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ARTICLE *in* JOURNAL OF ANDROLOGY · MARCH 2004

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Journal of Andrology, Vol. 25, No. 2, March/April 2004

Interaction of PDC-109, the Major Secretory Protein From Bull Seminal Vesicles, With Bovine Sperm Membrane Ca^{2+}-ATPase

SILVIA SÁNCHEZ-LUENGO,* GERHARD AUMÜLLER,* MARTIN ALBRECHT,* PARIMAL C. SEN,‡ KARL-HEINRICH RÖHM,† AND BEATE WILHELM*

*From the *Department of Anatomy and Cell Biology, Philipps-Universität, Marburg, Germany; ‡Department of Biochemistry, Philipps-Universität, Marburg, Germany; and †Department of Chemistry, Bose-Institute, Calcutta, India.*

ABSTRACT: PDC-109 is the prevalent secretory protein from bovine seminal vesicles that binds to the midpiece of sperm once they pass the ampulla of the vas deferens during emission. Thereby, the protein changes biophysical membrane properties, eventually resulting in increased sperm motility. To elucidate the underlying biochemical mechanism, we have studied the ion-pumping activity (Ca^{2+}-ATPase) in membrane preparations of bovine spermatozoa following in vitro incubation with the protein and analyzed whether PDC-109 influences sperm motility. PDC-109 was purified to homogeneity from bull seminal vesicle extracts using a newly described method. The effect of PDC-109 on sperm motility was analyzed using the CASA-method. These experiments clearly demonstrated that PDC-109 significantly increases sperm motility. Calcium-pumping mechanisms were analyzed by monitoring the effect of PDC-109 on various parameters of enzyme activity of Ca^{2+}-ATPase in epididymal sperm plasma membranes and were compared with Ca^{2+}-ATPase activities from other organs and from epididymal sperm of different species, respectively. Specificity studies were performed using different Ca^{2+}-antagonists. Enzyme activities of both Mg^{2+}-dependent and Mg^{2+}-independent Ca^{2+}-ATPases increased in a dose-dependent manner following the addition of the PDC-109 (range 5–20 μg). Preincubation of PDC-109 at temperatures above 37°C and pHs ranging from below 6.5 and above 8.5 led to the loss of the stimulatory effect. An analysis of enzyme kinetics pointed to irreversible, cooperative interaction of PDC-109 with the enzyme. The effect was organ-specific, that is, restricted to sperm ATPases, but it was not species-specific, as it could be elicited also in rat sperm.

Key words: Seminal vesicles, bovine sperm, plasma membrane Ca^{2+}-ATPase.

J Androl 2004;25:234–244

Seminal vesicles synthesize a variety of different products in an androgen-dependent manner (Aumüller and Riva, 1992), such as ions (eg, potassium) and low-molecular-weight substances (eg, fructose, prostaglandins), as well as peptides and proteins. The latter include structural proteins (eg, semenogelin [Lilja et al, 1987]), transport proteins (eg, transferrin), immunomodulators, enzymes (eg, 5′-nucleotidase [Fini and Cannistrado, 1990]), enzyme inhibitors, and sperm-modulating-proteins (eg, sperm-coating antigen, SCA [Abrescia et al, 1985]).

Electrophoretic studies have detected a number of basic proteins in the secretion of the bovine seminal vesicles, including a ''basic acrosin inhibitor'' (BUSI [Tamblyn, 1983]) and 5′-nucleotidase (Fini and Cannistrado, 1990).

Esch et al (1983) have purified three different major proteins from bovine seminal plasma named BSP I, BSP II, and BSP III. BSP II and III were later found to be identical and were designated PDC-109, referring to their composition of 109 amino acids. Calvete et al (1994) demonstrated that BSP I is the glycosylated form of PDC-109. The bull seminal proteins BSP-A1 and BSP-A2 described by Seidah et al (1987) and Major Protein (Scheit et al, 1988) were also identified as PDC-109. Structure analysis revealed that the peptide chain of PDC-109 is arranged in 2 antiparallel β-sheets, which lie perpendicular to each other, and 2 irregular loops supporting a large hydrophobic site (Constantine et al, 1992). The protein belongs to a group of fibronectin type II module proteins, present in the seminal secretions of several mammals (Saalmann et al, 2001) which display a particular protein-lipid binding ability.

PDC-109 binds to the choline group of phosphorylcholine-containing phospholipids in a very rapid process (Muller et al, 1998). Interaction of PDC-109 with artificial phosphorylcholine vesicles provoked permeability changes and partial disruption of the vesicles (Gasset et al, 2000). Protein crystallography revealed that the 2 fibronectin domains are separated by a shorter linker polypep-

This study was supported by the Deutsche Forschungsgemeinschaft (Graduiertenkolleg 533 Giessen-Marburg, Project A1) and the DAAD/DST (personal exchange project 422-PPP 1999/D/9915802).

Correspondence to: Prof Dr Gerhard Aumüller, Department of Anatomy and Cell Biology, Robert-Koch-Str 8, D-35033 Marburg, Germany (e-mail: aumuelle@mailer.uni-marburg.de).

tide that supports the clustering of the 2 domains (Wah et al, 2002). Thereby, PDC-109 stimulates cholesterol efflux after binding to the sperm membrane and phospholipid efflux in the early stages of capacitation (Gasset et al, 2000; Manjunath and Therien, 2002). Furthermore, it binds to choline lipids, thereby increasing the heparin-docking sites on the sperm surface (Therien et al, 1999). Greube et al (2001) have demonstrated that PDC-109 alters the membrane structure of lipid vesicles and likewise of bovine spermatozoa.

A comparable membrane effect has previously been suggested by our group (Aumüller et al, 1988), based on immunoelectron microscopic findings of the protein's being located underneath the plasma membrane of the sperm midpiece of epididymal sperm, once these were incubated with seminal vesicle secretion.

In the present study, we describe a novel biochemical feature of PDC-109 during its interaction with bovine epididymal sperm. Studying the activity of plasma membrane–bound calcium ATPases in bovine sperm, we found a strong stimulatory effect of PDC-109. This was analyzed in detail using conventional biochemical assays. Our results point to dose-dependent, saturable, and potentially irreversible cooperative effect of PDC-109 on sperm plasma membrane ATPases, resulting in increased motility of the spermatozoa.

