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·*Original Article*·

Destabilization of acrosome and elastase influence mediate the release of secretory phospholipase A_2 from human spermatozoa

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Abstract

Aim: To determine the cellular distribution of secretory phospholipase A_2 ($sPLA_2$) in dependence on the acrosomal state and under the action of elastase released under inflammatory processes from leukocytes. **Methods:** Acrosome reaction of spermatozoa was triggered by calcimycin. Human leukocyte elastase was used to simulate inflammatory conditions. To visualize the distribution of $sPLA_2$ and to determine the acrosomal state, immunofluorescence techniques and lectin binding combined with confocal laser scanning fluorescence microscopy and flow cytometry were used. **Results:** Although $sPLA_2$ was detected at the acrosome and tail regions in intact spermatozoa, it disappeared from the head region after triggering the acrosome reaction. This release of $sPLA_2$ was associated with enhanced binding of annexin V-fluoroscein isothiocyanate (FITC) to spermatozoa surfaces, intercalation of ethidium-homodimer I, and binding of FITC-labelled concanavalin A at the acrosomal region. Spermatozoa from healthy subjects treated with elastase were characterized by release of $sPLA_2$, disturbance of acrosome structure, and loss of vitality. **Conclusion:** The ability of spermatozoa to release secretory phospholipase A_2 is related to the acrosomal state. Premature destabilization of the acrosome and loss of $sPLA_2$ can occur during silent inflammations in the male genital tract. The distribution pattern of $sPLA_2$ in intact spermatozoa might be an additional parameter for evaluating sperm quality. *(Asian J Androl 2008 Nov; 10: 829–836)*

Keywords: acrosome reaction; elastase; human spermatozoa; inflammation; secretory phospholipase A_2

1 Introduction

The Ca^{2+}-dependent, membrane-bound enzyme secretory phospholipase A_2 ($sPLA_2$, EC 3.1.1.4) is thought to play a key role in membrane structure alterations and fusion events during the acrosome reaction and spermatozoa-egg fusion [1]. There are two further main groups of phospholipases A_2, cytosolic PLA_2 and calcium-independent PLA_2, that differ considerably in occurrence, calcium binding and other properties. The 14-kDa $sPLA_2$ is calcium-dependent in the millimolar range, whereas cytoplasmatic PLA_2 is a significantly heavier protein and depends on Ca^{2+} in the micromolar range. $sPLA_2$ is known to possess a catalytic dyad (histidine–aspartic acid), contrary to the catalytic triad of cytosolic PLA_2 containing a

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serine-residue in the active site [2, 3].

$sPLA_2$ is mainly detected in the acrosomal region of resting spermatozoa [4]. When $sPLA_2$ is released during the acrosome reaction, it initiates the formation of lysophospholipids (LP) and free fatty acids, either in the plasma membrane [5] or in the seminal plasma, resulting in an altered plasma membrane bilayer structure and increased membrane fluidity [4, 6]. The generated LP are well known as fusiogenic molecules implicated in membrane fusion events [7]. For successful fertilization of oocytes, spermatozoa have to reach the site of fertilization, in order to perform capacitation, to undergo the acrosome reaction on the surface of the oocyte and, subsequently, to complete membrane fusion. The LP lysophosphatidylcholine (LPC) may induce changes in the zona pellucida and in the oolemma, promoting spermatozoa-egg fusion. Based on these findings, it is suggested that sperm $sPLA_2$ and its product, LPC, may contribute to membrane fusion events in mammalian fertilization. Thus, $sPLA_2$ may be functionally relevant for the fertilizing capacity of spermatozoa. Therefore, the localization of $sPLA_2$ in the acrosome region can provide information about the functional quality of human spermatozoa. A disturbance of the $sPLA_2$ supply, such as premature or reduced release from the acrosome, may contribute to infertility because such spermatozoa are incapable of lysing egg envelopes. This situation is also imaginable as a result of genital tract inflammations, which may lead to an impaired acrosome reaction either by decreasing the inducibility of the acrosome reaction or by increasing the spontaneous acrosome reaction [8]. Therefore, we investigated the effect of elastase as one of the mediators of inflammation [9], as well as of the acrosome status, on $sPLA_2$ localization and on $sPLA_2$-release by confocal laser scanning fluorescence microscopy and fluorescence-activated cell sorting (FACS).

