Journal of Andrology, Vol. 24, No. 1, January/February 2003

Implication of Calmodulin-Dependent Phosphodiesterase Type 1 During Bovine Sperm Capacitation

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ABSTRACT: Phosphodiesterases (PDEs) are enzymes that degrade cyclic nucleotides. The calcium-calmodulin dependent PDE type 1 (PDE 1) and the cyclic adenosine monophosphate (cAMP)-specific PDE type 4 (PDE 4) have been implicated in sperm function. We tested the hypothesis that specific PDEs regulate capacitation of bovine sperm in a manner independent of those that mediate motility. Our objectives were to determine the effects of inhibiting PDE 1 and PDE 4 on capacitation and motility, and to compare these effects to those of heparin, which is necessary for capacitation of bull sperm in vitro. Fresh sperm were supplemented either with 15 μg/mL heparin (positive control) or the PDE inhibitors vinpocetine (specific for PDE 1) and rolipram (specific for PDE 4), and then incubated for 5 hours. At 0, 3, and 5 hours, samples were assayed for capacitation and motility parameters according to the chlortetracycline (CTC) fluorescent pattern B and computer-assisted sperm analysis, respectively. A higher percentage of CTC pattern B sperm relative to heparin controls was observed at 0 and 3 hours when sperm were incubated with vinpocetine. After 5 hours, the percentage of heparin- and vinpocetine-treated sperm showing pattern B did not differ ($P > .05$). Rolipram did not affect CTC patterns ($P > .05$; $n = 4$). Vinpocetine and heparin both reduced the percentage of progressively motile sperm after 3 and 5 hours, but vinpocetine reduced it more than heparin ($P < .05$; $n = 4$). Rolipram transiently increased linearity versus sperm with heparin ($P < .05$; $n = 4$). To further test the hypothesis that PDE 1 inhibition permits capacitation, we conducted in vitro fertilization. Vinpocetine did not support the ability of sperm to penetrate homologous oocytes ($n = 5$). Although cAMP regulation by PDE 1 may occur early during capacitation, downstream events appear to prevent full capacitation from occurring prematurely.

Key words: Vinpocetine, rolipram, cyclic AMP, in vitro fertilization, motility.

J Androl 2003;24:104–112

In mammals, freshly ejaculated sperm are not able to fertilize eggs. Sperm acquire this ability in vivo during their transit in the female reproductive tract toward the site of fertilization or in vitro in defined conditions (Yanagimachi, 1994). This time-dependent acquisition of functional competence was first documented (Austin, 1951; Chang, 1951) and designated as capacitation (Austin, 1952) 50 years ago. Capacitation is thus an extratesticular maturational event of mammalian sperm required for binding to the zona pellucida (Si and Olds-Clarke, 1999; Topper et al, 1999) and subsequent induction of the acrosome reaction (Saling et al, 1979; Florman and First, 1988). The precise mechanism of capacitation remains poorly understood but it involves changes in sperm intracellular ion concentrations, plasma membrane fluidity, metabolism, and motility (Yanagimachi, 1994 and references therein). The transmembrane and intracellular signaling events that regulate sperm capacitation appear to be common among mammalian sperm. Capacitation is associated with Ca^{2+} uptake (Handrow et al, 1989), decreased binding of proteins to calmodulin (Leclerc et al, 1989, 1990, 1992), increased cyclic adenosine monophosphate (cAMP; White and Aitken, 1989; Parrish et al, 1994; Parinaud and Milhet, 1996), activation of cAMP-dependent protein kinase A (PKA; Visconti et al, 1997), and changes in protein tyrosine phosphorylation levels (Visconti et al, 1995; Leclerc et al, 1996; Galantino-Homer et al, 1997; Aitken et al, 1998; Tardif et al, 2001). Cyclic AMP is implicated in many sperm functions such as capacitation (Stein and Fraser, 1984; Visconti et al, 1995), acrosome reaction (Garbers and Kopf, 1980; Kopf and Gerton, 1991), and initiation and maintenance of motility (Tash and Means, 1983; Lindemann and Kanous, 1989; Yanagimachi, 1994).

Cyclic nucleotide metabolism consists of a dynamic steady state between synthesis and degradation. Cyclic AMP is produced from adenosine triphosphate (ATP) by adenylyl cyclase (AC) and is degraded by cyclic nucleotide phosphodiesterases (PDE) to adenosine monophosphate (AMP). To date, 11 PDE families or types have

The Natural Sciences and Engineering Council of Canada supported this research. V.F. was a recipient of a graduate fellowship from the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche of Quebec.