Materials and Methods

Collection of Genital and Extragenital Organs

Organs (seminal vesicles, epididymis, liver, kidney, heart) were obtained from young mature bulls immediately after slaughter in the local slaughterhouse. Rat epididymis was dissected from sexually mature male Wistar rats (290–310 g body weight; Charles River Wiga GmbH, Sulzfeld, Germany), which were anesthetized, then killed by cervical dislocation. All tissues were immersed in PBS.

Purification of PDC-109

PDC-109 was purified from bovine seminal vesicles secretion, using a modified method described by Calvete et al (1996). To isolate the secretion, seminal vesicles were minced in tissue pieces (1 cm × 1 cm) and subsequently immersed in a solution containing 50 mM Tris-HCl buffer, pH 7.4, 150 mM NaCl, and 5 mM EDTA (ethylenediamine tetra-acetic acid). After the aqueous secretion extract was centrifuged at 12,000 × *g* for 15 minutes, the supernatant was applied to a Heparin Sepharose column (Amersham Pharmacia Biotech, Freiburg, Germany) equilibrated with 50 mM Tris-HCl buffer pH 7.4, containing 150 mM NaCl and 5 mM EDTA. Bound PDC-109 was eluted with 10 mM phosphorylcholine in 50 mM Tris-HCl buffer pH 7.4, 150 mM NaCl, and 5 mM EDTA. The Bio-Rad protein assay was used to determine protein concentrations (Bio-Rad Laboratories, Richmond, Calif). To check the purity of the fractions, tricine-SDS-PAGE was performed according to the method of Schagger and von Jagow (1987), using 12% separating slab gels. Subsequently, the gels were stained with Coomassie brilliant blue R-250.

Amino Acid Sequence Analysis

Purified PDC-109 (80 μg) was run on a preparative tricine-SDS-PAGE (12% separating slab gels) and transferred onto a PVDF membrane (Immobilon-P, Millipore) according to Kyhse-Andersen (1984). The blotted protein bands were stained with Coomassie brilliant blue for 1 minute. After destaining the membranes in 50% methanol and washing thoroughly in water, the 13 kDa and 14 kDa bands, respectively, were excised and cut into small pieces. N-terminal sequence analysis of both samples was carried out by Edman degradation, as described by Linder et al (1994).

Western Blotting Experiments

Isolated rat PDC-109 protein in native and in denatured form (SDS-PAGE strips) was used for immunization of rabbits. The immunization was carried out by Seramun (Berlin, Germany) using the standard immunization protocol. Polyclonal antisera raised in rabbits against native PDC-109 and denaturated PDC-109, respectively, were subsequently used for Western blotting. Western blotting was performed as previously described by Wilhelm et al (1998). Seminal vesicle secretion (15 μg) and purified PDC-109 (3 μg) were transferred from SDS-PAGE onto nitrocellulose membranes (Hybond; Amersham Pharmacia Biotech, Freiburg, Germany). The strips were incubated with Roti-block (Bio-Rad, Munich, Germany) to block nonspecific binding sites and were then incubated overnight at room temperature either with the antibody against native PDC-109 (1:20,000) or with the antibody against denaturated PDC-109 (1:1000). Detection of the immunoreaction with a peroxidase-labelled antibody against rabbit IgG (1:100,000; Pierce, Bonn, Germany) was performed using the SuperSignal system (Pierce, Bonn, Germany) according to the manufacturer's instructions.

Preparation of Microsomal Membranes

Microsomal membranes were prepared from epididymal sperm from bovine and rat organs as well as from different bovine tissues (liver, heart, kidney). Sperm were released from bovine and rat epididymides, respectively, by cutting the different epididymal segments several times with a sharp razor blade and swirling the tissue in 250 mM sucrose solution containing 5 mM Tris-HCl buffer pH 7.4, 1 mM EDTA, and 10 μm PMSF (phenylmethane sulfonyl fluoride). Sperm were isolated from the following epididymal regions: 1) distal corpus to proximal cauda, briefly termed epididymal sperm, 2) caput, 3) corpus, 4) cauda, and 5) ampulla of the vas deferens. The sperm were centrifuged for 10 minutes at 2500 × *g*. To obtain a higher amount of membrane protein, sperm were incubated in hyperosmotic swelling or HOS-buffer (65 mM saccharose, 65 mM sodium citrate, 1 mM EDTA, 10 μm PMSF) at 37°C for 45 minutes (Bohring and Krause, 1999). Samples were then centrifuged again at 2500 × *g* for 10 minutes and sperm were resuspended in homogenization buffer (500 mM imidazole, 1 mM EDTA, 1 mM DTE [1,4-dithioerythritol], and 250 mM sucrose, pH 8.5). The sperm, as well as the control tissues, were homogenized in homogenization buffer by 8 strokes in a glass potter. The homogenate was cen-

trifuged for 10 minutes at 2500 × *g* at 4°C, the supernatant was collected, and the pellet was resuspended in homogenization buffer and centrifuged again at 2500 × *g*. This step was repeated and the pooled supernatants were spun at 15 000 × *g* at 4°C for 15 minutes. The microsomal membranes were sedimented for 1 hour at 100 000 × *g* at 4°C. The pellet was resuspended in a small volume of homogenization buffer and the protein concentration was determined by using the Bio-Rad protein assay.

Ca^{2+}-ATPase Activity Assay

Mg^{2+}-dependent and Mg^{2+}-independent Ca^{2+}-ATPase activity assays, respectively, were performed according to Sikdar et al (1991). To assay the activity of Mg^{2+}-dependent Ca^{2+}-ATPase, 5 μg of the microsomal membrane preparation and 3 mM ATP were added to the reaction buffer containing 54.5 mM histidine hydrochloride, pH 7.5, 2 mM EGTA (ethylene glycol-bis[2-aminoethylether]-N,N,N′,N′-tetra-acetic acid), 0.2 mM DTE, 50 mM sucrose, 1 mM $MgCl_2$, and 4 mM $CaCl_2$ in a total volume of 250 μL. After incubation for 30 minutes at 37°C, the reaction was stopped by adding 70 μL of 30% ice-cold trichloroacetic acid (TCA); the sample was subsequently diluted with 550 μL of distilled water. To determine the amount of enzymatically released inorganic phosphate (P_i), a solution containing 100 μL of 1.75% ammonium molybdate and 100 μL of 2% ascorbic acid was added. After 10 minutes of incubation at room temperature, the complexed P_i was quantified using a spectrophotometer at 820 nm. Enzyme activity was expressed μM P_i/mg protein/h. The activity of the Mg^{2+}-independent Ca^{2+}-ATPase was measured in a buffer system containing 50 mM imidazole, pH 8.5, 2 mM EGTA, 0.2 mM DTE, 50 mM sucrose, and 4 mM $CaCl_2$, as described above. In both cases, Ca^{2+}-ATPase activity was defined as the difference of activity in the presence and absence of Ca^{2+}-ions.