2 Materials and methods

2.1 Selection criteria of semen samples

Semen samples, which were within semen parameters for normal values according to the World Health Organisation (WHO) reference ranges [10], were obtained from healthy volunteers (donors) following a period of 3–5 days of sexual abstinence and after their written informed consent, in accordance with the ethical standard guidelines of the University of Leipzig (Leipzig, Germany). The approval for this study was obtained from the ethical committee of the University of Leipzig. Semen samples with a sperm concentration of more than 20×10^6/mL, more than 15% morphologically normal cells and at least 50% progressive sperm motility were selected for the study. The lower limit for the percentage of spermatozoa with normal morphology was set at 15% according to the recommendations of the German Society of Andrology. The semen samples were collected by masturbation into sterile plastic Petri dishes. Semen samples with a positive mixed antiglobulin reaction test, i.e. >10% spermatozoa with adherent particles, were excluded. The computer-aided sperm motion analysis (CASA) was performed by Mika cell motion analysis (Version 2.0 for Windows NT 4.0, Mika Medical GmbH, Montreux, Switzerland). Aliquots of semen samples (5 μL) were placed into 10 μm deep disposable counting chambers (Stroemberg-Mika, Montreux, Switzerland) on a 36ºC microscope stage warmer. A minimum of 100 spermatozoa from at least four different fields were analyzed from each specimen [11].

The donors were free of clinical symptoms of genital inflammation such as urethritis, abnormal swelling of the epididymis, or painful prostate and epididymis or pelvic pain. No individual had been treated with antibiotics or anti-inflammatory drugs within 3 months before examination.

2.2 Semen preparation and processing

Spermatozoa were isolated with the density gradient medium Sil Select Plus (Santa Ana, CA, USA) with centrifugation at $400 \times g$ for 20 min. The sediment was washed twice in human tubal fluid (HTF) at $400 \times g$ for 8 min. The supernatants were discarded and the pellets were used for further experiments.

2.3 Acrosome reaction

To trigger the acrosome reaction, spermatozoa (15×10^6 cells) were incubated in 0.14 mol/L NaCl, 4 mmol/L KCl, 10 mmol/L glucose, 4 mmol/L HEPES, 1 mmol/L $CaCl_2$, pH 7.4 at 37ºC for 60 min. The calcium–ionophore calcimycin A23187 (2×10^{-5} mol/L, Sigma-Aldrich, Taufkirchen, Germany) was added and the samples were incubated at 37ºC for 60 min. The method varies from the standard method recommended by the WHO [10] in order to obtain a higher number of acrosome-reacted cells [12].

2.4 Incubation of human spermatozoa with elastase

To simulate inflammatory conditions in the human genital tract, human spermatozoa (15×10^6 cells) were incubated with elastase from human polymorphonuclear leukocytes (PMN; 3 μg/mL; Calbiochem, Merck, Schwalbach, Germany) at 37ºC for 2 h.

2.5 Detection of $sPLA_2$ in human spermatozoa

Immunohistochemical techniques were used to visualize secretory phospholipase A_2 in human spermatozoa. Spermatozoa were incubated with the primary antibody mouse-anti-human-secretory PLA_2 mIgG1 (200 μg/mL, 1 : 100, Research Diagnostics, RDI, Flanders, NJ, USA) for 1 h. After washing (three times with 10 mmol/L phosphate-buffered saline [PBS], pH 7.4, 400 × *g*, 5 min), spermatozoa were incubated with the secondary antibody (goat-anti-mouse-CY3 conjugated, 1 : 100, Dianova, Hamburg, Germany) for 45 min followed by three washing steps (10 mmol/L PBS, pH 7.4, 400 × *g*, 5 min). Controls to specify specific/unspecific binding were performed by only using the secondary antibody. Pre-immune serum was also used as control followed by the incubation with the secondary antibody. Using intact spermatozoa, we could not observe any staining.