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Received for publication May 29, 2001; accepted for publication July 25, 2002.

been identified (Beavo, 1995; Conti et al, 1995; Fisher et al, 1998a,b; Soderling et al, 1998a,b; 1999; Fawcett et al, 2000). The families differ in their amino acid composition, substrate specificities and affinities, their sensitivity to activators and inhibitors, subcellular distribution, and cell and tissue expression (Conti et al, 1995). Many studies have suggested the presence of PDE in male gametes (Gray et al, 1971; Hoskins et al, 1975; Stephens et al, 1979; Rossi et al, 1985) and their implication in sperm motility (McKinney et al, 1994; Jaiswal and Majumder, 1996; Nassar et al, 1999), capacitation (Visconti et al, 1995; Galantino-Homer et al, 1997; de Lamirande et al, 1997) and the acrosome reaction (Tesarik et al, 1992). Richter et al (1999) demonstrated that ejaculated human sperm contain an extended pattern of PDE messenger RNA (mRNA) transcripts corresponding to types 1, 2, 3, 4, 5, and 8. PDEs 1 and 4 are expressed in mammalian sperm and may play a special role in sperm function (Geremia et al, 1982, 1984; Naro et al, 1996). The PDE 1, calcium/calmodulin (Ca^{2+}/CaM)-dependent, preferentially cleaves cyclic guanosine monophosphate (cGMP) but can also degrade cAMP (Beavo, 1995). PDE 4 is cAMP-specific and may be regulated by phosphorylation (Conti et al, 1995). By using specific inhibitors, Fisch et al (1998) showed that in human sperm, PDEs 1 and 4 favor the acrosome reaction and motility, respectively.

Because cAMP and Ca^{2+} regulate capacitation and motility, it is possible that PDE 1 and PDE 4 are also involved (Tash and Means, 1983; Parrish et al, 1994). We hypothesize that separate cAMP pools regulate sperm capacitation and motility, such that one or more specific PDEs regulate capacitation without affecting sperm motility and vice versa. The objectives of this study were to determine the role of PDE 1 and PDE 4 on bovine sperm capacitation and motility using specific inhibitors. These effects are compared with those observed using heparin, which is recognized to induce bovine sperm capacitation in vitro (Parrish et al, 1988).

Materials and Methods

Reagents

Bovine serum albumin (BSA; fraction V, fatty acid free), polyvinyl alcohol (PVA; molecular weight 30 000–70 000 Daltons), chlortetracycline hydrochloride (CTC), heparin (from porcine intestinal mucosa, 175 USP units/mg), Percoll, and rolipram were purchased from Sigma Chemical Company (St Louis, Mo). Vinpocetine was obtained from Calbiochem (Darmstadt, Germany).

Culture Media

Two basic media were prepared as described by Galantino-Homer et al (1997): a modified Tyrodes Hepes-buffered medium (Talp-H; 100 mM NaCl, 3.1 mM KCl, 0.3 mM NaH_2PO_4, 21.6 mM sodium lactate, 0.4 mM $MgCl_2 \cdot 6H_2O$, 2.0 mM $CaCl_2 \cdot 2H_2O$, 40.0 mM Hepes, 1.0 mM pyruvate, 50 μg/mL gentamycin sulfate, 1 mg/mL PVA, and 10 mM $NaHCO_3$) and a modified Tyrodes bicarbonate-buffered medium (Sp-Talp; 100 mM NaCl, 3.1 mM KCl, 0.3 mM NaH_2PO_4, 21.6 mM sodium lactate, 0.4 mM $MgCl_2 \cdot 6H_2O$, 2.0 mM $CaCl_2 \cdot 2H_2O$, 10.0 mM Hepes, 1.0 mM pyruvate, 6 mg/mL BSA, and 25 mM $NaHCO_3$). The pHs were adjusted to 7.4 at 23°C and both media were sterilized by passage through a 0.22-μm pore filter (Millipore Corporation, Bedford, Mass).