To identify the specificity of enzyme activities, Ca^{2+}-antagonists such as nifedipine (0.2 mM), verapamil (0.2 mM), and trifluoperazine (TFP, 0.2 μM) were used. The stimulatory effect of PDC-109 on Mg^{2+}-independent and Mg^{2+}-dependent Ca^{2+}-ATPase activities, respectively, was determined by adding different amounts of PDC-109 (range: 5 μg to 80 μg) to the reaction mixture. Both PDC-109 and the respective antagonists were added to the reaction mixture prior to the addition of the microsomal enzyme preparation and the substrate (ATP).

Temperature and pH Sensitivity of PDC-109

Ca^{2+}-ATPase activities were measured as described above in the presence of 20 μg PDC-109. PDC-109 was preincubated at different temperatures (25°C, 33°C, 37°C, 41°C) or different pHs (6.6, 7.0, 7.5, 8.0, 8.5, 9.0) for 30 minutes. Activity determinations with and without addition of 20 μM PDC-109 were then performed as described above.

Mechanism of the Stimulatory Effect

In order to identify the substrate optimum for the stimulatory effect of PDC-109 on enzyme kinetics, ATPase activity was measured as a function of ATP concentrations at a ratio of 5 μM to 20 μg PDC-109. The dependence of the enzyme reaction velocity on substrate (ATP) concentration was analyzed using nonlinear regression to estimate [S] 0.5 (the substrate concentration at half maximal velocity) and V_{max} (describing the maximum velocity of the reaction at saturating substrate concentration).

In order to prove the reversibility/irreversibility of the stimulating effect of PDC-109 on Ca^{2+}-ATPase-activity, the microsomal fraction was incubated with PDC-109 for 30 minutes in a relation of 5 μg membranes: 20 μg PDC-109, and the Ca^{2+}-ATPase activity was estimated within an aliquot. In order to remove unbound PDC-109, a fraction of the incubated enzyme was centrifuged for 1 hour at 100 000 × *g* at 4°C, and the supernatant containing the unbound PDC-109 was removed. The pellet was resuspended and the ATPase activity in the resultant pellet was measured. The procedure of washing was repeated 6 times. The total experiment was repeated 3 times. To check the amount of membrane loss during the washing steps, a control sample was treated in the same way but without adding PDC-109. Furthermore, an aliquot (6 μg) of the pellets obtained was analyzed by SDS-PAGE (12% tricine-gel) as described above.

CASA-Method

To record the grade of motility, the computer-assisted, computer-aided software system (CASA type SM-CMA; Mika Medical, Montreal, Canada) method was used. CASA technologies calculate a number of parameters characterizing sperm motion. Sperm released from epididymis were washed by centrifugation in HBS-puffer at 300 × *g* during 10 minutes and the pellet was resuspended in HBS-buffer. Sperm concentration was adjusted to approximately 10^6 and spread in a Makler counting chamber with 10 μM volume. The chamber was maintained at 37°C temperature. The following parameters were measured by the CASA system: VCL = curvilinear velocity (μm/s), VSL = straight-line velocity (μm/s), and VAP = average path velocity (μm/s). For obtaining statistically significant results, 250 spermatozoa were counted in every experiment.

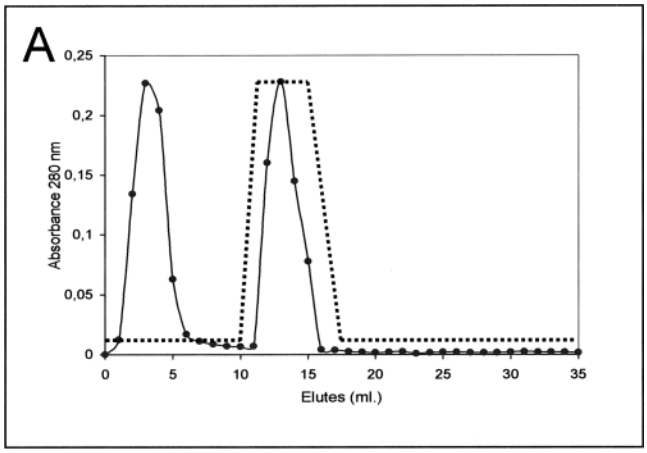
Statistical Evaluation

For the statistical evaluation, Statgraphics® plus software (Manugistics, Inc, Rockville, Md) was used. The data presented here were confirmed in at least 4 independent experiments. Statistical analyses performed were the one-way analysis of variance (ANOVA) and Wilcoxon's test for correlated data. In all the cases, a P-value $< .05$ was considered to have statistical significance.

Results

Purification of PDC-109

PDC-109 was purified to homogeneity from bovine seminal vesicle secretion using a single-step purification procedure on Heparin Sepharose. PDC-109 was eluted from the column using 10 mM phosphorylcholine. SDS-PAGE and Coomassie staining demonstrated a double band of about 13 and 14 kDa (Figure 1). To analyze whether both proteins represented PDC-109, an N-terminal amino acid sequence analysis was performed. The N-termini of both proteins were identical with the N-terminus of the mature PDC-109 (Asp-Gln-Asp-Glu-Gly-Val-Ser-Thr-Glu), corresponding to the amino acids 26–34 (Kemme and Scheit,



