Human Reproduction vol.13 no.5 pp.1248–1254, 1998

Enhancement of motility and acrosome reaction in human spermatozoa: differential activation by type-specific phosphodiesterase inhibitors*

J.D.Fisch[1,3], B.Behr[1] and M.Conti[2]

Divisions of [1]Reproductive Endocrinology and Infertility, and [2]Reproductive Biology, Department of Gynecology and Obstetrics, Stanford University School of Medicine, 300 Pasteur Drive, Stanford, CA 94305, USA

[3]To whom correspondence should be addressed

Inhibition of sperm phosphodiesterase (PDE) has been shown to increase cAMP concentrations and stimulate motility and the acrosome reaction. While several PDE genes exist in mammals, little is known about the physiological role of PDE forms expressed in human spermatozoa. Using type-selective inhibitors, we identified two of the PDE forms expressed in human spermatozoa and studied their involvement in sperm function. Selective inhibitors of calcium–calmodulin-regulated PDE1 (8-methoxy-isobutylmethylxanthine) and cAMP-specific PDE4 (RS-25344, Rolipram) were used to study PDE forms in human sperm extracts. 8-MeIBMX and Rolipram/RS-25344 inhibited sperm PDE activity by 35–40 and 25–30% respectively. Subcellular fractionation of the sperm homogenate suggests these pharmacologically distinct forms may be located in separate cellular regions. To evaluate the functional significance of different PDE forms, the effect of type-specific PDE inhibition on sperm motility and the acrosome reaction was examined. PDE4 inhibitors enhanced sperm motility over controls without affecting the acrosome reaction, while PDE1 inhibitors selectively stimulated the acrosome reaction. These data indicate at least two distinct PDE types exist in human spermatozoa. Our findings also support the hypothesis that PDE subtypes affect sperm function by regulating separate pools of cAMP and may ultimately offer novel treatments to infertile couples with abnormal semen parameters.

Key words: acrosome reaction/human spermatozoa/motility/phosphodiesterase inhibitors

Introduction

Cyclic nucleotides act as second messengers that control cell function. Phosphodiesterases (PDE) modulate the activity of cyclic nucleotides by regulating their degradation. There are seven known PDE families (Beavo, 1995; Conti *et al.*, 1995), which have been shown to control cyclic nucleotide concentrations in a variety of mammalian tissues. Animal studies have suggested the presence of specific PDE subsets in developing male gametes (Hoskins *et al.*, 1975; Tash and Means, 1982; Rossi *et al.*, 1985). Naro *et al.* (1996) have indicated that in addition to PDE type 1, type 4 PDE may also be expressed in rat spermatozoa. Several investigators, including Tash and Means (1983) and Yanagimachi (1988), have suggested that sperm motility and the acrosome reaction are regulated by cAMP. The type-4 PDE family is cAMP-specific and regulated by phosphorylation (Conti *et al.*, 1995). The type-1 PDE family is calcium–calmodulin dependent and has a high affinity for cGMP, but also degrades cAMP (Beavo, 1995). Because calcium influx is needed for initiation of the acrosome reaction (Zaneveld *et al.*, 1991; Visconti *et al.*, 1995), we speculate that the type 1 PDE family may be an important modulator of this sperm function.

While non-specific PDE inhibitors such as pentoxifylline and caffeine have been used for years to stimulate human sperm motility and the acrosome reaction (Levin *et al.*, 1981; Yovich *et al.*, 1990; Brennan and Holden, 1995), little is known about the presence, activity and subcellular location of specific PDE subtypes in human spermatozoa. Several previous reports have shown that methylxanthines such as pentoxifylline can stimulate sperm motility parameters (Rees *et al.*, 1990; Yovich *et al.* 1990; Pang *et al.*, 1993). However, the concentrations required to achieve this increase in activity (1–10 mM) also caused significant premature stimulation of the acrosome reaction. Since acrosome reacted cells are incapable of fertilization (Tesarik, 1992; Lanzafame *et al.*, 1994), non-specific PDE inhibition may decrease the benefit from motility enhancement.

Based on previous work in the rat (Rossi *et al.*, 1985; Naro *et al.*, 1996), we speculate that PDE types 1 and 4 are present in human spermatozoa. We further suggest that these distinct PDE forms may regulate separate pools of cAMP through differential localization. Finally, we propose that modulation of sperm function with type-specific PDE inhibitors can selectively enhance motility without stimulating the acrosome reaction. While the use of PDE inhibitors has previously been shown to increase fertilization rates in in-vitro fertilization (IVF) (Yovich *et al.*, 1990), the ability independently to manipulate human sperm function may ultimately lead to increased fertilization rates from less invasive procedures such as intrauterine insemination and may also help to identify patients likely to benefit from intracytoplasmic sperm injection (ICSI).

