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Fusion of Human Sperm to Prostasomes at Acidic pH

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Abstract. Prostasomes are membranous vesicles (150–200 nm diameter) present in human semen. They are secreted by the prostate and contain large amounts of cholesterol, sphingomyelin and Ca^{2+}. In addition, some of their proteins are enzymes. Prostasomes enhance the motility of ejaculated spermatozoa and are involved in a number of additional biological functions. The possibility that they may fuse to sperm has never been proved. In this work, we studied the fusion of sperm to prostasomes by using various methods (relief of octadecyl Rhodamine B fluorescence self-quenching, fluorescence microscopy and flow cytometry) and we found that it occurs at acidic pH (4–5), but not at pH 7.5. pH-dependent fusion relies on the integrity of one or more proteins and is different from the Ca^{2+}-stimulated fusion between rat liver liposomes and spermatozoa that does not require any protein and occurs at neutral pH.

We think that the H^+-dependent fusion of prostasomes to sperm may have physiological importance by modifying the lipid and protein pattern of sperm membranes.

Key words: Membrane — Seminal fluid — Rhodamine — Liposomes — Fluoresence

Introduction

Prostasomes are membranous vesicles secreted by the prostate gland (Ronquist & Brody, 1985). Their lipid composition is peculiar; cholesterol is present in high amounts as is sphingomyelin, whereas phosphatidiylcholine is less abundant (Ardivson et al., 1989). Therefore, these membranes differ amply from sperm plasma membranes (Mack et al., 1986) that contain less sphingomyelin and more phosphatidylcholine with a cholesterol: phospholipid ratio of 0.83 (Poulos & White, 1973). This may be interesting since cholesterol may have roles in sperm capacitation (Benhoff, 1993). Prostasomes are also rich in Ca^{2+}, GDP, ADP and ATP (Ronquist & Frithz, 1986; Fabiani, 1994), and many proteins at their surface possess a catayltic activity (Fabiani, 1994) or are involved in the immune response (Rooney et al., 1993; Fabiani et al., 1994). We would cite, among their physiological roles, the enhancement of sperm mobility (Stegmayr & Ronquist, 1982), the liquefaction of semen (Lilja & Laurell, 1984) and immunosuppression (Kelly et al., 1991; Skibinski et al., 1992).

It has been reported (Ronquist et al., 1990) that prostasomes ''interact'' with sperm but fusion has never been reported. On the other hand, fusion is necessary for a number of physiological functions, spanning from egg fecundation to the release of neurotransmitters. For this reason, many models have been proposed to study this phenomenon. Liposomes have extensively been used for this purpose and a pH-dependent fusion of spermatozoa to liposomes made with acidic phospholipid has been reported (Arts et al., 1993, 1994). Since the many membrane types in the eukaryotic cell retain their individuality, fusion must be a controlled phenomenon.

Prostasomes can be used to study fusion with two aims: (i) they are simple natural membranes although somewhat similar to certain types of liposomes and (ii) they may be related to the fertilizing capacity of human semen.

We thought that prostasomes may exert their effects through the fusion to sperm and the experiments reported here were done to test this hypothesis. Therefore, we studied the fusion capacity of sperm, prostasomes and liposomes and found that the fusion of spermatozoa to prostasomes has very different properties from the fusion

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to liposomes made with rat liver lipid extracts. We propose that the fusion of prostasomes to sperm may be somehow connected to fertility.

Materials and Methods

Materials

HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), Thesit (dodecylpoly(ethylenglycolether)$_9$), and MES (2-(N-morpholino)-ethanesulfonic acid) were produced by Boehringer-Biochemie (Mannheim, Germany). Sephadex G-50 and Sephadex G-200 were obtained from Pharmacia Fine Chemicals A B (Uppsala, Sweden), octadecyl Rhodamine B chloride (R_{18}) was purchased from Molecular Probes (Eugene, OR). Other reagents, all of reagent grade or better, were obtained from Carlo Erba (Milan, Italy), unless stated otherwise.