The distribution of $sPLA_2$ was detected by confocal laser scanning fluorescence microscopy (Leica DM IRBE, Leica, Wetzlar, Germany) using the software Leica Microsystems TCS SP2, and by flow cytometry (FACS) using a FACScan (Becton Dickinson, San Joes, CA, USA) and CellQuest software (Becton Dickinson). The flow cytometer device used an argon laser emitting at 488 nm. The following settings were acquired to obtain the data (10 000 events): forward scatter, voltage E00; side scatter, 288 V; fluorescence channel 1 for fluoroscein isothiocyanate (FITC)-fluorescence and channel 2 for Cy-3-fluorescence, 628 V. The influence of FITC in channel 2 was removed using a compensation of 25%. The primary $sPLA_2$ antibodies were validated by dot blot techniques and the mean fluorescence intensity for antibody binding were compared with the Cy-3 labelled second antibody conjugate controls using the CellQuest Software.

2.6 Detection of annexin V binding to phosphatidylserine (PS) of the spermatozoa

The binding of annexin V to PS epitopes was used to detect dead and apoptotic spermatozoa. Cells with externalized PS or deteriorated plasma membrane bind annexin V, whereas vital cells with intact membranes do not bind this protein. Spermatozoa samples were centrifuged (400 × *g*, 5 min) and resuspended in binding buffer (ApoAlert Annexin V-FITC apoptosis kit, Clontech Lab., Mountain View, CA, USA). After addition of 5 μL FITC-conjugated annexin V solution, samples were incubated in the dark at room temperature [13]. The samples were used for FACS analyses and fluorescence microscopy after washing and resuspending in PBS. A formalin-treated concanavalin A–FITC-marked sample (100 mg/mL; Biomeda, Foster City, CA, USA) was used as positive control.

2.7 Evaluation of the acrosomal state

The ability of concanavalin A-FITC to bind to glycoconjugates of the inner acrosomal membrane of spermatozoa [14] was used to determine the acrosomal state of the cells. Although spermatozoa with deteriorated plasma and acrosomal membranes have been reported to become fluorescently labelled over the anterior sperm head and equatorial regions, spermatozoa with intact membranes were impermeable to this lectin and did not produce fluorescence signal after exposure [14]. This feature has allowed the use of concanavalin A (Sigma-Aldrich, Taufkirchen, Germany; 100 mg/mL in PBS, pH 7.4, 30 min at room temperature, two washing steps, with distilled water) for the evaluation of acrosomal integrity in spermatozoa by fluorescence microscopy [14] as well as by flow cytometry [15]. Spermatozoa were not permeabilized at all.

2.8 Vitality assay with ethidium-homodimer I

Avital spermatozoa were labelled with ethidium-homodimer I [16]. Fifty μL of sperm suspension were mixed with 50 μL of a freshly prepared 2 mmol/L solution of ethidium-homodimer I (Sigma-Aldrich, Taufkirchen, Germany). After addition of 15 μL of the fixation solution (1 mmol/L Tris, 1.25 mol/L glutaraldehyde, pH 7.0), 10 μL of the suspension was investigated by fluorescence microscopy and also assessed by FACS.

3 Results

3.1 Effects of acrosome status and sperm viability on the localization of $sPLA_2$

To determine the relationship between the acrosome reaction and $sPLA_2$ release, we examined the localization of the enzyme in freshly isolated, non-acrosome-reacted and chemically acrosome-reacted, human sperma-

tozoa from healthy donors by means of immunocytochemistry and confocal laser scanning fluorescence microscopy.

A freshly ejaculated spermatozoa population from healthy volunteers produced with our modified method of detection of acrosome state only a weak fluorescence (Figure 1A, E, G; fluorescence classes 10–500). Thirty-nine percent of this population was recognized as acrosome reacted or as destabilized acrosome (Figure 1A, E, G; fluorescence classes 500–10 000). Nearly half of these cells also contained sPLA$_2$ (47%, Figure 1C, F, G; fluorescence classes 10–10 000). The secretory PLA$_2$ was localized at the acrosome and tail regions in approximately 75% of the cells. Figure 2A shows a typical distribution pattern in red fluorescence (Cy-3 staining) by the secondary sPLA$_2$ antibody. In all other cells, sPLA$_2$ was found only at the tail region, not inside the acrosome, only in small patches (Figure 2D). The tail staining of spermatozoa seems to be specific since controls only using the secondary antibody did not show any significant fluorescence labelling. Viability at freshly isolated cells amounted to 75%, which was slightly decreasing during the immunohistochemical procedure. The negative control with only the Cy-3 conjugated secondary antibody generated an insignificant fluorescence signal between 0 and 10 (autofluorescence). The concomitant analysis of these cells with annexin V-FITC revealed that the sPLA$_2$-positive cells were annexin V-FITC negative, whereas annexin V-FITC was able to bind to cells that did not contain sPLA$_2$ in the acrosome region (data not shown). Concanavalin A shows slight staining for cells containing sPLA$_2$ and bright staining for cells with only small patches