Collection and Preparation of Semen

Fresh bovine semen collected using an artificial vagina was generously donated by the Centre d'Insémination Artificielle du Québec (St Hyacinthe, QC, Canada). Semen was diluted 1:1 into egg yolk-Tris diluent without glycerol (0.25 M Tris, 60 mM citric acid, 69 mM fructose, 25% [v/v] egg yolk and an antibiotic cocktail containing tylosin, gentamicin, and lincospin) and was transported in a thermos (25°C) to the university laboratory within 2.5 hours of collection. The sperm were isolated by Percoll centrifugation as described by Parrish et al (1995). Two milliliters of semen were layered on the top of a gradient composed of 4-mL fractions each of 90% and 45% isotonic Percoll. After a 30-minute centrifugation at 700 × *g* (25°C), the supernatant was discarded and the pellet was washed twice at 280 × *g* for 10 minutes (38°C) with Talp-H. After the second centrifugation, the supernatant was removed and 3 mL of Sp-Talp were added. The concentration of sperm was estimated using a hemocytometer and adjusted with Sp-Talp to 50×10^6 sperm/mL for evaluation of capacitation and motility and to 1×10^6 sperm/mL for in vitro fertilization (IVF). The inhibitor treatments were added to the sperm suspension immediately before incubation. The sperm samples (1 mL) in 15-mL conical tubes were incubated in conditions to promote capacitation (39°C, 5% CO_2, 100% humidity) for up to 5 hours. At 0, 3, and 5 hours, 200-μL aliquots were removed for evaluation of capacitation, motility, and viability.

Eosin-Nigrosin Staining

Sperm viability was determined by eosin-nigrosin staining (Barth and Oko, 1989). Ten-microliter samples were mixed with 10 μL of modified eosin-nigrosin solution containing 0.5% (w/v) sodium citrate (Hancock, 1951) and 10 μL of this mixture was smeared onto a clean slide at room temperature. After drying, the slides were covered with Permount S-15 (Fisher, Montreal, QC, Canada) and mounted on a glass slide under a coverslip. At least 200 cells per slide were counted by light microscopy (400×) and the percentage of live sperm (unstained) was evaluated.

Evaluation of Capacitation by the Chlortetracycline Assay

The fluorescent antibiotic CTC was used to assess sperm capacitation. A modification of the technique described by Ward and Storey (1984) was used (Collin et al, 2000). The CTC solution contained 750 μM CTC and 5 mM DL-cysteine dissolved in 20 mM Tris and 130 mM NaCl (TN stock), and the pH was adjusted to 7.8. The CTC solution was prepared daily and kept at 4°C in

foil to protect it from the light. Fifteen microliters of CTC solution and 10 μL of spermatozoa were mixed in a warmed glass well; 0.5 μL of 12.5% glutaraldehyde in TN stock (pH 7.4) was then added. Ten microliters of this suspension were placed on a clean slide at 37°C. After mounting with a coverslip, the slides were stored in dark at 4°C until counting within 24 hours. The slides were assessed with a Nikon microscope equipped with phase contrast and epifluorescence optics; cells were observed under blue-violet illumination (excitation at 400–440 nm and emission at 470 nm). Two hundred spermatozoa per slide were counted and classified as one of three distinct CTC staining patterns as described by Fraser et al (1995). The precapacitated sperm had uniform fluorescence over the head (pattern F), capacitated sperm had a fluorescence-free band in the postacrosomal region (pattern B), and acrosome-reacted sperm had dull head fluorescence except for a thin band of fluorescence along the equatorial segment (pattern AR).

Motility Studies

To assess sperm motion, an HTM IVOS unit (Hamilton-Thorne Research, Beverly, MA) was used with MicroCell slides (20 μm deep; Conception Technologies, San Diego, Calif) essentially as described by Chamberland et al (2001). Ten microliters of sperm solution were deposited in the chamber (prewarmed to 37°C). The slide was placed on the stage of the motility analyzer and both chambers on each slide were evaluated as duplicates. Five fields per chamber were selected for computer-assisted analysis. The values of computer settings were determined by examining the sample of fresh bull sperm with the ''playback'' screen: frame acquired, 20 Hz; frame rate, 30/second; minimum contrast, 7; minimum size, 7; low and high size gates, 0.3 and 1.5, respectively; low and high intensity gates, 0.3 and 1.5, respectively; medium and low average path velocities, 80 μm/s and 20 μm/s, respectively; no motile head size, 14 pixels; no motile brightness, 14 pixels; threshold straightness, 80%; temperature, 38.5°C. Nine characteristics were evaluated: the percentage of motile sperm, progressive motility (PROG), average path velocity (VAP), curvilinear velocity (VCL), straight line or progressive velocity (VSL), linearity (LIN = VSL/VCL × 100), straightness (STR = VSL/VAP × 100), amplitude of lateral head displacement (ALH), and beat cross frequency (BCF).