Semen Samples and Sperm Preparation

Fresh human semen was obtained from donors and was left 30–40 min at room temperature. We used normospermic (WHO, 1987) samples and centrifuged the material (800 × *g* × 10 min) to harvest sperm. The supernatant (S_1) was used to prepare prostasomes. The pellet (P_1) was suspended in Tris 30 mmol.L^{-1} + NaCl 130 mmol.L^{-1} (adjusted to pH 7.6 with HCl). Sperm were purified by layering on 70% Percoll (Sigma Chemical, St Louis, MO) and by centrifuging at 600 × *g* × 30 min. The pellet was then washed twice with Tris 30 mmol.L^{-1} + NaCl 130 mmol.L^{-1} (adjusted to pH 7.6 with HCl). The final pellet was suspended in the same buffer and immediately used for further procedures, unless otherwise stated.

Preparation of Prostasomes

The supernatant (S_1) was diluted (1:1, by vol) with Tris 30 mmol.L^{-1} + NaCl 130 mmol.L^{-1} (adjusted to pH 7.6 with HCl) and was centrifuged at 1,000 × *g* × 20 min to eliminate cell debris and residual spermatozoa. The new supernatant was then centrifuged at 105,000 × *g* × 120 min. The supernatant was discarded and the pellet containing prostasomes and amorphous material (Ronquist & Brody, 1985) was suspended in Tris 30 mmol.L^{-1} + NaCl 130 mmol.L^{-1} (adjusted to pH 7.6 with HCl) to have about 1–1.5 mg prot.mL^{-1}.

Prostasomes were purified from amorphous material by chromatography on a Sephadex G-200 column (1.5 × 30 cm) preequilibrated with Tris 30 mmol.L^{-1} + NaCl 130 mmol.L^{-1} (adjusted to pH 7.6 with HCl) (Fabiani et al., 1994). Prostasomes were not retained by the column and were collected with V_0. They were finally harvested by centrifugation at 105,000 × *g* × 120 min and suspended in the same buffer.

Characterization of Prostasomes

The homogeneity of prostasome preparations was checked by quasi-elastic light-scattering (QELS) with a NICOMP Model 370/VHPL submicron particle sizer with very-high-power laser (75-mW air-cooled argon-ion 488 nm). In addition, prostasomes were characterized by measuring their lipid content. The extraction of lipid from membranes was performed according to Folch, Lees and Sloane-Stanley (1957). In some instances, chloroform extracts were used to determine the distribution of phosphorus among lipid classes. The chloroform phase was dried under a gentle stream of nitrogen and dissolved in known amounts of chloroform: methanol (2:1, v/v). Phospholipids were separated by two dimensional thin-layer chromatography (6.5 × 6.5 cm, PE SIL G 250 μm, Whatman Ltd., Maidstone, UK) with: (a) chloroform: methanol:1.6 mmol.L^{-1} ammonia (70:30:5, v/v) and (b) chloroform: acetone:acetic acid:methanol:water (75:30:15:15:7.5, v/v). Spots were visualized by exposure to I_2 vapors and identified with pure reference standards. After the sublimation of I_2, spots were scraped off the plate and their phosphorus content determined (Bartlett, 1959).

Preparation of Liposomes

Lipid extracts were prepared from rat liver or from human prostasomes following the method described by Folch *et al.* (1957). Proper amounts of lipids were dissolved in chloroform: methanol (2:1, v/v), the solvent removed under a gentle N_2 flux and lipsomes prepared suspending lipids (1.5 μmol.mL^{-1}) in a buffer (pH 7.0) containing 0.32 mmol.L^{-1} sucrose and 2 mmol.L^{-1} HEPES (SHB). Suspensions were sonicated to clearness in a MSE sonicating apparatus (Corazzi et al., 1989).