Materials and methods

Phosphodiesterase assays

The procedure described by Thompson and Appleman (1971) was used for assaying PDE activity. Briefly, 20 µl aliquots of each fraction

*Presented in part at the 53rd Annual Meeting of the American Society for Reproductive Medicine, October 18–22, 1997

were incubated in a final total volume of 200 µl with an incubation mixture containing 20 mM Tris–HCl, pH 8.0, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol and 1 µM ^{3}H-cAMP (~100 000 c.p.m./tube). The reaction was performed for 10–15 min at 34°C with and without PDE inhibitors. Rolipram (Schering AG, Berlin, Germany) and RS-25344 (Roche-Syntex, Palo Alto, CA, USA), selective inhibitors of the type-4 PDE family, and 8-methoxy-isobutyl-methylxanthine (8-MeIBMX; gift of Dr Jack N.Wells, Department of Pharmacology, Vanderbilt University, Nashville, TN, USA), a selective inhibitor of the type 1 family (Kramer *et al.*, 1977), as well as the non-specific inhibitor, IBMX, were used in the various experiments. The reaction was ended by adding a stop solution containing 200 µl 40 mM Tris–HCl, pH 7.4 and 10 mM ethylenediamine-tetra-acetic acid (EDTA) followed by heat denaturation of the enzyme at 100°C for 1 min. The samples were then incubated for 20 min at 34°C with *Crotalus atrox* venom (Sigma Chemical, St Louis, MO, USA) to cleave the labelled adenosine moiety from the 5′AMP. Columns containing AG 1-X8 resin (Bio Rad Laboratories, Hercules, CA, USA) were used to bind the unreacted ^{3}H-cAMP. The eluted fraction containing the labelled adenosine was analysed on a scintillation counter (LS5000TD; Beckman Instruments Inc., Fullerton, CA, USA). The results are reported as picomoles of cAMP hydrolysed per minute per 10^6 cells or as the percentage PDE activity of a sperm extract control incubated in the absence of inhibitor.

PDE inhibition assays

PDE assays performed as described above were used to generate enzyme inhibition curves to verify the presence of specific PDE families in human spermatozoa. A homogenate from normozoospermic, human spermatozoa (prepared as described below) and recombinant PDE 4A generated according to the protocol of Naro *et al.* (1996) were used as PDE sources. Specific inhibitors of PDE1 (8-MeIBMX) and PDE4 (Rolipram, RS-25344), were used at concentrations ranging between 10^{-11} to 10^{-3} M. From these data, optimal inhibitor concentrations for the remaining experiments were set at 10–100 times the compound's IC_{50}, in order to maximize type-specific inhibition, while avoiding non-specific interactions.

Subcellular localization

Normozoospermic, discarded human sperm samples were obtained by masturbation from healthy donors undergoing infertility treatment. Following analysis, the samples were either washed twice in Dulbecco's phosphate buffered saline (PBS) for 10 min at 600 *g* (later referred to as unwashed spermatozoa) or on a discontinuous density gradient (Percoll; Pharmacia Biotech AB, Uppsala, Sweden) at 300 *g* for 20 min followed by two PBS washes at 600 *g* for 10 min (washed spermatozoa). Sperm pellets were stored at –20°C until use. Spermatozoa were homogenized using the procedure described by Horowitz *et al.* (1984). Briefly, pellets were resuspended at a concentration of 100–200×10^6 cells/ml hypotonic homogenization buffer containing: 50 mM Tris–HCl pH 7.4, 12 mM KH_2PO_4, 58.5 mM NaCl, 4.8 mM KCl, 1 mM $MgCl_2$, 5 mM glucose, 0.5 µg/ml leupeptin, 0.7 µg/ml pepstatin, 4 µg/ml aprotinin, 50 mM benzamidine, 10 µg/ml soy bean trypsin inhibitor, and 2 mM PMSF. Spermatozoa were then homogenized with 30 strokes of a Dounce's homogenizer. An aliquot of the homogenate was saved at 4°C for PDE analysis. The remainder was centrifuged at 40 000 *g* at 4°C for 15 min. The supernatant was saved and will be referred to as the cytosol fraction. The pellet was resuspended in 1% Triton X-100 in homogenization buffer. The sample was incubated with gentle agitation for 10 min at 4°C and then centrifuged for 15 min at 40 000 *g*. The supernatant was saved on ice as the membrane fraction. The insoluble pellet was resuspended in homogenization buffer and rehomogenized. Aliquots of 20 µl from the four fractions were assayed for PDE activity in the presence and absence of 3 µM RS-25344 , 20 µM Rolipram, 40 µM 8-MeIBMX or 1 mM IBMXm as described above.

Motility studies

Discarded human sperm samples ($n = 30$) were obtained by masturbation from healthy donors presenting for an infertility evaluation. After analysis the samples were washed on a discontinuous density gradient (Percoll) at 300 *g* for 20 min, following the same procedure used to prepare spermatozoa for intrauterine insemination. The samples were analysed using a computer assisted semen analysis program (CASA, CellTrak/S, Motion Analysis Corp., Santa Rosa, CA, USA). Washed spermatozoa were resuspended in a capacitation media containing Ham's F10 with 2.5% human serum albumin (HSA). Aliquots containing approximately 10×10^6 cells were reacted with the type-specific PDE inhibitors: RS-25344, (10 µM final concentration) and 8MeIBMX, (100 µM final concentration). The inhibitors were dissolved in dimethyl sulphoxide (DMSO, Sigma Chemical). The control was incubated in 0.1% DMSO to match the concentration in the experimental samples. Incubations were performed at 37°C in 5% CO_2 in air. CASA was performed on each sample at 2 h and 24 h using 12 micron slide chambers (Microcell, Conception Technologies, San Diego, CA, USA), and a sampling rate of 60 frames per second. A minimum of 100 cells and three fields was analysed for each aliquot. All analyses were performed at room temperature (18–22°C).

