

Inhibition of the acrosome reaction by trypsin inhibitors and prevention of penetration of spermatozoa through the human zona pellucida

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In this study we evaluated the effect of several trypsin inhibitors (*p*-aminobenzamidine: pAB; *N*- α -*p*-tosyl-L-lysine-chloromethyl-ketone: TLCK and *p*-nitrophenyl-*p*'-guanidino-benzoate: NPGB) on sperm binding and penetration of the human zona pellucida. Motile spermatozoa, selected by a two-step Percoll gradient, were incubated at 1×10^7 cells ml⁻¹ at 37°C and in 5% CO₂ for 4.5 h. This was followed by the addition of 1 mmol pAB l⁻¹ or phosphate-buffered saline (control) for 30 min. Three to four non-viable human oocytes were then added to each sperm suspension and incubated for 3 h. The numbers of spermatozoa bound to the human zona pellucida and in the perivitelline space were determined by phase contrast microscopy. The results showed that pAB significantly inhibited zona penetration by spermatozoa ($56 \pm 8\%$ oocytes penetrated, control versus $0 \pm 0\%$ oocytes penetrated, pAB, mean \pm SEM), without modifying spermatozoa–zona pellucida binding. The inhibition of zona penetration was due to a block of the acrosome reaction normally induced by the human zona pellucida. In separate experiments, sperm suspensions pretreated with 1 mmol pAB l⁻¹ or 10 μ mol NPGB l⁻¹ exhibited a marked decrease in the percentage of acrosome reactions on the zona surface ($85 \pm 4\%$ and $76 \pm 3\%$ inhibition, respectively). In addition, the inhibitors prevented the acrosome reaction induced by human follicular fluid (percentage of acrosome-reacted spermatozoa: control 8 ± 2 ; follicular fluid 25 ± 3 ; pAB 6 ± 2 ; NPGB 8 ± 1 ; TLCK 12 ± 2). Electron microscope studies suggested a significant inhibition of the membrane fusion events of the acrosome reaction in the inhibitor-treated spermatozoa. These results are the first to show that trypsin inhibitors block sperm penetration of the human zona pellucida owing to an inhibition of the acrosome reaction. In addition, they suggest a role for a trypsin-like enzyme during the acrosome reaction of human spermatozoa.

Introduction

The interaction between spermatozoa and the zona pellucida is a crucial step during mammalian fertilization (Yanagimachi, 1988). This interaction involves several levels. First, the spermatozoa attach to the zona pellucida. This primary attachment is performed by either acrosome-intact or acrosome-reacted spermatozoa. In the mouse, however, only acrosome-intact spermatozoa can initiate binding to the zona pellucida (Florman and Storey, 1982; Saling and Storey, 1979). The initial binding is followed by either the zona-induced acrosome reaction or by secondary sperm binding and penetration of the zona pellucida (Yanagimachi, 1988). It is generally accepted that spermatozoa must be acrosome reacted to complete penetration of the zona. However, the exact site where the acrosome reaction occurs *in vivo* has not been determined. Various components of the zona pellucida involved in gamete

interaction have been identified and characterized. In the mouse, the zona pellucida is composed of three glycoproteins termed ZP1, ZP2 and ZP3. ZP3 is responsible for binding acrosome-intact spermatozoa to the zona pellucida and for stimulating the acrosome reaction (Wassarman *et al.*, 1989). ZP2 has been implicated in the secondary binding of acrosome-reacted spermatozoa to the zona (Bleil *et al.*, 1988). It has been suggested that a trypsin-like enzyme of spermatozoa could participate in several of these processes (Saling, 1981; Dravland *et al.*, 1984; Bleil *et al.*, 1988). For instance, there is evidence that sperm penetration through the zona pellucida involves localized proteolysis (McRorie and Williams, 1974). Before the morphologically visible human sperm acrosome reaction begins, limited amounts of activated, immunoreactive acrosin are mobilized to the sperm surface. The enzyme was detected on the plasma membrane covering the acrosomal area (Tesarik *et al.*, 1988, 1990). This finding supports a role for acrosin in spermatozoa–zona pellucida initial binding and in the early biochemical events of the human sperm acrosome reaction (De Jonge *et al.*, 1989). In non-human species, acrosin has been associated with

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the acrosome reaction, participating either in the dispersal of the acrosomal matrix or in the membrane events of the acrosome reaction, or in both (Shams-Borhan and Harrison, 1981; Dravland *et al.*, 1984). It has also been suggested that a trypsin-like enzyme may be involved in the binding of mouse spermatozoa to the zona pellucida (Saling, 1981; Benau and Storey, 1987).