In Vitro Fertilization

Bovine ovaries were collected at a slaughterhouse and transported to the laboratory within 2–3 hours in saline (0.9% w/v NaCl, 35°C). Cumulus-oocyte complexes (COCs) were aspirated from 1–5 mm follicles. After selection, isolated (intact) COCs were washed three times in Hepes-buffered Tyrodes medium (TLH; Bavister et al, 1983) supplemented with 10% heat-treated fetal calf serum (FCS; Flow Laboratories, McLean, Va), 0.2 mM pyruvate, and 50 μg/mL gentamicin (Sigma) then matured in vitro for 22–24 hours as described by Chian and Sirard (1995). The matured oocytes were used for IVF according to Blondin and Sirard (1995) and were modified to assess sperm fertility by Cormier et al (1997). Fresh semen was used for treatments and cryopreserved semen as an internal control to confirm oocyte quality. Treated sperm were added at 1×10^6/mL to each droplet containing five COCs and incubated for 6 hours at 39°C in fertilization medium (TALP containing 20 mM pyruvate, 1 mM hypotaurine, 2 mM penicillamine, 250 μM epinephrine, and 0.6% BSA ± 15 μg/mL heparin, ± 150 μM vinpocetine). After 6 hours, surplus sperm were removed and COCs were placed in FCS-enriched medium (TCM-199 supplemented with 10% FCS, 0.2 mM pyruvate, and 50 μg/mL gentamicin) for 12 hours. The COCs with cryopreserved sperm (internal control) were incubated in fertilization medium with 2 μg/mL heparin for a full 18 hours. This internal control assured the quality of the COCs and other experimental conditions. At the end of incubation (18 hours from the initial addition of sperm), the oocytes were denuded of the attached cumulus cells by gentle vortexing, fixed in ethanol:acetic acid (3:1), and stained with 1% orcein (w/v) in 40% acetic acid (v/v). An oocyte was judged as fertilized when either an enlarged spermatozoa head or two pronuclei were seen within cytoplasm by light microscopy (200×).

Statistical Analysis

For the CTC assay, motility, IVF, and the eosin-nigrosin staining, we conducted analysis of variance with the general linear models procedure using the Statistical Analysis System (SAS, 1990). The models included ejaculate; concentration of heparin, vinpocetine, or rolipram; and their interactions. When main effects were significant ($P < .05$), preplanned comparisons among treatments were conducted using the protected Fisher least significant difference test.

Results

Chlortetracycline Determination of Capacitation

Capacitation was evaluated by the percentage of spermatozoa exhibiting CTC pattern B (Ward and Storey, 1984; Fraser et al, 1995; Cormier et al, 1997). The effect of heparin (positive control) was compared with the addition of a specific PDE 1 inhibitor, vinpocetine, and a specific PDE 4 inhibitor, rolipram. Sperm suspensions were supplemented with either 15 μg/mL heparin or with vinpocetine (0, 50, 100, and 150 μM; Figure 1). Initially, vinpocetine (150 μM) increased the percentage of CTC pattern B sperm relative to heparin controls ($P < .05$). After 3 hours, the proportion of pattern B decreased with vinpocetine (150 μM) but was always higher than heparin ($P < .05$). After 5 hours, the percentage of heparin- and vinpocetine- (150 μM) treated sperm showing pattern B did not differ ($P > .05$). Rolipram did not affect the CTC fluorescence pattern at any time of incubation (Figure 2; $P > .05$).

Determination of Sperm Viability by Eosin-Nigrosin Staining

Sperm viability in the presence of vinpocetine or heparin is displayed in Table 1. After 3 hours of incubation, vinpocetine did not significantly affect the viability of sperm, however, more dead sperm were observed with heparin than with control or vinpocetine treatments ($P < .05$).