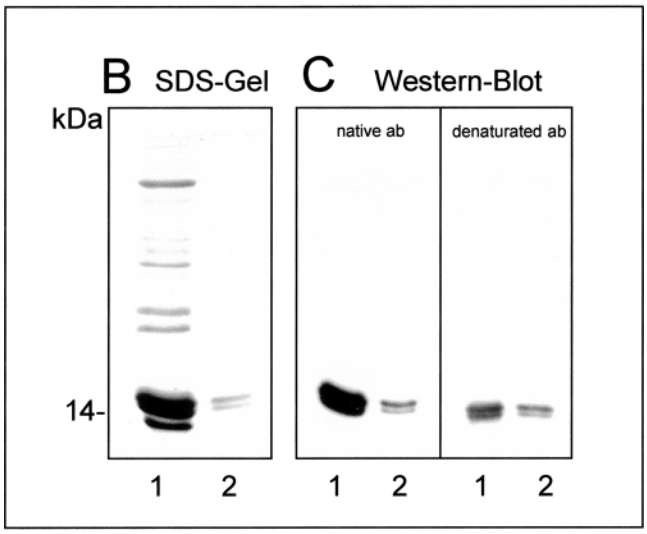


Figure 1. **(A)** Separation of secretory proteins of bull seminal vesicles using affinity chromatography on Heparin Sepharose. PDC-109 was eluted with 10 mM phosphorylcholine (second peak). The content of 10 mM phosphorylcholine in the running buffer is shown as dotted line. **(B)** Tricine-SDS-Page of bull seminal vesicle secretion (lane 1, 10 μg) and purified PDC-109 (lane 2, 2 μg) stained with Coomassie brilliant blue. **(C)** Western-blot analysis of secretory protein from the seminal vesicle (lane 1, 10 μg) and isolated PDC-109 (lane 2, 2 μg) using antibodies against native and denaturated PDC-109.

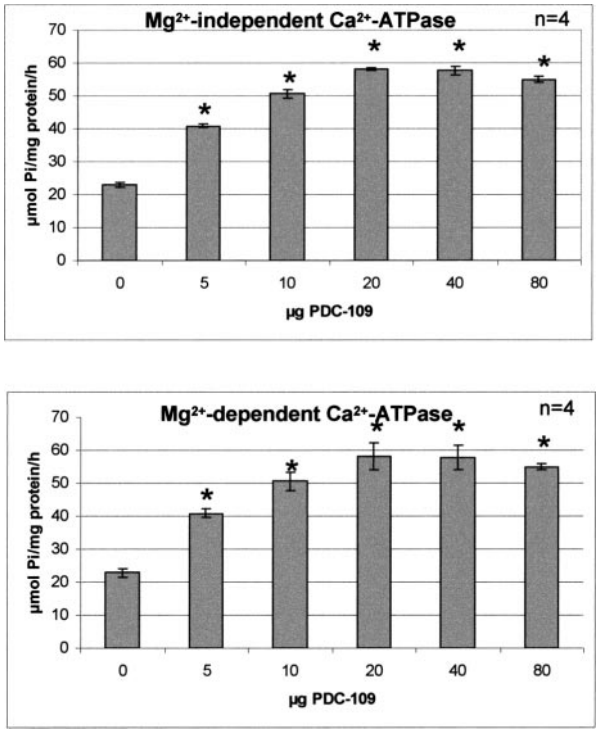


Figure 2. Effect of different concentrations of PDC-109 on Ca^{2+}-ATPase activity of epididymal sperm. Vertical bars show the standard error of the mean. *P* value $< .05$ (*).

1988). Performing a Western blot of seminal vesicle secretion using the polyclonal antibodies produced a double band at 13/14 kD, indicating that PDC-109 was immunoreactive in the extract (Figure 1).

Ca^{2+}-ATPase Activity in Bovine Epididymal Sperm

Both Mg^{2+}-dependent and Mg^{2+}-independent Ca^{2+}-ATPase activities were present in epididymal sperm (Figure 2, left column; Table 1). Specificity was proven as ATPase activities were inhibited by the specific Ca^{2+}-antagonists verapamil, TFP and nifedipine, respectively. Twenty-five μm of TFP inhibited the activity of Mg^{2+}-independent Ca^{2+}-ATPase by 56% and of Mg^{2+}-dependent Ca^{2+}-ATPase by 65%, while 0.2 mM verapamil decreased the Mg^{2+}-independent Ca^{2+}-ATPase by 65% and Mg^{2+}-dependent Ca^{2+}-ATPase by 76%. Addition of 0.2 mM ni-

Table 1. *Effects of PDC-109 on Mg^{2+}-independent Ca^{2+}-ATPase and Mg^{2+}-dependent Ca^{2+}-ATPase activity of microsomal membranes from epididymal sperm**

	Mg^{2+}-independent		Mg^{2+}-dependent	
Parameter	−PDC–109	+PDC–109	−PDC–109	+PDC–109
μmol P_i/mg protein/h	22.9 ± 2.6	58.2 ± 8	16.6 ± 1.3	41.45 ± 0.59
Relative Ca^{2+}-ATPase activity (%)	100	254	100	249
Analysis of variance (ANOVA), treatment vs. no treatment samples	. . .	$P < .05$	. . .	$P < .05$

* Assays were performed in presences and absence of 20 μg PDC−109.

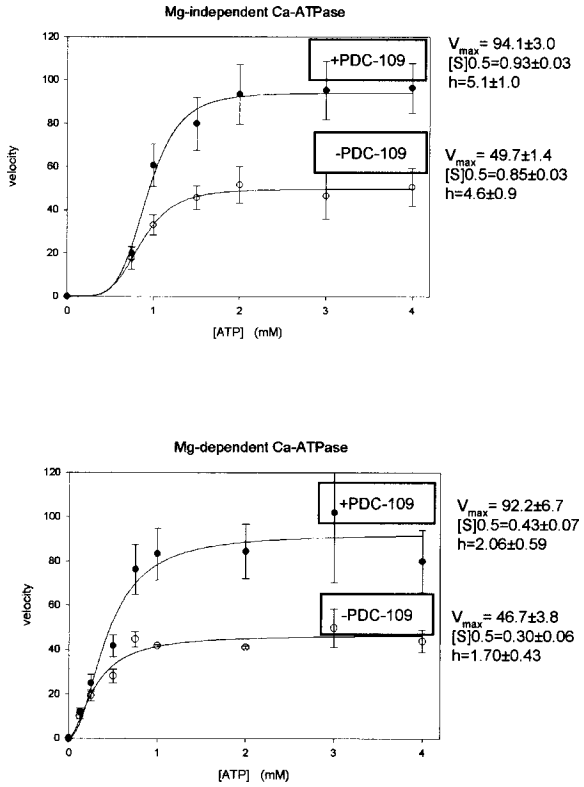


Figure 3. Enzyme kinetics of Ca^{2+}-ATPase: The dependence of velocity (μmol Pi/mg protein/h) to substrate concentration was analyzed with 20 μg (marked as +PDC-109) and without PDC-109 (marked as −PDC-109). Ca^{2+}-ATPase activities were assayed in the presence of different concentrations of ATP as described in the text. V_{max} = maximal velocity, [S] 0.5 = substrate concentration at half maximal velocity, h = Hill-coefficient. Vertical bars indicate SD.

fedipine inhibits 80% of the Mg^{2+}-independent Ca^{2+}-ATPase activity, and 77% of the Mg^{2+}-dependent Ca^{2+}-ATPase activity, respectively.