In the present study, we investigated the influence of several low molecular weight trypsin inhibitors on the interaction of human spermatozoa with zona pellucida. We performed experiments to test the effect of these inhibitors on the ability of spermatozoa to bind to the human zona pellucida, to undergo the acrosome reaction on the zona surface, to penetrate the human zona pellucida, and to undergo the acrosome reaction upon stimulation with human follicular fluid. Our results suggest a role for a trypsin-like enzyme in the mechanism by which human spermatozoa undergo the acrosome reaction, either induced by human zona pellucida or human follicular fluid. Part of these results has been presented elsewhere (Morales *et al.*, 1990).

Materials and Methods

Sperm preparation

Normal semen samples, obtained for diagnostic purposes at the Center for the Study of Reproductive Biology (CEBRE), of the P. Catholic University of Chile, were used. The specimens were obtained after two to four days of sexual abstinence and were allowed to liquefy for 30–60 min at room temperature. The samples were used within 1 h of collection. Motile spermatozoa were selected by centrifugation through a two-step Percoll gradient (Yudin *et al.*, 1988). The two steps of the Percoll gradient were 80% in the lower layer and 40% in the upper layer. Briefly, 1–2 ml aliquots of semen were layered over the upper layer of the Percoll gradient and then centrifuged for 20 min at 300 g. The pellet was washed twice by dilution with 10 ml of modified Tyrode's medium (Yudin *et al.*, 1988) supplemented with 0.3% bovine serum albumin (BSA, Fraction V, Sigma Chemical Co., St Louis, MO). The sperm suspension was then centrifuged for 10 min at 300 g. The final pellet was diluted in modified Tyrode's medium supplemented with 2.6% BSA. Sperm suspensions were adjusted to 1×10^7 cells ml^{-1} and incubated in 1 ml aliquots for various periods at 37°C in 5% CO_2 , 95% air.

Collection of human oocytes

Human oocytes were dissected from ovarian tissue obtained from cadavers, and stored at -80°C as previously described (Cross *et al.*, 1988). After thawing, the oocytes were freed of remaining cumulus cells by passing them through a narrow bore pipette. As a result of freezing and thawing, these oocytes were not viable.

Collection of follicular fluid

The fluids used in this study were collected from preovulatory follicles after gonadotrophin stimulation and were kept frozen in aliquots at -20°C until use. The human follicular fluid used

here was selected for its effectiveness in inducing acrosome reactions in previous experiments (Morales *et al.*, 1992).

Effect of inhibitors on the penetration of zona pellucida by spermatozoa

The effect of protease inhibitors on penetration of zona pellucida by spermatozoa was determined by incubating sperm suspensions for 5 or 20 h and then treating with 1 mmol *p*-aminobenzamidine l^{-1} (pAB) or with phosphate-buffered saline for 30 min. Subsequently, four human oocytes were added to each sperm suspension and the gametes were coincubated for an additional 3 h. The number of spermatozoa attached to the human zona pellucida was determined using phase contrast microscopy (the terms 'attached' and 'bound' will be used interchangeably to refer to any adherent spermatozoa, Cross *et al.*, 1988). Zona penetration was evaluated by aspirating oocytes in and out of a small-bore pipette several times to remove excess spermatozoa and they were then mounted between a glass slide and cover slip supported by four pillars of Vaseline-paraffin. Each human oocyte was repeatedly rolled between the slide and cover glass and the number of spermatozoa in the perivitelline space was determined (Lambert *et al.*, 1985).

Effect of inhibitors on the sperm acrosome reaction

The effect of protease inhibitors on the human sperm acrosome reaction was evaluated using intact zonae pellucidae or preovulatory human follicular fluid to induce the acrosome reaction, as described below. pAB and *N* α -*p*-tosyl-L-lysine-chloromethyl-ketone (TLCK) were dissolved in phosphate-buffered saline, pH 7.3. *p*-Nitrophenyl-*p*'-guanidino-benzoate (NPGb) was dissolved in dimethylsulfoxide. Inhibitor additions were made by adding 2 μl of the particular compound to 198 μl of capacitated spermatozoa; final inhibitor concentrations were 1 mmol pAB l^{-1} , 25 μmol TLCK l^{-1} and 10 μmol NPGb l^{-1} . The final concentration of dimethylsulfoxide in sperm suspensions was 0.1% (v/v). The concentrations of inhibitors used in the present study were the highest possible that did not inhibit sperm motility. The inhibitors were purchased from Sigma Chemical Co.