Insertion of R_{18} into Membranes

The insertion of R_{18} into vesicles (prostasomes or liposomes) was performed as described by Hoekstra *et al.* (1984). The probe was dissolved in ethanol (1 mg.mL^{-1}) and 50 μL of this solution were added to 1 mL of vesicle suspension. The mixture was then kept in the dark for 1 hr at room temperature. To eliminate noninserted R_{18}, vesicles were chromatographed on a Sephadex G-50 column (0.5 × 25 cm) and eluted with SHB (liposomes) or Tris 30 mmol.L^{-1} + NaCl 130 mmol.L^{-1} (adjusted to pH 7.6 with HCl) (prostasomes). The fluorescence of these preparations was stable for many hours.

Assay of Fusion

Fusion was tested by the relief of octadecyl-Rhodamine fluorescence self-quenching, which monitors lipid mixing. The assay was performed in a cuvette containing 0.32 mol.L^{-1} sucrose + 20 mmol.L^{-1} MES or 2 mmol.L^{-1} HEPES at the required pH (1.85 mL) and vesicles loaded with the probe (about 20 nmol lipid in 50 μL of SHB). The fusion process was started by adding unloaded sperm (about 30–50 nmol lipid P in 100 μL of SHB) and was monitored following the increments of fluorescence at 580 nm (excitation 560 nm) using a Shimadzu RF5000 spectrophotofluorimeter. Slit widths were set at 5 nm for both excitation and emission. The calibration of the assay was performed taking as 100% the fluorescence produced after the addition of 0.03% Thesit; indeed, fluorescence did not increase for further additions of the detergent. No increase of fluorescence was detected before the addition of unloaded vesicles. pH had no effects in the ranges used in this paper.

We investigated fluorescence self-quenching *vs.* surface density and always used concentrations comprised in the proportionality range; for surface densities ≤0.02 mol probe/mole lipid fluorescence quenching was linearly related to surface density. The extent of fusion (taking as 100% the complete intermixing of lipid phases) was calculated from the percent of fluorescence quenching relief using the following relationship (Corazzi et al., 1991): $F = PD(1 + r/l)$, were F = fusion, PD = percentage of fluorescence dequenching, r = amount of R_{18}-loaded lipid (in mol) and l = amount of unloaded lipid (in mol).

Fluorescence Microscopy

In some instances, the mixtures of fusing prostasomes and spermatozoa were examined with a microscope (Axiolab, Carl Zeiss, Oberkochen,

Germany) equipped with a filter set 15 (emission 590 nm). Photographs were taken with a 3600 ASA black and white film (Eastman-Kodak, Rochester, NY). Prostasome and sperm were mixed as described above in Assay of Fusion and incubated at 37°C for 10 min. Loosely associated vesicles were then removed after the process of fusion by centrifugation in the same buffer used for fusion (2 times at 600 × *g* × 5 min each time).

Cytofluorimetric Assessment

Samples were prepared as described above (in Fluorescence Microscopy). The observations were then performed with a FACS Analyzer flow cytometer (Becton-Dickinson, Sunnyvale, CA) and the signal emitted on the FL2 channel (585 ± 26 nm) analyzed with a Convert 30 program.

Analyses

Protein was determined as described (Bradford, 1976), cholesterol and phospholipid phosphorus assayed after digestion with 70% (w/w) perchloric acid (Bartlett, 1959) and cholesterol as described by Rudel & Morris (1973).

Abbreviations

SHB: Sucrose-HEPES-Buffer (a buffer containing 0.32 mol.L^{-1} sucrose, and 2 mmol.L^{-1} HEPES, pH 7.0) R_{18}: Octadecyl Rhodamine B chloride Thesit: dodecylpoly(ethylenglycolether)$_9$

Results

Characterization of Prostasomes

We checked the prostasome preparations employed in this work and concluded that the material we used was similar to that utilized by others (Ronquist & Brody, 1985; Arvidson et al., 1989) as demonstrated by the lipid composition (*see* legend to the Table) and by the size of the particles (about 150–200 nm, measured by quasi-elastic light scattering—QELS). QELS was performed either at pH 7.5 or at pH 5.0 to check that no aggregation occurred at the lower pH. Liposomes prepared with prostasome lipid extracts were also analyzed by QELS. Their diameter was about 120 nm and, therefore, comparable to that of prostasomes.