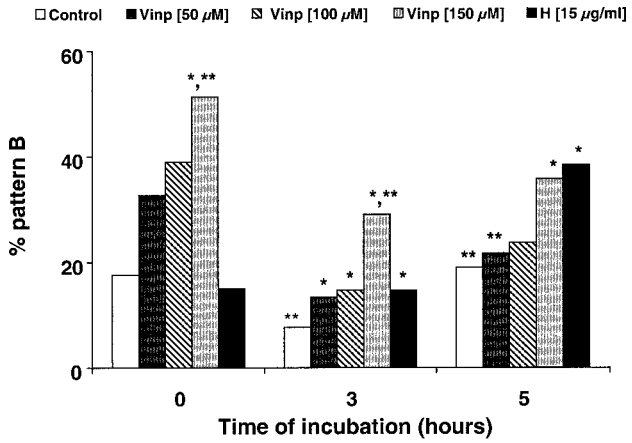

Figure 1. Evaluation of sperm capacitation in the presence of PDE 1 inhibitor, vinpocetine (Vinp), or heparin (H) as assessed by the percentage of sperm showing the CTC pattern B after an incubation of 5 hours (n = 3; SE ± 5%). Within each time, * indicates significant differences from negative control (Control); ** indicate significant differences from positive control (H, 15 μg/mL; $P < .05$).

Following 5 hours of incubation, sperm treated with either heparin or vinpocetine (100 and 150 μM) were less viable than control sperm ($P < .05$). Rolipram treatments did not affect sperm viability during incubation for 5 hours ($P > .05$; data not shown).

Evaluation of Sperm Motility

Among the nine characteristics of sperm motion evaluated by computer-assisted sperm analysis, the specific PDE inhibitors affected only PROG and LIN. The effects of the PDE 1 inhibitor vinpocetine on the percentage of progressive sperm compared to those of heparin are presented in Figure 3. At 3 hours, PROG with vinpocetine (100 and 150 μM) was different than that of controls but only the 150 μM dose was significantly lower than heparin ($P < .01$). After 5 hours of incubation, a decrease of the

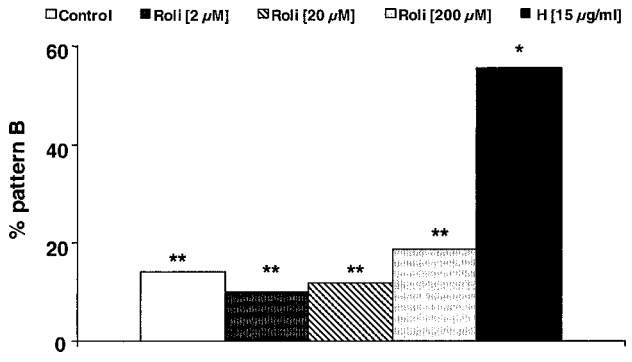

Figure 2. Evaluation of sperm capacitation in the presence of a PDE 4 inhibitor, rolipram (Roli), or heparin (H) as assessed by the percentage of sperm showing the CTC pattern B after an incubation of 5 hours (n = 4; SE ± 8%). Within each time, * indicates significant differences from negative control (Control); ** indicates significant differences from positive control (H, 15 μg/mL) ($P < .01$).

Table 1. *Evaluation of sperm viability in the presence of a PDE 1 inhibitor, vinpocetine, or heparin by eosin-nigrosin staining during an incubation of 5 hours (n = 3; SE ± 5%)*

Treatment	Time of incubation (hours)		
	0	3	5
Control	90	78†	78†
Vinpocetine (50 μM)	91	75†	71†
Vinpocetine (100 μM)	90	69†	67*†
Vinpocetine (150 μM)	91	68†	61*†
Heparin (15 μg/ml)	92	55*	47*

* SD from negative control (control) within time of incubation ($P < .05$).
† SD from positive control (heparin [15 μg/mL]) within time of incubation ($P < .05$).

percentage of PROG was observed with heparin and vinpocetine (100 and 150 μM), and PROG with 150 μM vinpocetine was still lower than that of heparin ($P < .01$). The PDE 4 inhibitor rolipram affected only the percentage of LIN sperm (Figure 4). After 3 hours of incubation, 200 μM rolipram increased the LIN of sperm ($P < .05$) in a manner observed in neither the negative nor positive (heparin) controls.