Acrosome reactions induced by human zona pellucida

Spermatozoa were incubated for 4.5 h and, separate aliquots were then incubated with 1 mmol pAB l^{-1} or 10 μmol NPGb l^{-1} for 30 min. Sperm suspensions pretreated with NPGb were washed and resuspended in fresh, inhibitor-free media before adding the zonae pellucidae. Control sperm suspensions were incubated with the appropriate solvents. Four human oocytes were then added to each aliquot and incubated for a further 30 min. The oocytes with adhered spermatozoa were then fixed in ethanol and the acrosomal status of the bound spermatozoa was determined by indirect immunofluorescence using a polyclonal antisperm antiserum (Cross *et al.*, 1986; Morales *et al.*, 1989).

Acrosome reactions induced by human follicular fluid

Spermatozoa were incubated for 20 h and separate aliquots were then pretreated with 1 mmol pAB l^{-1} , 10 μmol NPGb l^{-1}

or 25 $\mu\text{mol TLCK l}^{-1}$ for 30 min. Sperm suspensions pretreated with NPGB or TLCK were always washed and resuspended in fresh, inhibitor-free media before adding the human follicular fluid. Control sperm suspensions were incubated with the appropriate solvents. The spermatozoa were then treated with 20% human follicular fluid (v/v) for a further 15 min. The acrosome reaction was detected using fluoresceinated *Pisum sativum* agglutinin (Vector Laboratories, Inc., Burlingame, CA) as described previously (Cross *et al.*, 1986). *Pisum sativum* agglutinin and antisperm antiserum provide equivalent assessments of acrosomal status (Cross *et al.*, 1986, 1988), but *Pisum sativum* agglutinin was used for sperm suspensions because the method is simpler and more rapid. The antiserum was used for spermatozoa on the zona pellucida because *Pisum sativum* agglutinin binds to the zona pellucida and obscures the pattern of sperm labelling.

Electron microscopy

In other experiments, spermatozoa pretreated with inhibitors and then with human follicular fluid were processed for transmission electron microscopy. The spermatozoa were fixed in a solution of 3% glutaraldehyde prepared in albumin-free cacodylate buffer (0.25 mol l^{-1}) pH 7.4. The samples were then dehydrated in a series of increasing concentrations of acetone and embedded in Spurr's embedding medium Ted Pella, Inc., Redding, CA. Ultrathin sections were obtained in the MT 2B Porter Blum ultramicrotome. The grids with sections were stained with uranyl acetate and lead citrate. The numbers of acrosome-intact and acrosome-reacted spermatozoa were evaluated using a Siemens 1-AKV transmission electron microscope. One hundred spermatozoa were scored in each group.

Results

In the control group, about 60% of the oocytes were penetrated by one or more spermatozoa in the perivitelline space (2.1 ± 0.3 spermatozoa in the perivitelline space, mean \pm SEM, $n = 26$ penetrated oocytes). The ability of the spermatozoa to penetrate the human zona pellucida was totally inhibited by 1 mmol pAB l^{-1} (Fig. 1). There were no oocytes with spermatozoa in the perivitelline space in the pAB-treated group. This was the case whether the spermatozoa were incubated for 5 h or for 20 h. The number of spermatozoa bound to the human zona pellucida was unaffected by the presence of the inhibitor (120 ± 25 and 100 ± 30 sperm bound per zona, control and pAB-treated cells, respectively, $n = 11$). The inhibition of sperm passage through the human zona pellucida could be due to either an inhibition of the enzyme responsible for local digestion of the zona or an inhibition of the acrosome reaction itself. The latter was tested by determining the percentage of spermatozoa that could undergo the acrosome reaction on the surface of the human zona pellucida, in the presence or absence of trypsin inhibitors. After 30 min of coincubation of the gametes, the percentage of acrosome reaction among the zona-bound spermatozoa was between 40 and 50% in the control groups (Fig. 2). However, when the cells were pretreated with the inhibitors, the percentage of acrosome-reacted spermatozoa