Fusion as Measured by the Relief of Octadecyl-Rhodamine-B Fluorescence Self-quenching

As a first step, we studied the fusion of *spermatozoa to liposomes made with rat liver whole lipid extracts* (Table). Two types of fusion were present: one was active at pH 7.5 and depended on the presence of Ca^{2+} whereas the other required a decrease of pH to 6–5 (or less) and was usually measured at pH 5.0 (Table). The pH-dependent mechanism could be inactivated by exposure of spermatozoa to pronase (Table *B*) whereas the divalent cation-induced fusion was insensitive to the protease. With this type of liposomes, the pH-dependent mechanism was much less active than the Ca^{2+}-dependent one and the two systems may not sum their effects. Indeed, the pH-dependent fusion was abolished by the treatment of sperm with pronase; yet, in the presence of Ca^{2+} the effect of pronase was scarce or absent at pH 5.0.

We then studied the fusion of *sperm to prostasomes.* The addition of Ca^{2+} was without effect and fusion could be elicited only through the H^+-dependent mechanism (Fig. 1; Table). In addition, the fusion between these particles could be inactivated by exposing spermatozoa to the action of pronase. The inactivation due to the protease was not complete (Table). However, fusion of pronase-treated sperm could be reduced practically to

Table. Fusion of fresh sperm to R_{18}-labelled vesicles

A: Sperm not treated with pronase	1 mmol.L^{-1} Ca^{2+}	No Ca^{2+} added
Rat liver lipid liposomes		
pH 5.0	77.0 ± 8.2	14.8 ± 1.3
pH 7.5	26.9 ± 2.3	0.7 ± 0.3
Prostasome lipid liposomes		
pH 5.0	35.2 ± 3.4	33.6 ± 2.8
pH 7.5	1.9 ± 0.5	1.5 ± 2.1
Prostasomes		
pH 5.0	24.3 ± 1.7	26.0 ± 2.0
pH 7.5	1.9 ± 0.3	1.5 ± 0.4
B: Sperm treated with pronase		
Rat liver lipid liposomes		
pH 5.0	76.2 ± 7.9	0.4 ± 0.2
pH 7.5	30.0 ± 2.7	0.6 ± 0.3
Prostasome lipid liposomes		
pH 5.0	2.5 ± 0.3	2.7 ± 0.2
pH 7.5	1.3 ± 0.6	0.5 ± 0.3
Prostasomes		
pH 5.0	11.8 ± 1.6	11.8 ± 0.7
pH 7.5	1.4 ± 0.3	0.8 ± 0.4

(*A*) Sperm are fused for 10 min (no further increase of fluorescence was found thereafter) in the absence of Ca^{2+} or in the presence of Ca^{2+} with vesicle preparations loaded with R_{18}. Data are expressed as percentage of maximum fusion, as described (Corazzi et al., 1991) ± SE. Composition of prostasome lipid extracts (5 determinations): (14% phosphatidylserine, 8% phosphatidylinositol, 50% sphingomyelin, 11% phosphatidylcholine, 17% phosphatidylethanolamine on molar bases, with a cholesterol to phospholipid ratio of about 2). Composition of rat liver lipid extracts (5 determinations): 3% phosphatidylserine, 6% phosphatidylinositol, 6% sphingomyelin, 49% phosphatidylcholine, 29% phosphatidylethanolamine, 3% cardiolipin (cholesterol/phospholipid ratio of about 0.4). (*B*) The treatment with pronase was performed as described (Nilsson & Dallner, 1977), that is, sperm were suspended in 0.32 M sucrose, 2 mM Hepes (pH 7.5 and about 0.3 mg protein.L^{-1} and incubated with 0.03 mg.L^{-1} of pronase for 120 min. See (*A*) for additional information.