Determination of Capacitation by IVF

The effect of blocking sperm PDE 1 on IVF rates is shown in Table 2. The presence of heparin increased nearly fivefold the percentage of oocytes penetrated by sperm compared with controls ($P < .05$). Compared with the positive heparin controls, inclusion of the PDE 1 inhibitor vinpocetine (without heparin) did not promote fertilization because more than seven times fewer oocytes were penetrated ($P < .05$), which was similar to negative (heparin-free) controls. However, there was no difference in fertility between the combination of vinpocetine plus hep-

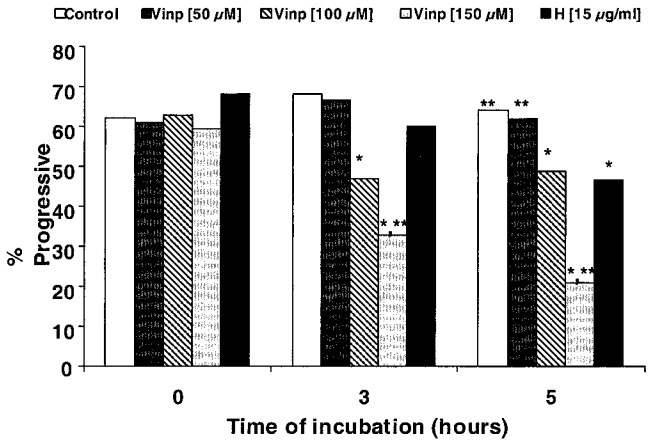

Figure 3. Effects of PDE 1 inhibitor, vinpocetine (Vinp), and heparin (H) on the percentage of progressively motile sperm during an incubation of 5 hours (n = 4; SE ± 10%). Within each time, * indicates significant differences from negative control (Control); ** indicates significant differences from positive control (H 15 μg/mL) ($P < .01$).

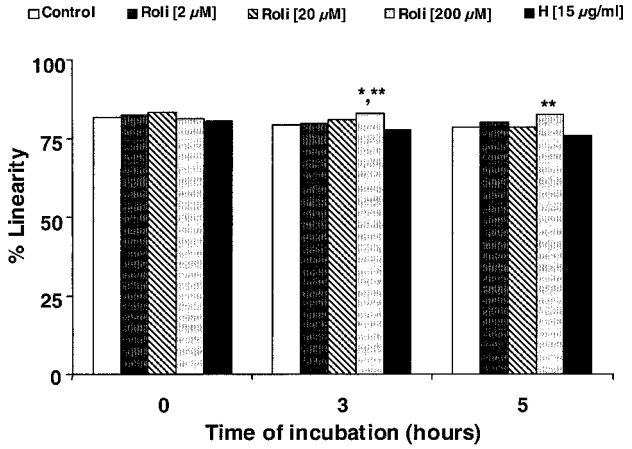

Figure 4. Effects of PDE 4 inhibitor, rolipram (Roli), and heparin (H) on the percentage of linear sperm during an incubation of 5 hours (n = 4; SE ± 3%). Within each time, * indicates significant differences from negative control (Control); ** indicates significant differences from positive control (H 15 μg/mL) ($P < .05$).

arin and heparin alone ($P > .05$), indicating that vinpocetine did not negatively affect oocyte function.

Discussion

This study shows that inhibition of a Ca^{2+}/CaM-dependent PDE 1 rapidly induces capacitation of bull sperm, reported by the CTC assay, and some, but not all motility changes that are similar to those observed with heparin, a known capacitation inducer of bull sperm. These data suggest that a Ca^{2+}/CaM-dependent PDE 1 is implicated in the onset of capacitation, but not its completion, because sperm treated with a PDE 1 inhibitor were unable to penetrate homologous oocytes as those treated with heparin.

It is recognized that sperm capacitation (Visconti et al, 1995, 1997; Leclerc et al, 1996; Galantino-Homer et al, 1997; Aitken et al, 1998) and motility (Tash and Means, 1983) are regulated by a cAMP-dependent pathway. Therefore, the control of cAMP levels in sperm is very important to properly regulate the effects of this messenger. In human sperm, the degradation of cyclic nucleotides by PDE occurs at a rate 9-fold to 600-fold faster than that of their synthesis by adenylyl cyclase (Cheng and Boettcher, 1982). It is thus probable that PDE plays a role in sperm functions. Fisch et al (1998) demonstrated the presence of two distinct PDE families in human sperm, PDE 1 and PDE 4, which appear to regulate the acrosome reaction and motility, respectively. These two PDE families may be implicated in these critical sperm functions by regulating separate pools of cAMP, which could explain why this second messenger can control several different sperm functions at different times, such as progressive motility (Tash and Means, 1983), capacitation (Visconti et al, 1998) and the acrosome reaction (Leclerc and Kopf, 1995).