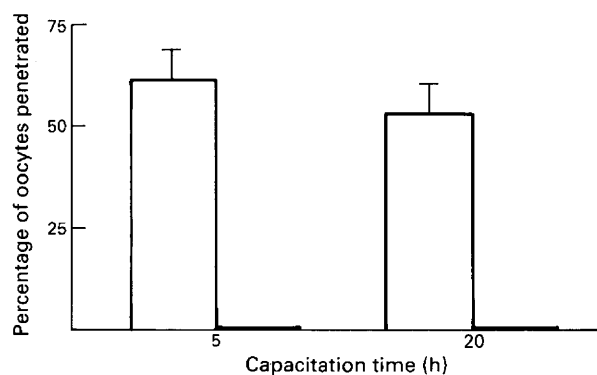


Fig. 1. Effect of 1 mmol *p*-aminobenzamidine l^{-1} (pAB) on the ability of human spermatozoa to penetrate the human zona pellucida. Results are means \pm SEM of seven experiments carried out after 5 h of sperm capacitation and four experiments carried out after 20 h of sperm capacitation. Four oocytes were used in each experiment. (□) Control and (■) pAB-treated groups.

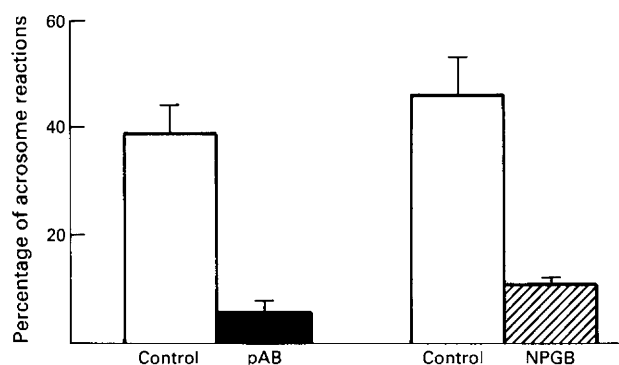


Fig. 2. Effect of 1 mmol *p*-aminobenzamidine l^{-1} (pAB) and 10 $\mu\text{mol p}$ -nitrophenyl-*p*'-guanidino-benzoate (NPGB) on the ability of human spermatozoa to undergo the acrosome reaction on the zona surface. Spermatozoa pretreated with NPGB were washed and resuspended in inhibitor-free media before adding the oocytes. Results are means \pm SEM of three experiments with each inhibitor. Four oocytes were used in each experiment.

on the zona surface was dramatically decreased ($85 \pm 4\%$ and $76 \pm 3\%$ inhibition for pAB- and NPGB-treated cells, respectively, $n = 3$). Again, the number of spermatozoa bound to the human zona pellucida was not different between the control and the treated groups (pAB-treated: 106 ± 18 spermatozoa bound per zona; NPGB-treated: 82 ± 18 spermatozoa bound per zona; control: 91 ± 20 spermatozoa bound per zona).

In additional experiments, we tested the ability of the various inhibitors to prevent the acrosome reaction induced by treatment with human follicular fluid (Fig. 3). Treatment with human follicular fluid induced a 3.5-fold increase in the percentage of acrosome-reacted spermatozoa, in comparison to the control group. Spermatozoa pretreated with pAB, NPGB or TLCK before the addition of human follicular fluid showed a 93 ± 7 , 90 ± 6 and $76 \pm 11\%$ inhibition of the acrosome reaction, respectively, in comparison with the human follicular fluid group

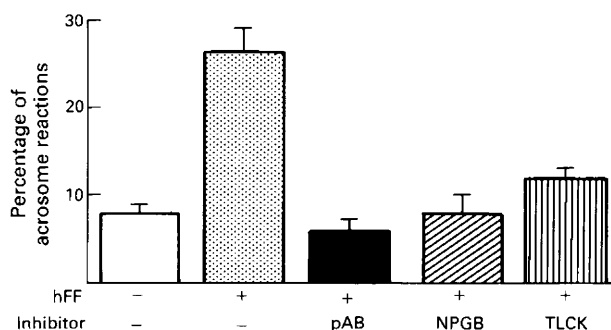


Fig. 3. Effect of 1 mmol *p*-aminobenzamidine l^{-1} (pAB), 10 μ mol *p*-nitrophenyl-*p*'-guanidino-benzoate l^{-1} (NPGB), and 25 μ mol *N* α -tosyl-L-lysine-chloromethyl-ketone l^{-1} (TLCK) on the ability of human spermatozoa to undergo the acrosome reaction upon stimulus with human follicular fluid (hFF). All the spermatozoa were treated with 20% human follicular fluid for 15 min, except the control group which received media alone. The various inhibitors were then added to the sperm suspensions. Spermatozoa pretreated with NPGB or TLCK were washed and resuspended in inhibitor-free media before adding the human follicular fluid. Results are means \pm SEM of eight experiments.