Evaluation of the acrosomal status

Discarded human sperm samples from healthy donors ($n = 6$) were washed on a discontinuous density gradient (Percoll) as above. CASA was performed and samples were standardized at 10×10^6 cells/ml Ham's F10 with 2.5% HSA. Aliquots of 200 µl were incubated with 10 µM RS-25344 or 100 µM 8-MeIBMX for 30 min at 37°C in 5% CO_2 in air. A calcium ionophore, A23187 (Sigma Chemical) at 10 µM final concentration, was used as a positive control and spermatozoa in 0.1% DMSO was used as a negative control. Following incubation, samples were washed in 5 ml PBS and centrifuged at 600 *g* for 10 min. Pellets were resuspended in 200 µl PBS and 20 µl aliquots were pipetted onto precleaned glass slides and allowed to air dry. Duplicate slides were made for each sample. Slides were fixed in 95% ethanol for 5 min at –20° C, were gently washed in PBS and were then labeled with 50 µl 100 µg/ml FITC-Concavalin-A (Sigma Chemical) for 60 min in a humidity chamber at room temperature in darkness. Slides were then washed in excess PBS and mounted with an anti-quenching agent, (Vectashield; Vector Laboratories, Burlingame, CA, USA). Slides were viewed at ×600 under oil on an epifluorescence equipped microscope with a 420–490 nm filter. Counts were validated by a blinded observer. A minimum of 200 cells was counted from each sample.

Institutional review

All experiments were performed on discarded human sperm samples which were coded to avoid any possible link to the donor. There was no effect on patient treatment or outcome. There was no need for informed consent nor for institutional review.

Statistical analysis

For the sperm function experiments, results were analysed using paired two-tailed Student's *t*-tests. Each sample was compared to its own control, incubated at the same time, as described above. The difference between treated and control for each sample was calculated and compared for significance against zero. The sample sizes and nature of the variables allowed adequate normality for computing

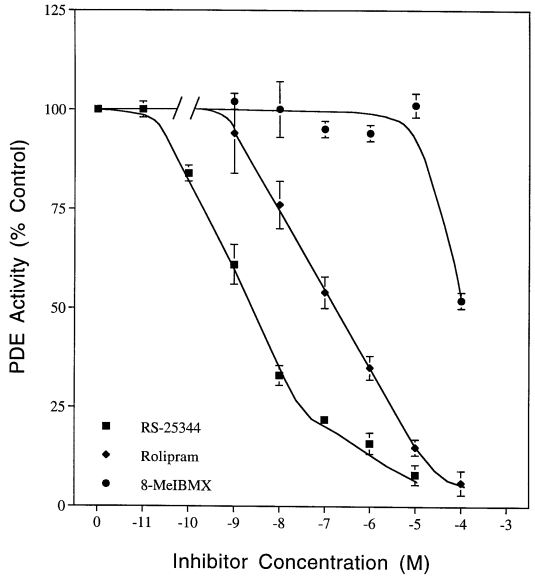

Figure 1. Effect of type-specific inhibitors on PDE activity in Percoll-washed human spermatozoa. A homogenate of Percoll-washed spermatozoa, prepared as described, was used as a source of PDE. Aliquots of the cell suspension were assayed for PDE activity using 1 μM cAMP as substrate in the presence of type 1 (8-MeIBMX) and type 4 (RS-25344) specific PDE inhibitors. Each point represents the mean and SE of three experiments performed in triplicate.

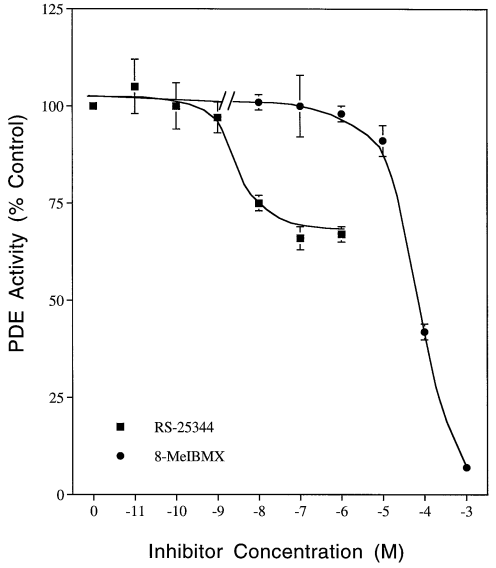

Figure 2. Effect of type-specific inhibitors on recombinant PDE4A activity. Recombinant PDE4A was obtained by transfection in MA-10 cells according to the methods of Naro *et al.* (1996). Aliquots of the cell extract were assayed for PDE activity using 1 μM cAMP as substrate in the presence of type 1 (8-MeIBMX) and type 4 (Rolipram, RS-25344) specific PDE inhibitors. Each point represents the mean and SE of six observations.

P values based on the normal approximation. Data are presented as means and standard errors. The *P* value for each test is given. *P* values are interpreted in light of the modest numbers of tests done.