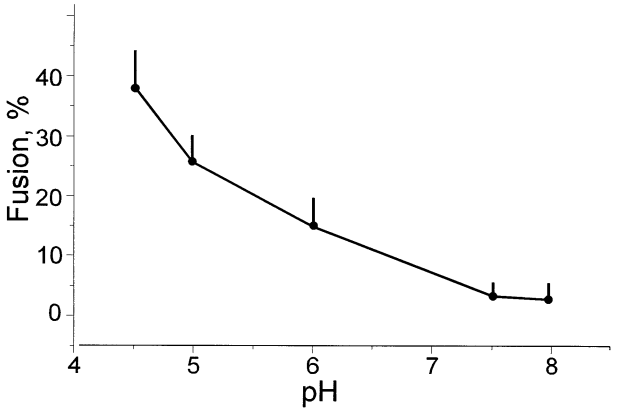

Fig. 1. pH-dependent fusion of prostasomes to spermatozoa (no Ca^{2+} added). For lipid composition, see the Table. The percentage of fluorescence was calculated as reported in Materials and Methods (*see* Corazzi et al., 1991). Vertical bars indicate the SE. The desired pH was achieved by using the following buffer: 0.32 mol.L^{-1} sucrose + either 20 mmol.L^{-1} MES or 2 mmol.L^{-1} HEPES.

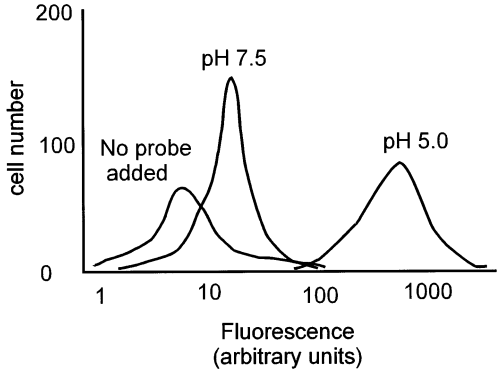

Fig. 2. Flow cytometry of spermatozoa after different treatments. The graph reported in this figure is one of the several obtained with this technique. Spermatozoa were mixed with R_{18}-loaded prostasomes, they were kept at 37°C at the desired pH (*see* legend to Fig. 1) and the fluorescence was measured 10 min afterwards.

zero by one of these procedures (i) by treating also prostasome with pronase, (ii) by using boiled prostasomes and (iii) by using liposomes made with prostasomal extracts (*see also below*). Therefore, the fusion between sperm and prostasomes was due to two components: one bound to sperm and the other to prostasomes. In no cases could we obtain fusion of sperm/prostasome mixtures by adding divalent cations (Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+} in the range 0.1–2 mmol.L^{-1}) at pH 7.5 or increase it by adding the same cations at pH 5.0. The possibility of a cation-dependent fusion between sperm and prostasomes was therefore ruled out by these observations.

We also assessed the fusion of *spermatozoa to liposomes made with prostasomal lipids.* These particles could fuse to sperm at acidic pH, but the process was not stimulated by Ca^{2+}. The fusion of prostasomal liposomes was ever more extensive than the fusion of native prostasomes (Table). This may be due to the removal of compounds other than lipid during lipid extraction from prostasomes and liposome preparation.