Regarding the effects on capacitation, rolipram, a PDE 4 inhibitor, has no effect, whereas vinpocetine, a specific inhibitor of the Ca^{2+}/CaM-dependent PDE 1, increased the rate of capacitation according to CTC pattern B fluorescence in a dose-response manner. The Ca^{2+}/CaM complex regulates a large number of enzymes in many cell types (Cheung, 1980; Means et al, 1982). Calmodulin is present in both the acrosomal region and the flagellum of sperm (Manjunath et al, 1993; Leclerc and Goupil, 2000) and is necessary for PDE 1 activity. The implication of PDE 1 in capacitation is supported by the observation that CaM intracellular concentration decreases during heparin-induced capacitation of bull sperm (Leclerc et al, 1992). As sperm CaM declines during capacitation, PDE 1 would not be activated to hydrolyze cAMP, and is thus in agreement with an increased cAMP pool observed during capacitation (White and Aitken, 1989; Parrish et al, 1994; Parinaud and Milhet, 1996).

PDE 1 inhibition induced a rapid appearance of the CTC pattern B in a dose-response manner supporting the hypothesis that this enzyme family mediates membrane changes associated with capacitation. The rapid appearance of pattern B was not an artifact due to a fluorescent interaction between CTC and the vinpocetine because this compound is not fluorescent at the wavelengths used (data not shown). Our results suggest that an increase in sperm cAMP concentration by PDE 1 inhibition is responsible for the rapid appearance of CTC pattern B. Harrison and

Table 2. *Effect of blocking sperm PDE 1 by vinpocetine on in vitro fertilization rates (n = 3 to 5)*

Sperm Treatment	Presence of Heparin (15 μg/mL)	Fertilization Rate	
		Number of Oocytes (Penetrated/Total)	Percentage
Negative control	−	21/159	15.3†
Positive control	+	99/137	72.5*
Vinpocetine (150 μM)	−	12/103	9.8†
Vinpocetine (150 μM)	+	36/53	68.9*

* SD from negative control ($P < .05$).
† SD from positive control ($P < .05$).

Miller (2000) proposed that plasma membrane lipid architecture in boar sperm during capacitation is controlled via a target protein phosphorylated by PKA and is dephosphorylated by a protein phosphatase type 1. The PKA can be activated by the high cAMP level and increase membrane fluidity leading to capacitation. Furthermore, in boar sperm, these changes are extremely rapid, resulting in increased membrane fluidity within minutes and in the present study, vinpocetine increased the proportion of bull sperm showing pattern B (capacitated) fluorescence immediately. The mechanism of the CTC assay reflects the location of membrane calcium, which in turn is dependent on the characteristics of bilayer phospholipids. Therefore, in the presence of an inhibitor of a capacitation-specific PDE 1 family (according to our hypothesis), any PKA-mediated plasma membrane modifications would be reflected in the elevated percentage of sperm displaying CTC pattern B even at the beginning of the incubation.

In contrast, sperm treated with high concentrations of the PDE 1 inhibitor vinpocetine did not penetrate COCs as those treated with heparin. Our findings resemble those of Lefièvre et al (2000), who observed that sildenfafil, an inhibitor of PDE 5, a cGMP-dependent PDE, promoted the sperm protein tyrosine phosphorylation that is associated with capacitation in humans, but is inadequate to promote the acrosome reaction. Clearly, PDE inhibition favors certain events associated with capacitation, but not all of them. A possible explanation for the results of the present study (Figure 1 and Table 2) is that the regulation of cAMP by PDE 1 occurs early during capacitation, however, full capacitation is prevented from prematurely occurring by downstream events. For example, the dephosphorylation of capacitation-related molecules by phosphatases that regulate lipid architecture (Harrison and Miller, 2000) or of tyrosine-phosphorylated sperm proteins (Visconti et al, 1998) could prevent premature capacitation. The aim of PDE 1 inhibition is to increase the cAMP concentration, which itself represents an important step of capacitation, yet on its own, elevated cAMP may be insufficient to support complete capacitation. Alternatively, perhaps another type of PDE is activated in the sperm to compensate for the inhibition of PDE 1 in an effort to restrict premature cAMP elevation. Capacitation has been reported to be a reversible phenomenon, largely due to the ability of components from the male reproductive tract to restabilize the membranes of capacitated sperm after their reintroduction (discussed in Yanagamachi, 1994). However, based on the observations that PDE 1 inhibition leads to membrane changes that resemble capacitation (as indicated by the pattern B staining), one can also speculate that some internal process, perhaps a phosphatase or another PDE type, rapidly reversed the vinpocetine-induced capacitation. Consequently, vinpocetine would not be sufficient to induce functional capacitation in its entirety because its effects are too transient to be functional (ie, oocyte fertilization during IVF). However, this is a weak speculation; if vinpocetin even temporarily induced functional capacitation, the oocyte penetration rate would, in theory, be greater than that of the negative control sperm, which did not happen (Table 2).