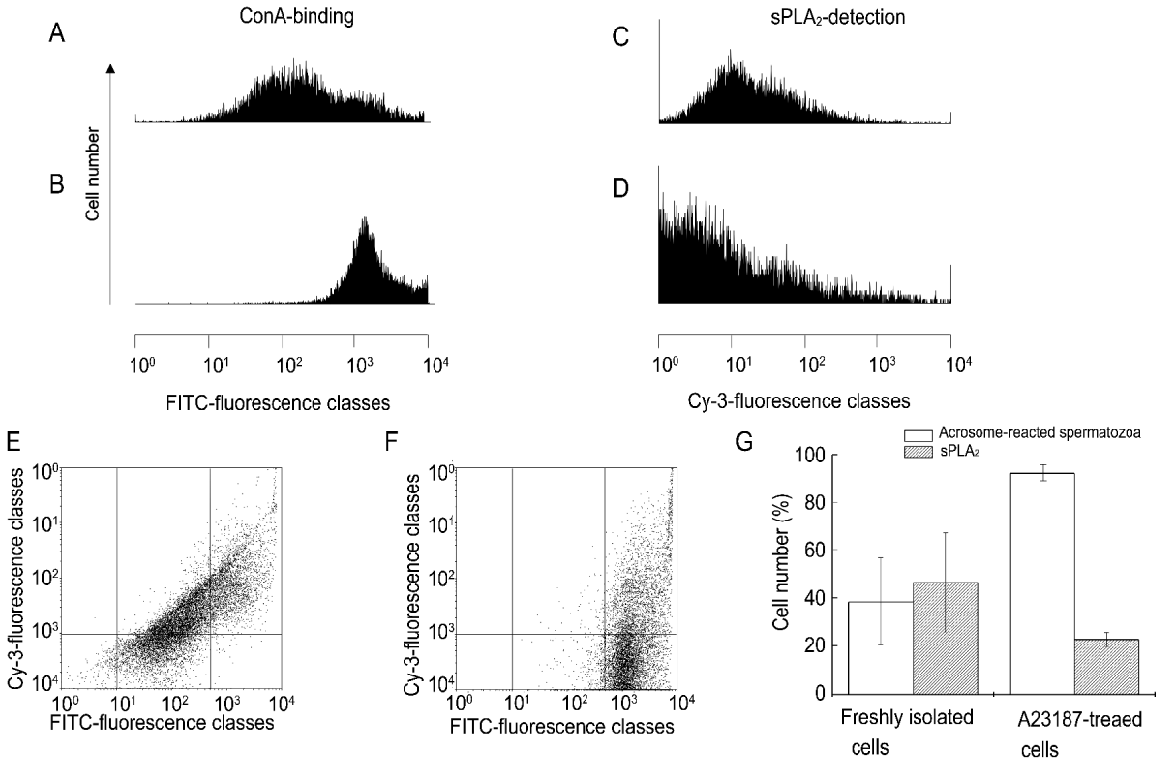

Figure 1. The quantitative estimation of secretory phospholipase A$_2$ (sPLA$_2$)-release and acrosome reaction in freshly isolated human spermatozoa from healthy donors determined by flow cytometry. The working voltages were E00 (forward-angle light scattering, FSC), 288 V (side-angle light scattering, SSC) and 628 V (fluoroscein isothiocyanate [FITC]- and Cy-3-fluorescence). Two different populations were shown, distinguishable by different fluorescence intensities after concanavalin A (ConA)-staining (A; E, FITC-fluorescence classes). With initiation of the acrosome reaction, the cell number of the population with high fluorescence intensity increases (B, F, ConA-FITC-fluorescence). The spermatozoa contained sPLA$_2$ (C, E, Cy-3-fluorescence classes), which can be released by the calcimycin-triggered acrosome reaction (D, F, decreasing Cy-3-fluorescence). Viability of all freshly isolated spermatozoa approximately amounted 75% with a slight decrease during the immunohistochemical procedure. All results are combined in a diagram (G).