Flow Cytometry

Flow cytometry permitted us to obtain additional data on fusion between prostasomes and sperm. The addition of prostasomes to sperm at pH 7.5 produced a very low increase of sperm fluorescence that might be due to a small, aspecific transfer of the probe. Upon repeating the procedure at pH 5.0, the fluorescence of sperm was about 100 times as high as the basal fluorescence at pH 7.5 and it presented very small overlaps (about 3%, Fig. 2). This means that the fusion process involved most sperm and was strictly dependent on pH. Therefore the results obtained by flow cytometry were in good agreement with those reported above by using the relief of R_{18} self-quenching and with those obtained by fluoresence microscopy (*see* below). In addition, they indicated that most spermatozoa fused with prostasome at acidic pH in 10 min.

Microscopic Examination

Fused sperm cells were evaluated with a fluorescence microscope. In a first set of experiments, we examined sperm fused to rat liver lipid liposomes at pH 7.5 and at pH 5.0 in the presence of Ca^{2+}. As expected, the stain was better taken up after fusion at pH 5.0, but it was also evident at pH 7.5. We also tested frozen sperm and found that these were stained more heavily than the fresh material.

The behavior of prostasomes or of liposomes made with prostasome lipids was different from that of rat liver liposomes. In our study, the label was very low at pH 7.5 and was present only in the neck zone of the sperm. Yet, it was very well evident at pH 5.0 (Fig. 3). At this pH, the fluorescence was distributed uniformly on the cell surface and we could not find any differences related to the treatment of the sperm (frozen *vs.* fresh).

Discussion

Prostasomes are natural vesicles secreted by the prostate gland into human semen. They are claimed to have a number of functions, among which the stimulation of sperm motility (Stegmayr & Ronquist, 1982) and immunosuppression (Kelly et al., 1991). Although they have been reported to interact with sperm (Ronquist et al., 1990), no reports of their fusion to sperm have appeared in the literature. On the other hand, they possess a pe-

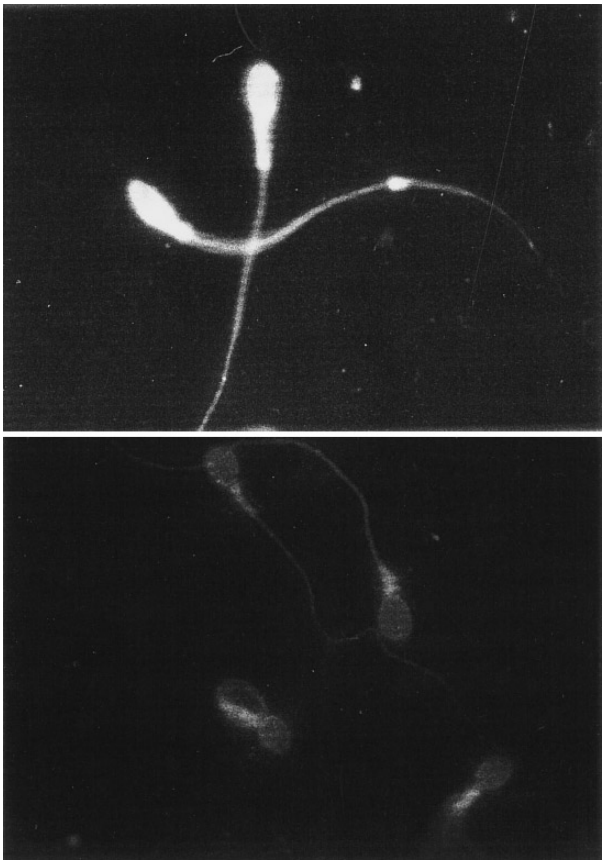

Fig. 3. Appearance of a mixture of spermatozoa and R_{18}-loaded prostasomes, kept for 10 min at pH 7.5 (*a*) or at pH 5 (*b*). Magnification was 1,000×. The time of exposure was 25 sec in (*a*) and 3 sec in (*b*). Samples were prepared as described in Material and Methods.

culiar lipid composition, a number of proteins and contain several small molecules (Fabiani, 1994). Therefore, they may deliver any of these components to sperm, so modifying its properties.