Influence of PDC-109 on Ca^{2+}-ATPase Activity in Epididymal Sperm

To prove the hypothesis of a stimulatory effect of PDC-109 on sperm membrane Ca^{2+}-ATPases, the influence of different concentrations of PDC-109 on Mg^{2+}-independent and Mg^{2+}-dependent Ca^{2+}-ATPases, respectively, in microsomal membranes of epididymal sperm was measured. A concentration-dependent activity increase was observed for both Mg^{2+}-independent and Mg^{2+}-dependent Ca^{2+}-ATPase following the addition of PDC-109. A plateau of enzyme stimulation was reached at a concentration of about 20 μg PDC-109 (Figure 2), that is, addition of amounts of PDC-109 above 20 μg did not further increase enzyme activity. Following the addition of 20 μg PDC-109, Mg^{2+}-independent Ca^{2+}-ATPase was increased by 154%, whereas Mg^{2+}-dependent Ca^{2+}-ATPase activity was increased by 149% (Table 1). Effects were statistically significant ($P < .05$). As deduced from these experiments, the PDC-109 concentration for the following experiments was chosen at 20 μg.

Temperature effects on the activation of Ca^{2+}-ATPase by PDC-109 were studied by preincubation of PDC-109 at 25°C, 33°C, 37°C, and 41°C for 30 minutes prior to adding the protein to the ATPase assay. Incubation at 37°C revealed no change in the stimulatory effect of PDC-109 on ATPase activity. Preincubation above 37°C led to a decrease of the stimulatory effect. It was completely lost after heating the protein up to 40°C.

Effects of the pH on the stimulation of Ca^{2+}-ATPase activity by PDC-109 were studied by preincubation of PDC-109 at different pH values ranging from 6.5 to 9. Incubation at pH ranging from 7.5 to 8.5 revealed no change of ATPase activity stimulation. Incubation at pH values below pH 6.5 and above pH 8.5 led to the decrease of the stimulatory effect.

Mechanism of Stimulation

The kinetic parameters [S] 0.5 (substrate concentration at half maximal velocity) and V_{max} (maximal velocity) for the substrate ATP were determined by varying substrate concentrations (range 5 μM to 4 mM, final concentration) in the ATPase assays. The experiments were performed with and without 20 μg PDC-109. The results are depicted in Figure 3. The addition of PDC-109 to the assay did not influence the affinity of the enzyme to the substrate (ATP). The [S] 0.5 values were nearly identical in both assays, that is, with and without PDC-109; however, different values were shown for Mg^{2+}-independent (0.9 mM) and Mg^{2+}-dependent Ca^{2+}-ATPase (0.35 mM) activities, respectively. In contrast, V_{max} was increased double by addition of PDC-109 to the Mg^{2+}-dependent and -independent activity assays.

To check the reversibility/irreversibility of the interaction between PDC-109 and Ca^{2+}-ATPase, microsomal membranes from epididymal sperm were incubated for 30 minutes with PDC-109. Following the incubation time, Ca^{2+}-ATPase activity was determined showing enzyme activation up to 150%. To separate the presumed Ca^{2+}-ATPase/PDC-109-complex, a fraction of the reaction mixture was diluted in buffer and then centrifuged at 100 000 × *g*. This washing procedure was performed several times. Enzyme activity of the pellets formed was measured again and showed the same stimulating effect, compared to the respective control (without PDC-109), even after 6 washing steps. Furthermore, SDS-PAGE of the resulted pellets clearly demonstrated that PDC-109 was still present in the membrane samples (Figure 4), suggesting that binding of PDC-109 to the enzyme is irreversible.

Organ and Species Specificity of the Stimulatory Effect of PDC-109 on Ca^{2+}-ATPase

In order to measure the potential activating effect of PDC-109 (20 μg) on Ca^{2+}-ATPase of sperm membranes from

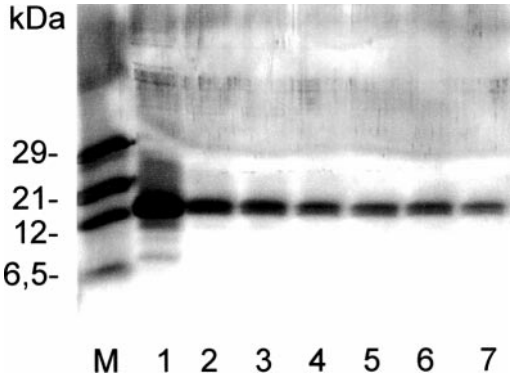


Figure 4. To determine whether the interaction of PDC-109 and Ca^{2+}-ATPase is reversible, microsomal sperm membranes were incubated with PDC-109 at a relation of 5 μg: 20 μg (w:w). After centrifugation at 100 000 × *g* and dilution of the pellet obtained, an aliquot (6 μg) was run on tricine-SDS-PAGE using 12% separating gels. The centrifugation and dilution procedure was repeated 6 times. The gel clearly indicated that PDC-109 was still present in all aliquots. M indicates marker; 1, starting sample; 2–7, following 6 pellets after subsequent centrifugation and dilution.

different parts of the epididymis (caput, corpus, cauda) and the ampulla of the vas deferens, microsomal membrane fractions were prepared from the respective sperm samples. In membranes prepared from caput sperm, the activity of Mg^{2+}-independent Ca^{2+}-ATPase was stimulated by PDC-109 by 66%, in those from corpus sperm by 177%, from cauda sperm by 292%, and from ampulla sperm by 137%. Mg^{2+}-dependent Ca^{2+}-ATPase activity was increased in membranes obtained from caput sperm by 70%, from the corpus sperm by 196%, from cauda sperm by 111%, and from ampulla sperm by 78%. The highest stimulatory effect was hence observed in sperm membrane ATPase of sperm obtained from the cauda and corpus region of the epididymis, both in Mg^{2+}-dependent and Mg^{2+}-independent ATPases (Table 2 and Figure 5).