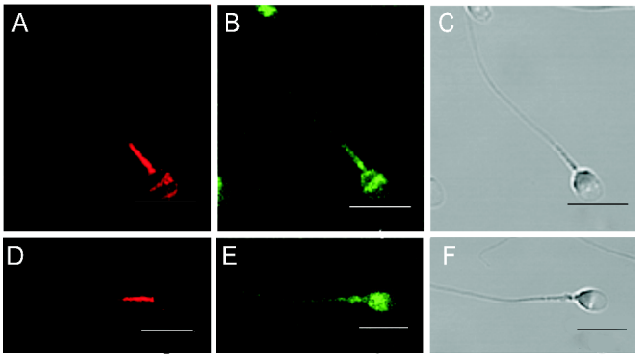

Figure 2. Distribution of secretory phospholipase A_2 ($sPLA_2$) in freshly isolated human spermatozoa from healthy donors detected by antibody-techniques and confocal laser scanning fluorescence microscopy. Most cells contain $sPLA_2$ in the whole cell body, especially in the acrosome region (A, red staining), whereas approximately 25% of freshly isolated human spermatozoa contain $sPLA_2$ only in the tail region and the midpiece (D). For both conditions the acrosomal states (B, E) and the phase contrast pictures (C, F) are shown; (B) secretory phospholipase containing cells are show only slight concanavalin A (ConA)-staining, whereas cells not containing the enzyme are ConA-positive (E). Bars = 10 μm.

of the enzyme at the tail region (Figure 2B, E). The phase contrast pictures are added for better recognition.

The induction of the acrosome reaction in human spermatozoa by calcimycin induced the release of $sPLA_2$ from the acrosome. In acrosome-reacted spermatozoa, $sPLA_2$ was only detected at the tail region, partially at middle portion and in small patches at the plasma membrane in the head region of cells. Interestingly, the acrosome region was free of $sPLA_2$ (Figure 3A, B, C, D). Acrosome-reacted spermatozoa were also annexin-V positive, as revealed by co-staining with FITC-labelled annexin-V (Figure 3B) and concanavalin A-FITC at the acrosomal region, which reveals the availability of the inner acrosomal membrane (Figure 3C). This change during the course of the acrosome reaction could also be verified by quantitative detection with flow cytometry. With initiation of the acrosome reaction by calcimycin, the concanavalin A-FITC fluorescence increased dramatically (92%, Figure 1B, F, G), whereas the relative number of $sPLA_2$-containing spermatozoa decreased to 23% (Figure 1D, F, G). These results suggest a strong association of $sPLA_2$ release with the acrosome reaction.

Ethidium-homodimer I verifies the vitality loss of cells subsequent to the acrosome reaction (Figure 3D, false colour staining, turquoise).