Results

Enzyme inhibition assays

Using a recombinant PDE4A similar to that expressed in rat male germ cells (Naro *et al.*, 1996), as a target and 1 μM cAMP as substrate, the IC_{50} of RS-25344 was determined to be 1–10 nM. The IC_{50} of Rolipram was determined to be 100 nM, and the IC_{50} for 8-MeIBMX was in the range of 100 μM (Figure 1). At concentrations of 10 μM or less, 8-MeIBMX had no effect on type-4A PDE activity. The fact that at 100 μM there is some inhibition of type-4 PDE by 8-MeIBMX indicated that this inhibitor may not be completely type-specific at these concentrations. Using a homogenate of Percoll-washed spermatozoa as the source of PDE, two distinct inhibition curves were generated (Figure 2). The IC_{50} for each compound was similar to that obtained using recombinant PDE4A. RS-25344 was shown to be a potent inhibitor of sperm PDE activity, with an effective concentration of 0.3 nM and reaching maximum inhibition in the range of 100 nM. Approximately 25–30% of the PDE activity was RS-25344-sensitive, indicating that type 4 PDE is present in human spermatozoa. A similar conclusion was reached using the less potent PDE4 inhibitor, Rolipram (data not shown). 8-MeIBMX was effective from 0.1 μM and reached maximal inhibition at 1 mM. Again, at concentrations above 50–100 μM, this inhibitor is probably no longer completely specific for PDE1, making it difficult to determine the exact percentage of PDE activity attributable to this subtype. Nevertheless, on the basis

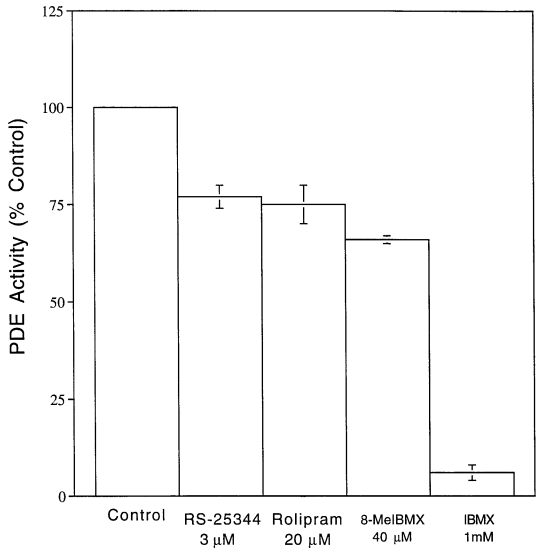

Figure 3. Effect of type-specific PDE inhibitors on a homogenate of Percoll-washed human spermatozoa. A homogenate of human spermatozoa was prepared as described. Aliquots of the total homogenate were then assayed for PDE activity in the presence of type-specific inhibitors. Data for the type 1 and type 4 inhibitors represent the mean and SE of 15 observations. Data for IBMX represent the mean and SE of six observations.

of these experiments, we estimate that ~35–40% of human sperm PDE is type 1, being inhibited by 8-MeIBMX, while another 25–30% is inhibited by Rolipram/RS-25344 and is therefore a PDE4 form (Figure 3). Together, PDE types 1 and 4 accounted for ~60–70% of the PDE activity seen. Approximately 95% of the PDE activity was sensitive to the non-specific inhibitor, IBMX (Figure 3). Preliminary data indicate that 10–15% of human sperm PDE may be sensitive

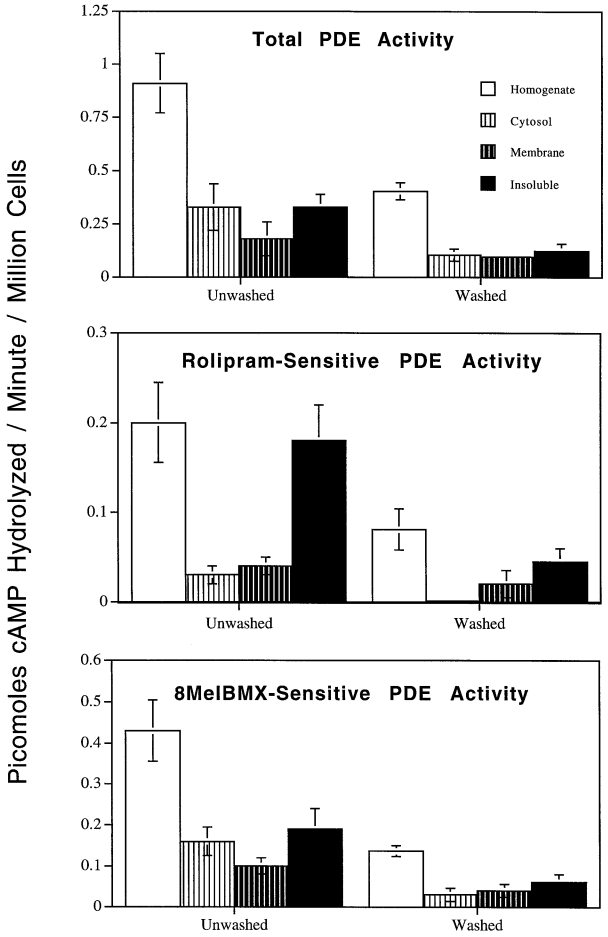

Figure 4. Distribution of PDE activity in human spermatozoa. A homogenate of human spermatozoa (unwashed and washed) was prepared as described in the methods. Differential centrifugation and extraction were used to generate cytosol, membrane and insoluble fractions. Aliquots of cell suspension from each fraction were assayed for PDE activity using 1 μM cAMP as substrate, with and without the type-specific inhibitors. Final concentrations of 40 μM 8-MeIBMX and 20 μM Rolipram were used. The unwashed sperm data represent the mean and SE of four independent experiments and 12 observations. The washed sperm data represent the mean and SE of three independent experiments and nine observations.

to cilostamide, a type 3 specific inhibitor (data not shown) and experiments are underway to investigate this possibility further.