Membrane fusion is required for many biological events and various methods have been claimed to assess it. In this paper, we explored the properties of octadecyl Rhodamine-B (R_{18}) (Hoekstra et al., 1984). The dye dissolves into the lipid core of the membrane, exhibits fluorescence self-quenching and, for this reason, the fluorescent signal increases upon dilution, as it happens following the fusion of two membrane populations whose only one is loaded.

When the relief of R_{18} self-quenching is used to measure the fusion between liposomes, the extent of the phenomenon is evaluated from the maximal theoretical fluorescence, that is the fluorescence obtained when the probe is evenly distributed among all lipids in the sample, as after the addition of proper amounts of detergent (Hoekstra et al., 1984; Corazzi et al., 1991). However, this method may not give quantitative results in our case because sperm are much larger and more complex than liposomes, and the probe might not reach membranes other than plasma membrane after fusion. It is therefore clear that when we express fusion extent as a percentage of total fusion, we introduce a potential error (underevaluation).

Another fact that could lead to an underevaluation of fusion is the possibility that lateral diffusion of lipid may be limited on the sperm plasma membrane. It has been reported that in certain instances the fused lipid accumulates in some zones of the membrane (Arts et al., 1993, 1994) and that the lipid is patched in gel domains in this structure (Wolf, 1995). Of course, this would limit the dilution of the probe and therefore the relief of fluorescence self-quenching.

Sperm is able to distinguish between two types of liposomes, at least: those made with rat liver lipid extract and those assembled with lipids extracted from human prostasomes. Since all vesicles tested in this paper (liposomes from total liver lipid extracts, liposomes from prostasomal lipids or prostasomes) may use the pH-dependent fusion, it is not yet possible to state the requirements of this process. On the other hand, lipid composition and/or vesicle size may be responsible for the lack of cation-dependent fusion between sperm and prostasomes (or liposomes made with prostasomal lipids). In a previous paper, Arts et al. (1993) found that human fresh sperm cannot fuse with liposomes made of acidic lipid. In this paper, we find that the characteristics of liposomes are extremely important for fusion and that different results are expected when challenging different types of vesicles.

The pH-dependent fusion requires one (or more) protein(s) on the sperm surface. On the prostasomal surface there should be a protein that helps fusion. Indeed, to abolish fusion completely both prostasomes and spermatozoa must be treated with pronase. Yet, liposomes made with prostasomal lipid fuse to a greater extent than prostasomes. This might be due to the presence of other components that may hinder the fusion process. We think that the main fact reported here is the pH-dependent fusion of sperm to prostasomes.

Prostasomes contain CD59 (Rooney et al., 1993), CD55 and CDw52 (Hale et al., 1990). These proteins can be transferred to cells through different mechanisms (Rooney et al., 1996). Yet, the phenomenon described in this paper is certainly different from those reported above because it also happens after treatments able to destroy or alter prostasomal protein (boiling, treatment with pronase) or to remove it (preparation of liposomes with prostasomal lipids).

One may wonder whether this phenomenon possesses any physiological significance. The first point to consider is pH. Usually, we measure fusion at pH 5, but it may also occur at higher pH values (Fig. 1). For this reason, although human seminal plasma has a high buff-

ering capacity, it is possible that the pH of vaginal content is low enough to allow fusion to occur, also considering the low pH of prostatic secretions.

The fusion with prostasomes may interest sperm particularly exposed to an acidic milieu following the incomplete mixing of seminal plasma and vaginal secretions. From this point of view, the fusion with prostasomes may be a mechanism to protect sperm.

In this paper, we studied the behavior of lipids. However, prostasomes also contain protein and a number of small molecules. Any of these could modify the properties of human sperm upon fusion with prostasomes.

The pH-dependent fusion with prostasomes or with liposomes made by prostasomal lipid reported in this paper may be interesting from different points of view. Indeed, it represents a model of fusion between two natural membranes and it may also have a physiological role in the fecundation process.

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