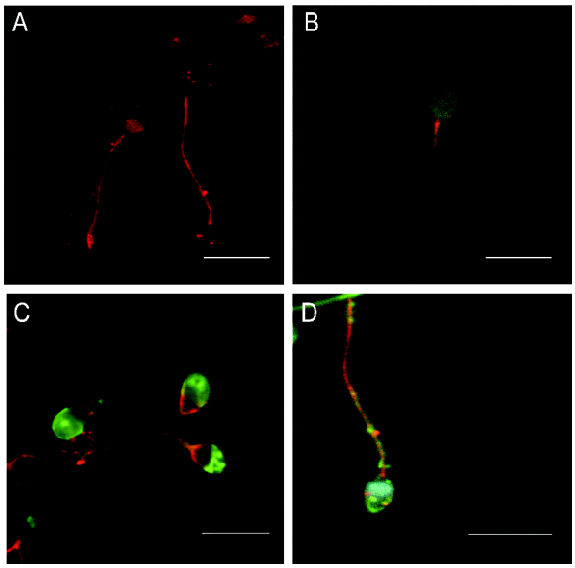

Figure 3. The distribution of secretory phospholipase A_2 ($sPLA_2$) in human spermatozoa after artificial acrosome activation triggered by the calcium-ionophore calcimycin A23187. The acrosome reaction leads to a loss of $sPLA_2$ in the sperm head, especially at the anterior acrosome region (A, red fluorescence). Subsequently, acrosome-reacted spermatozoa lose their vitality as evidenced by annexin V-fluoroscein isothiocyanate (FITC) co-staining (B, green fluorescence) and ethidium-homodimer I intercalation (D, false colour representation, turquoise). Capacitation and acrosome reaction were detected by concanavalin A (ConA)-FITC-co-staining (C, green fluorescence). The triple-staining (D) presents the $sPLA_2$-release from the head (red fluorescence) during the acrosome reaction (green fluorescence), and subsequent loss of vitality (false colour representation, turquoise). Bars = 10 μm.

3.2 Effect of elastase on $sPLA_2$ release

A premature $sPLA_2$ loss seems to occur during a premature acrosome reaction, which takes place in the presence of elastase that is used as a model for inflammatory conditions in the male genital tract (Figure 4A, concanavalin A-FITC staining, immunofluorescence techniques for $sPLA_2$). Numerous polymorphonuclear leukocytes (PMN) are known to accumulate at inflammatory loci and to secrete elastase [16]. Incubation of human spermatozoa with PMN elastase (3 μg/mL) caused a premature triggering of the acrosome reaction. The ability of concanavalin A able to bind to the inner acrosomal membrane (Figure 4A, concanavalin A-FITC staining) indicated the loss of the outer acrosomal membrane. $sPLA_2$ could no longer be detected at the acrosome region (Figure 4A). Figure 4B shows the phase contrast picture.

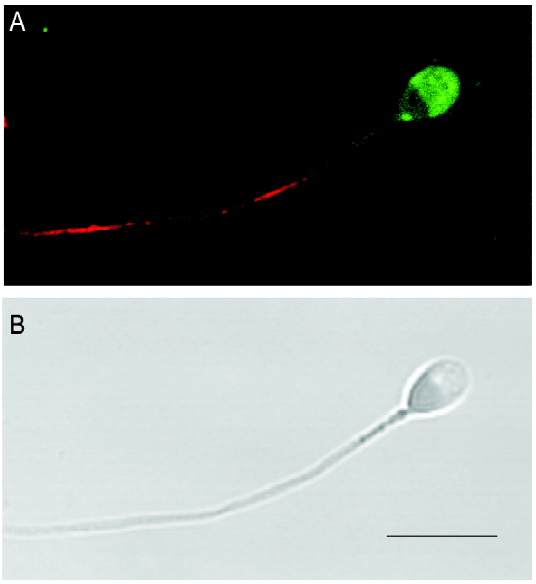

Figure 4. The localization of sPLA$_2$ in human spermatozoal cells from healthy donors after elastase incubation (A). The phase contrast picture is shown in (B). An inflammatory model, incubating the human neutrophil elastase (3 μg/mL) with human spermatozoa, emphasizes the impact of premature acrosome reaction (concanavalin A [ConA]-fluoroscein isothiocyanate [FITC] staining, green fluorescence) on the release of sPLA$_2$ (F, red fluorescence) from human spermatozoa. Bar = 10 μm.

The quantitative assessment of the correlation of sPLA$_2$ release from the spermatozoal cells to the initiation of the acrosome reaction was confirmed by flow cytometry (Figure 1).

4 Discussion

In the present study, we demonstrated the concomitance of sPLA$_2$ release with chemically induced deterioration of the acrosome with a calcium ionophore, as well as under inflammatory conditions. The enzyme disappeared from the acrosome region in acrosome-reacted spermatozoa as well as in spermatozoa after treatment with elastase. On the other hand, some portion of sPLA$_2$ is localized at the tail region of spermatozoa. Apparently, this portion is only hardly changed during acrosome reaction. It remains unknown to us, during which stage of sperm maturation, sPLA$_2$ appears on these epitopes.

sPLA$_2$ is known to induce the formation of LP, which results in increased membrane fluidity [4, 6] and mediation of membrane fusion events [7]. Thus, sPLA$_2$, provided at the appropriate time might promote the fertilization process and, on the other hand, premature sPLA$_2$ release could contribute to impaired fertilizing capabilities.

