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Involvement of trypsin-like activity in binding of mouse spermatozoa to zonae pellucidae

(cell–cell interaction/protease inhibitors/fertilization *in vitro*/gamete recognition)

PATRICIA M. SALING*

The Population Council, 1230 York Avenue, New York, New York 10021

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ABSTRACT **Work from a number of laboratories has shown that fertilization is blocked in the presence of protease inhibitors, although the specific site of inhibition has not been identified. The present experiments were designed to discriminate between sperm binding to zonae pellucidae as opposed to sperm penetration through zonae, so as to assess the effect of protease inhibitors on these two distinct events. Exposure of capacitated mouse spermatozoa to a variety of protease inhibitors directed against trypsin blocked sperm binding to zonae in a concentration-dependent manner. A chymotrypsin-directed inhibitor was not capable of blocking sperm binding to zonae. The trypsin inhibitors did not affect sperm penetration through zonae nor gamete membrane fusion if the sperm had established a firm association with the zona surface before addition of the inhibitors. Previous incubation of zona-intact eggs with the inhibitors did not lead to a reduction in sperm binding, indicating that the activity affected by the inhibitors is borne by spermatozoa. Interaction between spermatozoa and the zona surface appeared to be the specific locus of inhibition; sperm binding to zona-free eggs (i.e., binding to the egg plasma membrane) was unaltered by the trypsin inhibitors. These results suggest a reevaluation of the function of proteases in fertilization focusing on their role in initial sperm contact with the zona pellucida.**

Mammalian spermatozoa must traverse several investments before reaching their ultimate target, the plasma membrane of the egg. The final layer for the fertilizing spermatozoon is the zona pellucida, a glycoprotein envelope surrounding the egg, which the spermatozoon first binds to and then penetrates. The activity of a trypsin-like enzyme, acrosin (EC 3.4.21.10), localized in the acrosomal region of the sperm cell, has long been thought to be responsible for zona penetration (1–4). The essential role of this enzyme in fertilization appeared to be confirmed by the ability of a variety of trypsin inhibitors to block fertilization, both *in vivo* (5, 6) and *in vitro* (4, 7, 8). Aspects of several reports suggest, however, that acrosin may not be the functional zona penetrant (9–13). Since the specific site of the trypsin inhibitor-induced block to fertilization was not identified in the studies demonstrating the trypsin inhibitor block to fertilization, we have designed experiments to separate sperm binding to the zona from sperm penetration through the zona. The basic conclusion from these experiments, that a trypsin inhibitor-sensitive site is responsible for zona binding, suggests reevaluation of the function of acrosomal proteases.

MATERIALS AND METHODS

Medium. A complete culture medium [CM; 119.4 mM NaCl/4.8 mM KCl/1.7 mM $CaCl_2$/1.2 mM KH_2PO_4/1.2 mM $MgSO_4$/25.1 mM $NaHCO_3$/25 mM Na lactate/1 mM Na pyruvate/5.6 mM glucose/0.001% phenol red containing bovine serum albumin (20 mg/ml), penicillin G (60 units/ml), and streptomycin (60 μg/ml)] that supports both capacitation and fertilization of mouse gametes *in vitro* was used (14, 15). This modified Krebs–Ringer bicarbonate medium contains a variety of energy sources and serum albumin. The CM was adjusted to pH 7.4–7.5 with 1.0 M NaOH, sterilized by filtration (0.2-μm filter), and equilibrated with 5% CO_2/95% air immediately prior to use.

Gametes. All gamete manipulations were conducted at 37°C under a layer of sterile silicone oil (dimethylsiloxane, 20 cs; Contour Chemical, North Reading, MA) previously equilibrated with 5% CO_2/95% air. Superovulated tubal eggs from randomly bred Swiss mice (Taconic Farms, Germantown, NY) were recovered at 13–15 hr after human chorionic gonadotropin injection. Cumulus cells were removed by incubation for 10 min in CM/0.1% hyaluronidase (type 1-S); any remaining cumulus cells were removed mechanically. Completely denuded zona-intact eggs were washed twice and stored in CM under silicone oil until egg collection was complete, generally accomplished within 20 min. When required, zonae pellucidae were removed manually by forcing the cumulus-free eggs through narrow-bore micropipettes; such zona-free eggs were washed and maintained similarly in CM. Insemination of mouse eggs *in vitro* was accomplished according to the method of Wolf and Inoue (15). Unless otherwise indicated, a final sperm concentration of 1–5 × 10^5 cells per ml, estimated with a hemocytometer, was used. For a given experiment, all test points contained equal concentrations of sperm.

Mouse sperm suspensions were prepared by rupturing the excised caudae epididymides of mature (≥10 weeks) mice in CM. For each epididymis, 0.2 ml of CM was used; two animals were used per experiment. After 10–15 min for sperm dispersal, an aliquot of the epididymal suspension was diluted 1:5 in CM. Sperm were first incubated for 90–120 min at 37°C to allow for the occurrence of capacitation (15, 16). In some experiments, these sperm were added directly to the eggs without further manipulation. In other experiments, intended to examine the effects of various trypsin inhibitors on sperm function, previously incubated sperm were exposed to inhibitors as specified for 15–20 min at 37°C; the sperm concentration was maintained at 1–5 × 10^5 cells per ml. Control sperm suspensions were in-

Abbreviations: CM, complete culture medium; Me_2CONH_2, dimethylformamide; LBTI, lima bean trypsin inhibitor; NPGB, nitrophenylguanidinobenzoate; $PhMeSO_2F$, phenylmethylsulfonyl fluoride; OMTI; ovomucoid trypsin inhibitor; SBTI; soy bean trypsin inhibitor; $TosLysCH_2Cl$; N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (TLCK); $TosPheCH_2Cl$: L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK).