(Fig. 3). We did not detect intermediate stages of the acrosome reaction in the inhibitor-treated groups (Fig. 4). The percentage of acrosome-reacted spermatozoa in the group treated with human follicular fluid was 48%, in the pAB-treated groups was 16%, and in the NPGB-treated group was 18%. The basal percentage of acrosome reacted spermatozoa was between 8 and 12%.

Discussion

This study is the first to show that trypsin inhibitors prevent human sperm penetration through the zona pellucida of human oocytes. We suggest that the inhibition of zona penetration was caused by a block of the acrosome reaction induced by human zona pellucida. The ability of the sperm cells to bind to the human zona pellucida was not affected by the inhibitors. In addition, the inhibitors used in this study were able to block the human follicular fluid-induced acrosome reaction. These observations support a role for an endogenous trypsin-like enzyme from spermatozoa in the human sperm acrosome reaction, induced by either human follicular fluid or human zona pellucida. Our observations, however, do not support a role for a trypsin-like enzyme from spermatozoa in the primary spermatozoa–zona binding, as reported for mouse gametes (Saling, 1981; Benau and Storey, 1987).

It is not known at what level this trypsin-like enzyme acts during the sperm acrosome reaction. It has been suggested, for non-human species, that a trypsin-like activity may be involved in the membrane events of the acrosome reaction (Dravland *et al.*, 1984), or in the dispersal of the acrosomal matrix (Shams-Borhan and Harrison, 1981; Perreault *et al.*, 1982). Pillai and Meizel (1991) showed that trypsin inhibitors block the membrane events of the human sperm acrosome reaction. Our electron microscopy results also suggest that the inhibitors blocked the membrane fusion events of the acrosome reaction. However, the possibility that dispersal of the acrosomal matrix

was also prevented by the inhibitors cannot be ruled out by the present data.

It has long been recognized that a trypsin-like enzyme from spermatozoa has a role in zona pellucida penetration, through a limited proteolysis of the zona pellucida glycoproteins (Fraser, 1982; Dunbar *et al.*, 1985). The sperm trypsin-like enzyme implicated in gamete binding and recognition has been suggested to be acrosin, or its zymogen form proacrosin (Töpfer-Petersen and Henschen, 1987; Jones *et al.*, 1988). However, it is not clear whether the enzyme or zymogen would participate in the primary or in the secondary sperm binding to the zona, or in both. In the mouse, it was suggested that acrosin may be involved in both types of binding (Saling, 1981; Benau and Storey, 1987; Bleil *et al.*, 1988). It has been also shown that before the morphological events of the acrosome reaction, immunoreactive acrosin appears on the surface of the plasma membrane overlying the human sperm acrosome (Tesarik *et al.*, 1988, 1990). Thus, the authors proposed a role for acrosin in the primary binding of human spermatozoa with zona pellucida. Our results suggest that a trypsin-like enzyme may be involved in the human sperm acrosome reaction, but not in the primary binding between spermatozoa and human zona pellucida. The observation that treatment of spermatozoa with the inhibitors pAB or NPGB did not decrease the number of cells bound to the zona supports this assumption. However, we cannot draw any conclusion regarding a role for this trypsin-like enzyme during the secondary binding of spermatozoa to the human zona pellucida.

Although the identity of this human sperm trypsin-like enzyme is not clear, the best known mammalian sperm trypsin-like enzyme is acrosin. In this study, we used various low molecular weight serine protease inhibitors, known to inhibit trypsin-like enzymes through different mechanisms (Mares-Guia and Shaw, 1965; Chase and Shaw, 1970). The most probable action of these inhibitors in the sperm cell could therefore be related to proacrosin or acrosin inhibition (De Jonge *et al.*, 1989). Indeed, during extraction of this enzyme from spermatozoa it was shown that these inhibitors prevented the conversion of proacrosin to acrosin (Goodpasture *et al.*, 1981). It has also been shown that inhibitors of this type at concentrations above 10 μ mol l^{-1} block acrosin activity in whole human spermatozoa (Kaminski *et al.*, 1987). Nevertheless, the possibility that these inhibitors could be acting on hydrolytic enzymes other than acrosin cannot be excluded. Somatic cell phospholipase A_2 can be a potential target for serine protease inhibitors (Hesse *et al.*, 1984) and phospholipase A_2 has also been implicated in the process of acrosome reaction (Llanos *et al.*, 1982). However, a concentration of NPGB as high as 1 mmol l^{-1} did not inhibit this enzyme (Anderson *et al.*, 1988). In our study the low inhibitor concentrations used should therefore have precluded any significant effect on phospholipase A_2 .