Subcellular localization

A total of 0.9 ± 0.28 pmol/min/10^6 cells of PDE activity was isolated from unwashed human spermatozoa (average of four experiments). Consistent with the above data, ~25–30% of this activity was Rolipram-sensitive, while ~35–40% was 8-MeIBMX sensitive. After differential centrifugation and extraction, roughly one-third of the total activity was recovered from each of the three fractions: cytosol, membrane and insoluble pellet (Figure 4). However, the Rolipram-sensitive activity was recovered mostly in the particulate fraction, while the 8-MeIBMX-sensitive activity was more evenly distributed. When washed on a discontinuous Percoll gradient, only 0.4 ± 0.04 pmol/min/10^6 cells could be recovered, one-half of the activity measured in the unwashed spermatozoa (average of three experiments). While the total recovered PDE activity was decreased, the subcellular distributions remained similar (Figure 4). Total PDE recovery in the fractions was ~80–90% of the homogenate in all groups studied.

Table I. Effect of type-specific PDE inhibitors on motility of washed human spermatozoa. Aliquots of Percoll-washed spermatozoa standardized to 10×10^6 cells were incubated in the presence of type 1 (100 μM 8-MeIBMX) and type 4 (10 μM RS-25344) specific PDE inhibitors. CASA was performed at 2 and 24 h. Significance tests for each sample are compared to the control for that sample

Time	Sample	% Motility (mean ± SE)	*P* value
2 h:			
	Control (n = 27)	71.1 ± 1.9	
	RS-25344 (n = 27)	76.1 ± 1.3	
	Difference	5.1 ± 1.1	0.0001
	Control (n = 17)	70.9 ± 2.4	
	8-MeIBMX (n = 17)	72.5 ± 3.2	
	Difference	1.6 ± 2.2	0.4
24 h:			
	Control (n = 30)	42.5 ± 4.2	
	RS-25344 (n = 30)	47.8 ± 4.1	
	Difference	5.3 ± 2.2	0.023
	Control (n = 15)	36.8 ± 5.2	
	8-MeIBMX (n = 15)	34.0 ± 5.4	
	Difference	–2.9 ± 2.8	0.4

Motility enhancement

A small but significant increase in motility was seen in the RS-25344-treated group at both the 2 h (n = 27) and 24 h (n = 30) incubations (Table I). At 2 h, the average motility of the control was 71.1 ± 1.9%, while that of the RS-25344-treated samples was 76.1 ± 1.3%. The difference between the treated group and control was 5.1 ± 1.1 (P = 0.0001). At 24 h, the average motilities of the control and RS-25344-treated samples were 42.5 ± 4.2 and 47.8 ± 4.1% respectively. The difference, 5.3%, was of borderline statistical significance, P = 0.023. At 2 h, the average motility of the 8-MeIBMX treated group (n = 17) was 70.9 ± 2.4% and at 24 h, 36.8 ± 5.2%. There was no significant difference in motility parameters: straight line velocity (VSL), curvilinear velocity (VCL), linearity (LIN), average lateral head displacement (ALH) or velocity of average path (VAP) noted between groups at either incubation.

When the groups were stratified based on WHO criteria (Table II) for count and motility at the initial analysis, ($>20\times10^6$ cells/ml and >50% motility) the group with abnormal parameters treated with RS-25344 (n = 12) had significantly increased motility at 2 h compared to control (74.3 ± 1.5 versus 65.6 ± 2.0%; difference: 8.7 ± 1.4) (P = 0.00008). No significant difference was seen between treated and control in the normal group at 2 h or in either group at 24 h. Again, the 8-MeIBMX treated samples were not significantly different from control in either group and no increase in VSL, VCL, LIN, ALH or VAP was noted.

Acrosome reaction

The type 4A PDE inhibitor RS-25344 at 10 μM did not significantly affect the acrosome reaction, while the type 1

Table II. The effect of a type 4-specific PDE inhibitor on motility of washed human spermatozoa based on WHO criteria. Semen samples from the experiments in Table I were stratified based on WHO criteria (>20 $\times 10^6$ cells/ml, >50% motility) at the initial analysis. Significance tests for each sample are compared to the control for that sample

Time	WHO Criteria	Sample	% Motility (mean ± SE)	*P* value
2 h:				
	Normal	Control (n = 14)	77.1 ± 2.4	
		RS-25344 (n = 14)	79.4 ± 2.3	
		Difference	2.3 ± 1.3	0.10
		Control (n = 8)	73.5 ± 3.4	
		8-MeIBMX (n = 8)	73.6 ± 4.8	
		Difference	0.1 ± 3.4	0.97
	Abnormal:	Control (n = 12)	65.6 ± 2.0	
		RS-25344 (n = 12)	74.3 ± 1.5	
		Difference	8.7 ± 1.4	0.00008
		Control (n = 7)	67.9 ± 3.2	
		8-MeIBMX (n = 7)	71.0 ± 3.1	
		Difference	3.1 ± 3.2	0.36
24 h:				
	Normal:	Control (n = 15)	35.3 ± 6.5	
		RS-25344 (n = 15)	41.7 ± 6.8	
		Difference	6.5 ± 3.3	0.07
		Control (n = 8)	28.4 ± 6.3	
		8-MeIBMX (n = 8)	29.1 ± 7.5	
		Difference	0.8 ± 4.6	0.9
	Abnormal:	Control (n = 15)	49.6 ± 4.7	
		RS-25344 (n = 15)	53.2 ± 4.8	
		Difference	3.6 ± 3.3	0.29
		Control (n = 7)	49.3 ± 6.6	
		8-MeIBMX (n = 7)	42.6 ± 7.2	
		Difference	–6.7 ± 3.1	0.08

WHO = World Health Organization.

