece as well as sperm head, could have similar downstream effects.

There is a need to optimize sperm function in assisted reproductive techniques, e.g., for intracervical insemination, intrauterine insemination, and IVF. Our results indicate that incubation of mouse sperm with ATPe significantly increases the success of IVF, likely by altering sperm motility via purinergic receptor-mediated ion fluxes. As discussed, similar results have been reported in human IVF [8], and we have previously shown that ATPe alters motility in human sperm [22]. It has been estimated that at least 40% of infertile couples have difficulty conceiving because of problems attributed to the male, of which >80% show an absence or a reduced motility (asthenozoospermia) [36]. There are few established clinically utilized protocols that enhance sperm fertilization capacity in vivo or in vitro. These studies suggest that modulation of sperm function by ATPe or other reagents that affect purinergic receptors may be beneficial for artificial reproductive techniques used in human and other animal species.

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