In order to determine whether the enzyme activating effect of PDC-109 is restricted to sperm Ca^{2+}-ATPase, activity measurements were performed with membrane preparations from different bovine organs. The respective membrane fractions were isolated from heart, kidney, and liver. A stimulatory effect on membrane-bound Mg^{2+}-independent Ca^{2+}-ATPase activity was lower or insignificant for all three organs, while in the case of Mg^{2+}-dependent Ca^{2+}-ATPase activity, a stimulatory effect of PDC-109 was detected in the case of liver plasma membranes (50%), while a minor effect was observed in case of heart and kidney membrane enzymes (Table 3).

In order to investigate the species specificity of the stimulatory effect of PDC-109 on sperm ATPases, rat epididymal sperm were treated in exactly the same way as bovine sperm, and enzyme activity was measured in these samples. Again, following the addition of PDC-109 to the reaction mixture, enzyme activity was significantly increased in both Mg^{2+}-independent (131%) and -dependent (96%) Ca^{2+}-ATPase activities (Figure 6).

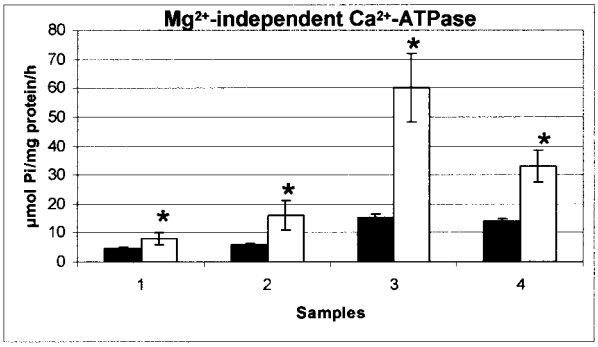
Effect of PDC-109 on Sperm Motility

The CASA method detects curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s) and average path velocity (VAP, μm/s) in freshly prepared native epididymal sperm. VSL values increased from a basal value of 9.6 ± 0.14 to 42.4 ± 0.10 following the addition of 2

Table 2. *Modulating effect of PDC-109 on Ca^{2+}-ATPase activity in microsomal membranes of sperm obtained from different epididymal regions and the ampulla of the vas deferens**

	Caput		Corpus		Cauda		Ampulla	
Samples	−PDC-109	+PDC-109	−PDC-109	+PDC-109	−PDC-109	+PDC-109	−PDC-109	+PDC-109
A. Mg^{2+}-independent Ca^{2+}-ATPase activity								
μmol P_i/mg protein/h	4.8 ± 0.7	8 ± 2.1	5.8 ± 0.7	16.1 ± 2.2	15.3 ± 2.4	60 ± 11.2	13.9 ± 2.1	33 ± 2.2
Relative Mg^{2+}-independent Ca^{2+}-ATPase activity (%)	100	166	100	277	100	392	100	237
Analysis of variance (ANOVA), treatment vs no treatment samples		$P < .05$		$P < .05$		$P < .05$		$P < .05$
B. Mg^{2+}-dependent Ca^{2+}-ATPase activity								
μmol P_i/mg protein/h	10.3 ± 1.7	17.6 ± 4.5	18.4 ± 3.7	54.5 ± 0.2	48.7 ± 13.9	103 ± 23.8	50.7 ± 8.6	90.3 ± 16.7
Relative Mg^{2+}-dependent Ca^{2+}-ATPase activity (%)	100	170	100	296	100	211	100	178
Analysis of variance (ANOVA), treatment vs no treatment samples		$P < .05$		$P < .05$		$P < .05$		$P < .05$

* Assays were performed without and with 20 μg PDC-109.



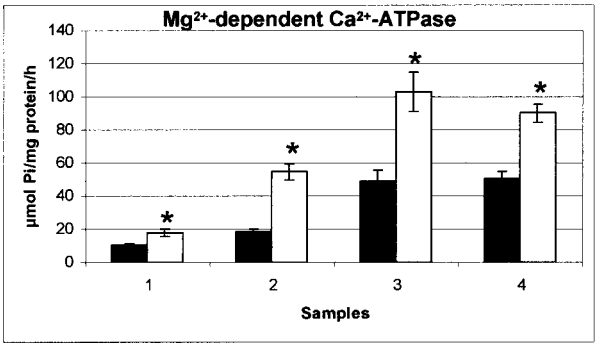


Figure 5. Modulating effect of PDC-109 on Ca^{2+}-ATPase activity on microsomal membranes of sperm obtained from different epididymal regions and the ampulla of the vas deferens. Ca^{2+}-ATPase activities were measured without (black columns) and with (white columns) 20 µg PDC-109. **(1)**, caput; **(2)**, corpus; **(3)**, cauda; and **(4)**, ampulla. (*) denotes statistical significance ($P < .05$). Vertical bars indicate SEM.

µM of PDC-109. VAP values increased from a basal value of 19.2 ± 0.07 to 52.2 ± 0.11 after adding 2 µM protein. VCL values ranged from 44.5 ± 0.13 without protein to 100.9 ± 0.23 after adding 2 µM of PDC-109. The velocity was not further enhanced by increasing the amount of PDC-109 to 4 µm (Figure 6). The relative number of spermatozoa with enhanced motility was not increased after addition of PDC-109.

Discussion

In a previous study we demonstrated by immunoelectron microscopy (Aumüller et al, 1988) that the major seminal vesicle protein (PDC-109) is located underneath the plasma membrane of the sperm midpiece of epididymal sperm once these get into contact with seminal vesicle secretion. Therefore, a functional role of PDC-109 on sperm motility was presumed. Sperm functions obligatory for the fertilization process of the egg are motility, capacitation, and acrosomal reaction. These processes are known to be regulated by intracellular calcium concentrations (Yanagimachi and Usui, 1974; Babcock et al, 1979; Breitbart et al, 1985). Breitbart and coworkers have suggested that Ca^{2+}-ATPase has a functional role in regulating sperm motility. After inhibition of Ca^{2+}-ATPase, the motility of ram spermatozoa was reduced (Breitbart et al, 1985). This effect was also shown in human spermatozoa (Kanwar et al, 1993; Khanduja et al, 2001) using different inhibitors. Furthermore, it was shown that membranes from bull sperm head possess Ca^{2+}-ATPase activity (Vijayasarathy et al, 1980; Breitbart et al, 1984). In the present study we have isolated sperm plasma membranes in order to examine whether they exhibit Mg^{2+}-dependent and -independent Ca^{2+}-ATPase activities, respectively, that are dependent on PDC-109. In a second set of experiments, we analyzed whether PDC-109 influences motility of bull sperm isolated from cauda epididymis.