Successful fertilization of the oocyte can only take place with spermatozoa that are capable of passing through capacitation, acrosome reaction, binding and penetration through the oocyte surrounding complex. This sequence of fertilization events may be impaired by silent genital tract inflammations, which are often not recognized because of the mostly asymptomatic course of the disease [17], but may be associated with reduced sperm motility and vitality as well as with sperm apoptosis [17, 18]. Increased levels of the leukocyte protease elastase as well as cytokines as TNFα, IL 1β or IL 8 are often found at areas of inflammation [16, 19, 20, 21]. Since we chose elastase as model for inflammatory processes, it is crucial that elevated elastase concentrations in infertile men are frequently associated with the number of peroxidase-positive cells and negatively correlated with sperm vitality, motility and intact DNA. Therefore, the influence of elastase on the acrosome and PLA$_2$ release was of practical interest. Besides the leukocytes, bacteria themselves, as well as bacterial endotoxins, may also have a damaging effect on spermatozoa by stimulating the production of reactive oxygen species (ROS) in leukocytes [22]. Oxidative stress increases the risk of lipid peroxidation and impairs the biological function of sperm membranes [23]. These two inflammatory mediators, bacteria and leukocytes, play a mutual role in deepening the harmful effect of oxidative stress on spermatozoa [22]. Inflammations in the male genital tract may affect two pathways critical for fertilization, intracellular apoptosis associated signals [24] and premature acrosome destabilization. Apoptotic spermatozoa are characterized by scramblase-activation [25], increased amounts of ROS and sPLA$_2$ activation [26]. A premature acrosome reaction or deterioration of the acrosome during silent inflammations results in a loss of lytic ability before contact with the egg, as well as impairment of fertilizing ability, since the capability of these spermatozoa to fuse with oocyte membranes is highly reduced. These cells are removed from the pool of fertilizing spermatozoa and the consequence may be reduced fertility. The concomitance of the acrosome reaction and sPLA$_2$ release suggests that the sPLA$_2$-distribution pattern can be regarded as one indicator for the ability of sperma-

tozoa to proceed through a successful fertilization sequence.

The calcimycin-induced acrosome reaction in human spermatozoa led to $sPLA_2$ release from the sperm head, as evidenced by immunohistochemical techniques and visualization by confocal laser-scanning fluorescence microscopy and flow cytometry. This chemically induced situation can be used as model for the natural occurring incidences in order to determine the relationship between acrosome status and $sPLA_2$ effects [1, 27]. The monoclonal $sPLA_2$ antibody specifically detects $sPLA_2$ mostly in the acrosome and in the tail regions of intact spermatozoa, whereas acrosome-reacted populations are characterized by the presence of only small patches of $sPLA_2$ in the head region demonstrating the localization of the enzyme at the acrosomal membranes released by the membrane loss. Since permeabilization of cells was not performed it can be evidenced that $sPLA_2$ is localized at the sperm surface but not in the acrosomal content.

The determination of the acrosomal state of spermatozoa by chlorotetracycline, pisum sativum agglutinin or concanavalin A as well as the distribution of specified molecules with confocal laser scanning fluorescence microscopy provides the ability to scan objects in slices and therefore establishes the possibility of extremely accurately determining the localization of specific molecules or attributes [28, 29, 30]. The high precision of the performed visualization of the estrogen receptor β, the androgen receptor and binding of myeloperoxidase at spermatozoa [31, 32] induced us to use this method to estimate the location of $sPLA_2$ during different acrosomal states in the cells.

Our suggested conclusion is that the concomitance of disintegration of acrosome and $sPLA_2$ release represents a functional relationship and may allow $sPLA_2$-distribution patterns to be regarded as indicators for the ability of spermatozoa to fuse with the oocyte. The existence of invading polymorphonuclear leukocytes at inflammatory loci and specific PMN products, such as elastase, might prevent spermatozoa from supplying $sPLA_2$ for the fertilization process.

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