Table III. Effect of type-specific PDE inhibitors on induction of the acrosome reaction. Aliquots of Percoll-washed spermatozoa containing 2×10^6 cells from six healthy donors were incubated for 30 min in the presence of type 1 and type 4-specific PDE inhibitors. Approximately 200 000 cells from each sample were stained with FITC-labelled Concavalin A and viewed under oil at ×400 on an epifluorescence equipped microscope. A minimum of 200 cells was examined from each sample. Results were verified by a blinded observer. Significance tests are compared to control

Compound	PDE inhibited	% reacted cells (mean ± SE)	*P* value
Control (0.1% DMSO)		25.3 ± 3.3	
A23187 (10 μM)		70.5 ± 6.3	
RS-25344 (10 μM):	Type 4	27.9 ± 3.5	0.15
8-MeIBMX (100 μM):	Type 1	56.1 ± 3.8	0.0013

PDE inhibitor 8-MeIBMX at 100 μM had clear stimulating effects. In an average of six experiments, the percentage of acrosome reacted spermatozoa in the negative control was 25.3 ± 3.3%. The positive control, treated with 10 μM A23187, demonstrated an average of 70.5 ± 6.3% reacted cells. The RS-25344-treated group had an average of 27.9 ± 3.5% reacted, while 56.1 ± 3.8% of the 8-MeIBMX-treated group were reacted (P = 0.0013) (Table III).

Discussion

It has long been thought that sperm motility is regulated by a cAMP-dependent pathway (Tash and Means, 1983) and that the acrosome reaction is regulated by calcium–calmodulin (Yanagimachi, 1988; De Jonge *et al.*, 1991). First generation PDE inhibitors such as pentoxifylline have been used to stimulate sperm motility in infertility patients (Yovich *et al.*, 1990). The concentrations used to achieve this increase in motility, however, may be accompanied by an increase in initiation of the acrosome reaction due to nonselective inhibition (Lanzafame *et al.*, 1994). Because acrosome-reacted human spermatozoa are incapable of fertilization, it is important that this reaction is not initiated until the spermatozoa are in close proximity to the egg (Tesarik, 1989). When used for IVF procedures, this non-specific stimulation is not detrimental, but when used to enhance motility of fresh or cryopreserved spermatozoa intended for insemination, the increased percentage of acrosome-reacted spermatozoa becomes significant.

Newer, second-generation PDE inhibitors that are type-specific have recently been developed (Beavo, 1995). We postulate that selective inhibition of specific PDE families could allow enhancement of sperm motility without stimulating the acrosome reaction. While we do not identify all of the PDE types present in human spermatozoa, our pharmacological studies demonstrate the existence of at least two distinct PDE forms. This conclusion is supported by our fractionation studies, which indicate that different PDE subtypes may be located in distinct subcellular compartments in human spermatozoa. The majority of type 4 PDE activity was recovered in the insoluble fraction, suggesting interaction with the flagella, mid piece and cytoskeletal structures, while type 1 PDE was more evenly distributed, with activity recovered in all three fractions. Preliminary data using immunocytochemical staining indicate that type 4 PDE localizes mainly to the sperm mid-piece, while type 1 PDE is found more prominently in the sperm head (manuscript in preparation). These observations support our hypothesis that differential PDE localization within the spermatozoon can allow for selective modulation of sperm function through the regulation of distinct pools of cAMP.

Less total PDE activity was recovered from the Percoll-washed spermatozoa. One possibility to explain this discrepancy is that the increased PDE activity seen in the unwashed spermatozoa could be caused by contamination from lymphocytes, round spermatids or retained cytoplasmic droplets, which are removed after treatment on a density gradient. Because of their relatively large size, the presence of even a small percentage of immature germ cells in a sperm sample could significantly alter the recovery of PDE activity after washing. Aitken (1997) describes the stimulatory effect of reactive oxygen species (ROS) generated from retained cytoplasmic droplets and seminal plasma lymphocytes on sperm capacitation. De Lamirande *et al.* (1997) speculate that these ROS may activate adenylyl cyclase, leading to increased cAMP concentrations. Although elevated cAMP concentrations might lead to an increase in PDE activity, this mechanism has never been demonstrated in human spermatozoa. Tash and Means (1982) have demonstrated increased cAMP protein kinase concentrations in cytoplasmic droplets. It is possible that these droplets which are normally shed into the seminal plasma of mature spermatozoa also contain PDE activity. A density gradient would select against lymphocytes, round cells and

cells with abnormal morphology, such as retained cytoplasmic droplets. Also, to our knowledge, previous localization studies have used epididymal spermatozoa from animal models which were not washed on a density gradient. It is therefore unknown whether our findings are truly discordant from those of previous authors.