Table 3. *Stimulating effect of PDC-109 (20 µg) on Ca^{2+}-ATP activity in microsomal membranes of different bovine organs**

Organ	−**PDC-109** (µmol P_i/mg protein/h)	+**PDC-109** (µmol P_i/mg protein/h)	Analysis of Variance (ANOVA), Treatment vs No Treatment Samples
A. Mg^{2+}-independent Ca^{2+}-ATPase activity			
Heart	19.7 ± 3.0	21.1 ± 3.2	$P > .05$
Liver	35.2 ± 4.3	36.7 ± 9.0	$P > .05$
Kidney	39.7 ± 8.6	43.1 ± 5.5	$P > .05$
B. Mg^{2+}-dependent Ca^{2+}-ATPase activity			
Heart	13.3 ± 6.0	13.4 ± 2	$P > .05$
Liver	22.8 ± 2.7	34.4 ± 7.4	$P > .05$
Kidney	28.5 ± 3.7	27.3 ± 5.8	$P > .05$

* $P < .05$ denotes statistical significance.

First, we analyzed whether plasma membranes of epididymal sperm show Ca^{2+}-ATPase activity. We could identify both Mg^{2+}-dependent and -independent Ca^{2+}-ATPase activities in epididymal sperm. Both activities were also shown for goat spermatozoa (Sikdar et al, 1991; Sikdar et al, 1993; Sikdar et al, 1999). Sperm Ca^{2+}-ATPase has been identified in microsomal membranes in some other species revealing the influence of this sort of pumps in calcium transport (Sikdar et al, 1991; Bhattacharyya and Sen, 1998; Sikdar et al, 1999). The plasma membrane Ca^{2+}-ATPase is an essential membrane protein controlling the intracellular calcium concentration in cells and tissues (Carafoli, 1992; Carafoli and Stauffer, 1994). In most organs Ca^{2+}-ATPase requires Mg^{2+} for its activation (Robinson, 1976; Niggli et al, 1979). This Mg^{2+}-dependent Ca^{2+}-ATPase is called plasma membrane Ca^{2+}-ATPase (PMCA). Four distinct isoforms and several splice variants of the PMCA are described (PMCA1–4) so far (Carafoli, 1992; Carafoli and Stauffer, 1994; Guerini, 1998).

To analyze the potential effect of PDC-109 on the Ca^{2+}-

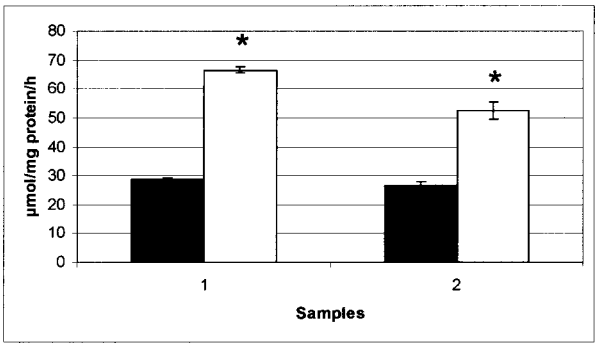


Figure 6. Stimulatory effects of PDC-109 on Ca^{2+}-ATPase activity on microsomal membranes of rat epididymal sperm. Mg^{2+}-independent Ca^{2+}-ATPase activity **(1)** and Mg^{2+}-dependent Ca^{2+}-ATPase activity **(2)** was measured without (black column) and with 20 µg PDC-109 (white column). (*) denotes *P* value $<$ .05. Vertical bars show SEM.

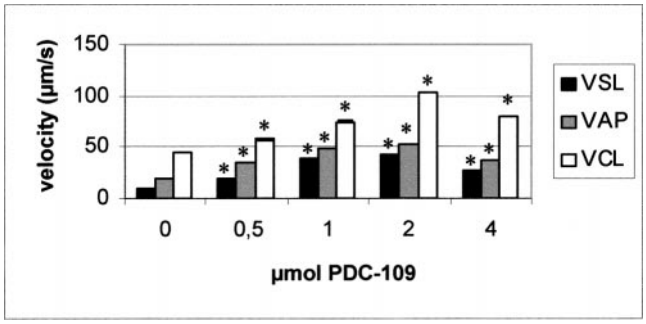


Figure 7. Effect of PDC-109 on sperm motility. The CASA method was used to analyze the influence of PDC-109 on straight line velocity (VSL), average path velocity (VAP), and curvilinear velocity (VCL) of epididymal bovine sperm. VSL, VAP, and VCL of bovine sperm were increased significantly by PDC-109 in a concentration-dependent manner. (*) denotes *P* value $<$ .05. Vertical bars show SEM.

ATPase activity, we purified PDC-109 from the secretion of bovine seminal vesicles to homogeneity, using a novel, highly efficient purification procedure developed by Calvete et al (1996). The purified protein showed a double band at 13/14 kD on SDS-PAGE. Upon sequencing, the N-termini of both protein bands were found to be identical with the N-terminus of mature PDC-109 (Esch et al, 1983; Kemme and Scheit, 1988). This is in agreement with investigations performed by Calvete and coworkers (1996). They could show that the PDC-109 preparation contained a mixture of 2 species with molecular weights of 12.789 and 13.44 kDa, respectively. Both proteins had the identical N-terminus of PDC-109, the same amino acid composition, and the same tryptic fingerprint (Calvete et al, 1996). The difference in the molecular weight corresponds to the nonglycosylated and O-glycosylated isoform (Calvete et al, 1994).

After incubation of microsomal sperm membranes with bovine PDC-109, we observed an increase on Ca^{2+}-ATPase activity, dependent on the concentration of the seminal protein, until it reached a level of about 20 µg/250 µL where activity remained constant. This stimulatory effect of about 150% was shown for both Mg^{2+}-dependent and Mg^{2+}-independent ATPase activities in epididymal sperm membranes. Interestingly, Sen and coworkers described another modulator protein from rat brain that stimulated the Mg^{2+}-dependent Ca^{2+}-ATPase activity in goat sperm but inhibited Mg^{2+}-independent Ca^{2+}-ATPase (Bhattacharyya and Sen, 1998).