Numerous studies have demonstrated that the addition of heparin is essential to induce bovine sperm capacitation in vitro (Parrish et al, 1988; Miller et al, 1990; Galantino-Homer et al, 1997; Chamberland et al, 2001). In this study, heparin reduced sperm viability relative to vinpocetine, the PDE 1 inhibitor. Capacitation is an ongoing destabilization process and sperm become increasingly fragile owing to various alterations in the plasma membrane (Harrison, 1996). In this sense, a drop in sperm survival can be an indirect indicator of full capacitation such that the changes induced by the PDE 1 inhibitor do not fully correspond to complete capacitation (as heparin) because vinpocetine maintained sperm viability. By these observations, the present study shows that the CTC assay is representative of sperm physiology indicating the early stages of a series of molecular changes leading to capacitation. However, the CTC pattern B does not necessarily indicate full capacitation because the sperm treated with the PDE 1 inhibitor showed a CTC pattern B yet could not penetrate COC, as discussed earlier.

During incubation with heparin, sperm progressive motility is reduced. Vinpocetine induced similar change in the percentage of progressively motile sperm, supporting the hypothesis that PDE 1 mimics heparin. Although the mechanisms of the motility changes in sperm due to heparin are unclear, an increase of cAMP during capacitation may lead to hyperactivation. Heparin elevates cAMP and intracellular Ca^{2+} in bull sperm (Handrow et al, 1989; Parrish et al, 1989, 1994) and these molecules are directly implicated in the regulation of sperm motility (Garbers and Kopf, 1980; Tash and Means, 1983; Garty and Salomon, 1987). Chamberland et al (2001) observed greater BCF and ALH in bull sperm treated with heparin. These parameters are believed to be related to sperm hyperactivation that occurs in vivo and favors sperm oocyte penetration (Suarez et al, 1991; Suarez and Dai, 1992; Stauss et al, 1995). In the present study, however, the PDE 1 inhibitor did not affect these parameters. The inhibition of PDE 1 altered sperm motility but contrary to heparin, it did not affect the parameters associated with hyperactivation. PDE 1 clearly does not affect all molecular changes implicated in motility that favors fertilization.

The inhibition of PDE 4 with rolipram slightly changed motility parameters, but in a manner different from heparin. Rolipram induces a minor but significant increase in the percentage of linear motile sperm, although the high

concentration required to induce these changes renders the physiological relevance of the results somewhat questionable. Tash and Means (1983) postulated that in highly motile noncapacitated sperm (such as the heparin-free fresh sperm used in this study), the PKA activity is already at maximal levels. Thus it is possible that using PDE inhibitors to increase cAMP has no additional effect on PKA activity in the absence of heparin. This may be why rolipram did not markedly alter sperm motion, however, it would be interesting to repeat this experiment using poor motility sperm like those from cryopreserved semen.

In summary, the present study demonstrates that PDE 1 inhibition mimics certain effects of heparin (the membrane modifications reported by CTC pattern B fluorescence and motility changes) but can not support complete capacitation (fertilization of homologous oocytes during IVF). Our experiments do support the hypothesis that multiple PDE families in sperm affect different sperm functions by regulating separate pools of cAMP (Fisch et al, 1998). This study also supports the role of a cAMP-dependent signaling pathway in capacitation (Visconti et al, 1995; Leclerc et al, 1996; Galantino-Homer et al, 1997; Aitken et al, 1998) via increased cAMP levels. Furthermore, these results reinforce the hypothesis that a loss of calmodulin accompanies capacitation of bull sperm (Leclerc et al, 1992), thereby favoring the increased cAMP levels necessary to drive the signaling events associated with this phenomenon.

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