Following from the observation of at least two distinct PDE forms, which may be located in separate subcellular regions, we attempted selectively to manipulate sperm function through inhibition of these specific PDE subtypes. Several previous reports have shown successful enhancement of motility using pentoxifylline and caffeine. Levin *et al.* (1981) report a three-fold increase over baseline, but they use an indirect method to measure the percent motility. Others such as Hoskins *et al.* (1975) report data based on ATP-reactivated spermatozoa. Using CASA to measure sperm activity, no investigator has, to our knowledge, been able to achieve more than a 5–10% increase in motility. Our data are consistent with these previous observations (Rees *et al.*, 1990; Pang *et al.*, 1993; Brennan and Holden, 1995). While only a small difference in percentage motility was seen between the RS-25344 -treated and control groups, the enhancement was significant and highly reproducible. Conversely 8-MeIBMX had no apparent effect on sperm motility at 100 μM concentration. Although we have demonstrated some PDE4 inhibition occurs at this concentration in a cell-free system, the intracellular concentration of the inhibitor in intact spermatozoa is probably <100 μM. However, while the data suggest type 1 PDE inhibitors have no effect on motility, our sample size limits our ability to make a definitive statement.

When stratified based on WHO criteria, those patients with abnormal parameters in the initial sample seemed to have most of the significant benefit in motility enhancement from type-specific PDE inhibition. Several of the previous studies have only examined patients with normal semen analyses (Levin *et al.*, 1981; Rees *et al.*, 1990; Pang *et al.*, 1993). Our data support the findings of Yovich *et al.* (1990), who suggest that PDE inhibitors may be especially suited for patients with abnormal semen parameters. Tash and Means (1983) suggest that highly motile spermatozoa may have a maximally stimulated protein kinase A and that a further increase of cAMP using PDE inhibitors would have no additional effect.

Previous reports show an increase in parameters of hyperactivation: (VCL, LIN, ALH) (Pang *et al.*, 1993). Our measurements do not show a significant change in these parameters. While, again, our sample size is not large enough to make a definitive statement, one explanation for this discrepancy may be our use of 12 micron counting chambers which could limit the freedom of spermatozoa motion in three dimensions. In addition, our samples were analysed at room temperature, which is known to slow sperm motility, and which may have masked the observation of hyperactivation. Nevertheless, our data are in line with the work of earlier investigators who report that the most commonly seen effect of PDE inhibitors is an increase in the proportion of motile cells (Tash and Means, 1983). Also reported in some studies is a rapid decline in motility over time (Pang *et al.*, 1993). Levin *et al.* (1981) cite an almost immediate response to pentoxifylline which peaks at 2 h. Under our experimental conditions, we did not begin to see significant differences in activity until 2 h (data not shown) and while total motility decreased over time, the effect of motility enhancement persisted up to 24 h. Another important point is that non-specific inhibitors such as caffeine, pentoxifylline and IBMX, have been used at concentrations in the 1–10 mM range to achieve a clinically recognizable result. Second generation PDE4 inhibitors are effective at concentrations in the 1–10 μM range. This potential for lower concentration dosing may provide an extra margin of safety when considering possible teratogenic effects.

Regarding the effect on induction of the acrosome reaction, our data also support the presence of two functionally separate subpopulations of PDE. We consistently found the percentage of acrosome reacted spermatozoa in Percoll-washed sperm samples treated with the type-4 inhibitor was not different than the control, while the percentage treated with the PDE1 inhibitor was significantly increased over baseline. While our findings regarding the percentages of acrosome reacted cells in the control and A23187 groups may seem high, they are consistent with those of Mortimer *et al.* (1989).

The ability selectively to enhance sperm motility without affecting the acrosome reaction may be clinically useful. In the future, it might be possible to increase the motility of sub-optimal sperm specimens by using type-specific PDE inhibitors. This could potentially increase the probability of fertilization with less invasive treatments such as intrauterine inseminations, thereby avoiding more involved procedures such as IVF or ICSI. Conversely, type-specific PDE inhibitors could also be used as a diagnostic tool. The group of patients in which the acrosome reaction cannot be stimulated with a type-1 inhibitor may have an intrinsic defect in the acrosome. These patients may have poorer fertilization rates in conventional IVF and may be better suited for ICSI. Future clinical trials may help to test these hypotheses.

In summary, our pharmacological studies demonstrate the presence of type 1 and 4 PDE activity in human spermatozoa. Subcellular localization studies suggest that these two PDEs are located in distinct regions of the cell. We have also shown that type-specific PDE inhibitors can be employed selectively to manipulate sperm motility and the acrosome reaction, suggesting that different PDE forms regulate distinct pools of cAMP. These findings may lead to novel, cost-effective clinical applications for the treatment of infertile couples.

Acknowledgements

We would like to acknowledge Dr Michele Salanova, Linda Lan, Douglas Moore and Janice Gebhardt for their input. We would also like to thank Dr Byron Brown in the Department of Biostatistics for his advice on statistical analysis. Finally, we wish thank Dr Robert Alvarez of Syntex (now Roche) for the gift of RS25344 and Dr Jack Wells for the gift of 8-MeIBMX. This research was supported in part by NIH-NICHD Grants # HD 31544 and 1P50-HD 31398.