To analyze the dependence of the velocity and the substrate concentration, detailed kinetic experiments were performed. The results show that the affinity of the enzyme to the substrate ATP is higher in case of Mg^{2+}-dependent Ca^{2+}-ATPase activity rather than in case of Mg^{2+}-independent activity in microsomal membranes of epididymal sperm, as the resulting [S] 0.5 value of Mg^{2+}-dependent Ca^{2+}-ATPase activity is lower. The affinity of the enzyme to the substrate was the same after adding of PDC-109, while the maximal velocity was increasing. PDC-109 and the enzyme substrate obviously do not compete for the same binding site; conversely, there is a positive cooperativity. Therefore, it can be concluded that the binding site of PDC-109 and the binding site for the substrate ATP on the enzyme are different. Furthermore, the effect on the ATPase activity was not reduced after dilution, repeated centrifugation, and washing of the stimulated sample. This indicates that the binding of PDC-109 and ATPases is irreversible. Different binding sites for substrate and modulator, yet reversibility of the modulator binding, was shown for the brain-modulator protein that stimulates Ca^{2+}-ATPase activity in goat spermatozoa (Bhattacharyya and Sen, 1998). The stimulatory effect of PDC-109 is quite sensitive to temperature and to pH. Preincubation at 41°C and at pH between 6.5 to 7.5 and at pH above 8.5 completely abolishes the Ca^{2+}-ATPase activation ability. This effect might be due to disturbing its secondary structure by unfolding monomers or by dissociation of oligomers. Heat-induced denaturation of PDC-109 oligomers has been reported to lead to an irreversible melting transition at 36°C (Gasset et al, 1997).

Both Ca^{2+}-ATPases were inhibited by Ca^{2+}-antagonists such as nifedipine, TFP, and verapamil (Scharff and Foder, 1984; Kim and Raess, 1988; Sikdar et al, 1991; Olorunsogo and Bababunmi, 1992; Kanwar et al, 1993; Bhattacharyya and Sen, 1998). Actually, there are no reports whether Mg^{2+}-dependent and Mg^{2+}-independent ATPases are 2 different enzymes or just 1 with 2 different catalytic sites. Since their behavior in terms of inhibition with Ca^{2+}-antagonists and stimulation with PDC-109 is the same, it is conceivable that they resemble just 1 enzyme with 2 different binding sites. Sen and coworkers favored the model of Ca^{2+}-ATPase being only 1 enzyme molecule with different catalytic sites (Sikdar et al, 1993).

We analyzed whether the Ca^{2+}-ATPase is stimulated to

the same degree in sperm membranes present in the different epididymal regions. Our results clearly demonstrate that Ca^{2+}-ATPase activity grows from caput sperm to cauda sperm and the stimulatory effect of PDC-109 increases, too. Spermatozoa undergo a maturation process during their transport from the testis to the cauda epididymidis (Jones, 1989; Cooper, 1995). Therefore, changes in ATPase activity as well in the response to the stimulatory protein are conceivable.

In order to determine whether the activation of PDC-109 is a species-specific event, ATPase activities in microsomal membranes of rat epididymal sperm were analyzed. Our results clearly indicate that the Ca^{2+}-ATPase activity of rat as well as human and goat (unpublished observation) epididymal sperm is also activated by bovine PDC-109. This result indicates that the Ca^{2+}-ATPase in the different species exhibit a high homology resulting in a similar functional response. Ca^{2+}-ATPase activity in microsomal membrane preparations of different organs was checked to determine whether enzyme activation exists in extra genital tissues or only in sperm cells. A stimulatory effect was shown for Mg^{2+}-dependent ATPase activity only in the case of bovine liver microsomes but was less pronounced compared with sperm preparations. In the case of heart and kidney microsomes, no ATPase stimulation by PDC-109 was observed.

To date, it is not known which PMCA isoform is present in sperm. Wennemuth et al (2003), using an antibody against all 4 isoforms, demonstrated in mouse sperm that PMCA is located in the sperm tail. Different commercially available antibodies against PMCA isoforms showed ambiguous results in the bovine samples, maybe due deficient cross-reactivity with the respective bovine antigen(s). Performing RT-PCR using total RNA from human testis and specific primers for the PMCA isoforms 1 through 4, we demonstrated that the isoforms PMCA1 and PMCA4 are expressed in human testis, while PMCA2 and PMCA3 are not expressed (unpublished data). PMCA1 and PMCA4 are known to be ubiquitously transcribed, while PMCA2 and 3 are expressed in a more restricted manner, predominantly in brain and striated muscle; PMCA 2 is present in addition in kidney and liver (Greeb and Shull, 1989; Stahl et al, 1992; Stauffer et al, 1993; Carafoli and Stauffer, 1994; Howard et al, 1994; Keeton and Shull, 1995). As PDC-109 acts predominantly on ATPase in sperm, it is likely that a particular splice variant of PMCA1 or 4 is present in sperm membranes.

Besides activating Ca^{2+}-ATPases, we showed that PDC-109 accelerates sperm motility in a dose-dependent manner. Furthermore, it was shown from different groups that PDC-109 binds to the choline group of phospholipids and provokes cholesterol- and phospholipid-efflux from the membranes. The loss of membrane cholesterol is an important event in sperm capacitation (Muller et al, 1998; Moreau et al, 1999; Moreau and Manjunath, 2000). Therefore, Majunath and coworkers propose that PDC-109 may play an important role in the process of capacitation too (Manjunath and Therien, 2002). Therefore, PDC-109 seems to be a multifunctional protein. Further experiments are necessary to clarify whether the 2 effects of PDC-109 shown in our study and the effects shown by other groups are joint or independent events. Motility and Ca^{2+}-ATPase activity being interdependent events was already predicted by studies of Breitbart et al (1985; cf. Kanwar et al, 1993; Khanduja et al, 2001). They have suggested from inhibition experiments that Ca^{2+}-ATPase has a functional role in the regulation of sperm motility. In contrast, studies performed in the group around Suarez (Ho et al, 2002; Ho and Suarez, 2003; Suarez and Ho, 2003) indicated that increased free intracellular Ca^{2+} plays a major role in regulating hyperactivated motility. They further showed that although hyperactivation and capacitation occur simultaneously, both events are regulated by different pathways (Ho and Suarez, 2001).

Our data show that PDC-109 increases sperm motility and the pumping efficiency of plasma membrane Ca^{2+}-ATPases in an irreversible, cooperative manner. The effect is tissue specific but not species specific.

Acknowledgment

The authors gratefully acknowledge the excellent technical assistance of Anne Henkeler.

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