Several investigators, using species other than humans, have proposed a role for a trypsin-like enzyme from spermatozoa in the acrosome reaction (Meizel and Lui, 1976; Dravland *et al.*, 1984). Our results with human spermatozoa suggest that human zona pellucida and follicular fluid induce the human sperm acrosome reaction through a common mechanism, mediated by a trypsin-like activity. It has been shown that the acrosome reaction induced by human follicular fluid involves a

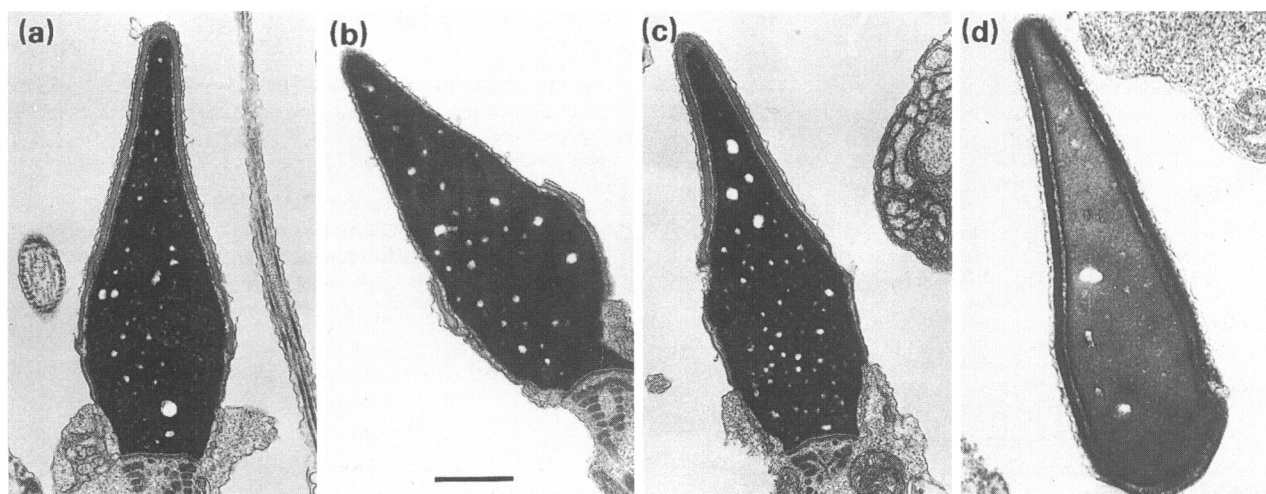


Fig. 4. Electron micrographs of human spermatozoa showing: (a) an acrosome-intact cell in the control group; (b) a typical acrosome-reacted spermatozoa from the human follicular fluid-treated group; (c) a representative, acrosome-intact spermatozoa from the human follicular fluid plus *p*-aminobenzamidinium (pAB) group; (d) a representative, acrosome-intact spermatozoa from the human follicular fluid plus *p*-nitrophenyl-*p*'-guanidino-benzoate (NPGb) group. The percentage of acrosome-reacted spermatozoa was 10% in the control group, 48% in the human follicular fluid-treated group, 16% in the human follicular fluid plus pAB group, and 18% in the human follicular fluid plus NPGb group. The bar represents 1 μ m.

rapid and transient influx of extracellular calcium into human spermatozoa (Thomas and Meizel, 1988; Blackmore *et al.*, 1990). Similar findings have been reported during the mouse and bovine zona pellucida-induced acrosome reaction (Lee and Storey, 1988; Florman *et al.*, 1989). Pillai and Meizel (1991) reported that trypsin inhibitors block the progesterone-induced acrosome reaction by preventing calcium influx, suggesting a role for a trypsin-like enzyme in increasing intracellular calcium of spermatozoa during the early events of the acrosome reaction. These findings and those of Tesarik *et al.* (1990), which demonstrate the presence of active acrosin on the surface of the human spermatozoa before the acrosome reaction, support a role for acrosin before stimulation of calcium entry. We propose that a trypsin-like enzyme, presumably acrosin, is playing a key role in the molecular events of the human sperm acrosome reaction. The enzyme could be activating other systems through proteolytic cleavages and thus committing the capacitated spermatozoa to the irreversible morphological events of the acrosome reaction.

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