References

Aitken, R.J. (1997) Molecular mechanisms regulating human sperm function. *Mol. Hum. Reprod.*, **3,** 169–173.

Beavo, J. (1995*)* Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. *Am. J. Physiol.*, **75,** 725–748.

Brennan, A. and Holden, C. (1995) Pentoxifylline-supplemented cryoprotectant improves human sperm motility after cryopreservation. *Hum. Reprod.*, **10,** 2308–2312.

Conti, M., Nemoz, G., Sette, C. and Vicini, E. (1995) Recent progress in understanding the hormonal regulation of phosphodiesterases. *Endocrin. Rev.*, **16,** 370–389.

De Jonge, C.J., Han, H.I., Lawrie, H. *et al.* (1991*)* Modulation of the human sperm the acrosome reaction by effectors of the adenylate cyclase/cyclic AMP second-messenger pathway. *J. Exp. Zool.*, **258,** 113–125.

De Lamirande, E., Leclerc, P. and Gagon, C. (1997) Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction. *Mol. Hum. Reprod.*, **3,** 175–194.

Horowitz, J.A., Toeg, H. and Orr, G.A. (1984) Characterization and localization of cAMP-dependent protein kinases in rat caudal epididymal sperm. *J. Biol. Chem.*, **259,** 832–838.

Hoskins, D.D., Hall, M.L. and Musterman, D. (1975). Induction of motility in immature bovine spermatozoa by cyclic AMP phosphodiesterase inhibitors and seminal plasma. *Biol. Reprod.*, **13,** 168–176.

Kramer, G.L., Garst, J.E., Mitchel, S.S. and Wells, J.N. (1977) Selective inhibition of cyclic nucleotide phosphodiesterases by analogues of 1-methyl-3-isobutylxanthine. *Biochemistry*, **16,** 3316–3321.

Lanzafame, F., Chapman, M.G., Guglielmino, A. *et al.* (1994) Pharmacological stimulation of sperm motility. *Hum. Reprod.*, **9,** 192–199.

Levin, R.M., Greenberg, S.H. and Wein, A.J. (1981) Quantitative analysis of the effects of caffeine on sperm motility and cyclic adenosine 3′, 5′-monophosphate (AMP) phosphodiesterase. *Fertil. Steril.*, **36,** 798–802.

Mortimer, D., Curtis, E.F., Camenzind, A.R. and Tanaka, S. (1989) The spontaneous acrosome reaction of human spermatozoa incubated *in vitro*. *Hum. Reprod.*, **4,** 57–62.

Naro, F., Zhang, R. and Conti, M. (1996) Developmental regulation of unique adenosine 3′,5′-monophosphate-specific phosphodiesterase variants during rat spermatogenesis. *Endocrinology*, **137,** 2464–2472.

Pang, S.C., Chan, P.J. and Lu, A. (1993) Effects of pentoxifylline on sperm motility and hyperactivation in normozoospermic and normokinetic semen. *Fertil. Steril.*, **60,** 336–343.

Rees, J.M., Ford, W.C.L. and Hull, M.G.R. (1990) Effect of caffeine and of pentoxifylline on the motility and metabolism of human spermatozoa. *J. Reprod. Fertil.*, **90,** 147–156.

Rossi, P., Pezzotti, R., Conti, M. and Geremia, R. (1985) Cyclic nucleotide phosphodiesterases in somatic and germ cells of mouse seminiferous tubules. *J. Reprod. Fert.*, **74,** 317–327.

Tash, J.S. and Means, A.R. (1982) Regulation of protein phosphorylation and motility of sperm by cyclic adenosine monophosphate and calcium. *Biol. Reprod.*, **26,** 745–763.

Tash, J.S. and Means, A.R. (1983) Cyclic adenosine 3′, 5′ monophosphate, calcium and protein phosphorylation in flagellar motility. *Biol. Reprod.*, **28,** 75–104.

Tesarik, J. (1989) Appropriate timing of the acrosome reaction is a major requirement for the fertilizing spermatozoon. *Hum. Reprod.*, **4,** 957–961.

Tesarik, J., Mendoza, C. and Carreras, A. (1992) Effects of phosphodiesterase inhibitors caffeine and pentoxifylline on spontaneous and stimulus-induced acrosome reactions in human sperm. *Fertil. Steril.*, **58,** 1185–1190.

Thompson, W.J. and Appleman, M.M. (1971) Multiple cyclic nucleotide phosphodiesterase activities from rat brain. *Biochem. N.Y.*, **10,** 311–316.

Visconti, P.E., Moore, G.D., Bailey, J.L. *et al.* (1995) Capacitation of mouse spermatozoa II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. *Development*, **121,** 1139–1150.

Yanagimachi, R. (1988) Mammalian fertilization. In Knobil, E. and Neill, J. (eds), *The Physiology of Reproduction.* Raven Press, New York, pp. 135–185.

Yovich, J.M., Edirisinghe, W.R., Cummins, J.M. and Yovich, J.L. (1990) Influence of pentoxifylline in severe male factor infertility. *Fertil. Steril.*, **53,** 715–722.

Zaneveld, L.J.D., De Jonge, C.J., Anderson, R.A. and Mack, S.R. (1991) Human sperm capacitation and the acrosome reaction. *Hum. Reprod.*, **6,** 1265–1274.

Received on August 21, 1997; accepted on February 6, 1998