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* Present address: Laboratories for Cell Biology, Department of Anatomy, The School of Medicine, University of North Carolina at Chapel Hill, 111 Swing Building, 217 H, Chapel Hill, NC 27514.

cubated similarly in CM or, if dimethylformamide (Me_2COHN_2) was used as the solvent in the experimental suspension, in 0.5–1.0% Me_2CONH_2 in CM. Zona-intact eggs were then added to these sperm suspensions to assay the effects of the inhibitors on sperm binding. To determine whether these inhibitors might affect the egg rather than the spermatozoon, eggs were first incubated in CM, SBTI, or $TosLysCH_2Cl$, washed three times in CM, and added to previously incubated sperm that had not been exposed to the inhibitors. Throughout this study, sperm motility was evaluated subjectively with an inverted microscope by using interference optics (Nikon).

Sperm-binding Assay. Quantitative assessment of sperm bound to eggs was accomplished by using the stop-fix technique (16), consisting of centrifugation through a discontinuous dextran gradient during which sperm not bound specifically to eggs are removed, while bound sperm are fixed to the egg due to glutaraldehyde in the lower layer of the dextran gradient (16). By using this technique, eggs were recovered at 15–30 min after sperm addition, mounted on slides, stained with aceto-lacmoid (17), and examined with phase-contrast optics. The average number of sperm bound per egg was calculated from the total number of sperm and the total number of eggs in the sample.

Fertilization *in vitro*. To assay the effects of the inhibitors on zona penetration and fertilization, a variation of the stop-fix technique was used in which glutaraldehyde was omitted entirely from the dextran gradient. The inseminating sperm concentration was adjusted in these experiments to result in 1–3 sperm bound per egg after centrifugation. These eggs were recovered from the centrifuge tubes and placed immediately in fresh CM in the presence or absence of inhibitor. The gametes were recovered after 4 hr at 37°C in 5% CO_2/95% air. The eggs were fixed in 2.5% glutaraldehyde, mounted on slides, stained and examined as above. Eggs were scored as penetrated if sperm were found within the perivitelline space or vitellus or both and as fertilized only if both the sperm head (or pronucleus) and sperm tail were identified in the vitellus.

Chemicals. Other than the exceptions noted, all chemicals were purchased from Sigma and used without further purification. Benzamidine·HCl, *p*-aminobenzamidine·HCl, lima bean trypsin inhibitor (LBTI; type II; 1 mg inhibits ≈1.6 mg of trypsin), ovomucoid trypsin inhibitor (OMTI; type III; essentially free of ovoinhibitor; 1 mg inhibits ≈1.1 mg of trypsin), soybean trypsin inhibitor SBTI; type I; 1 mg inhibits ≈1.9 mg of trypsin), and N^{α}-*p*-tosyl-L-lysine chloromethyl ketone·HCl ($TosLysCH_2Cl$) were dissolved in CM directly, whereas Me_2CONH_2 (Fisher) was used as the solvent for nitrophenylguanidinobenzoate (NPGB), phenylmethylsulfonyl fluoride ($PhMeSO_2F$), and L-1-tosylamide-2-phenylethyl chloromethyl ketone ($TosPheCH_2Cl$).

The statistical significance of the data was evaluated by Student's *t* test for matched pairs of observations.

RESULTS

When capacitated mouse sperm were exposed to either natural or synthetic trypsin inhibitors for 15 min and then added to cumulus-free eggs, the sperm displayed greatly reduced binding levels despite the maintenance of motility equivalent to that observed in control suspensions (Table 1). $TosLysCH_2Cl$, an alkylating agent and trypsin active-site inhibitor (18), was the most effective inhibitor. In contrast, $TosPheCH_2Cl$, a closely related compound that is also an alkylating agent and protease inhibitor but directed toward the active site of chymotrypsin rather than that of trypsin (19), did not have a significant effect on sperm binding levels to the zona (Table 1). Me_2CONH_2, used as the solvent for $TosPheCH_2Cl$, was similarly ineffective in reducing sperm binding levels. Marked inhibition (66–78%) was found with all of the trypsin inhibitors tested, which included both synthetic (*p*-aminobenzamidine, benzamidine) and natural (SBTI, LBTI, OMTI) inhibitors (Table 1).

Table 1. Effect of protease inhibitors on sperm binding to zonae pellucidae

Treatment	Eggs, no.	Sperm bound, no.	Sperm:egg ratio Mean	Sperm:egg ratio % of control
None	142	1127	7.9	100
Solvent alone (1.0% Me_2CONH_2)	71	569	8.0	101
1.5 mM *p*-Aminobenzamidine	154	406	2.6	33
1.5 mM Benzamidine	90	153	1.7	22
0.5 mM $TosLysCH_2Cl$	108	159	1.5	18
0.5 mM $TosPheCH_2Cl$ (0.5% Me_2CONH_2)	55	369	6.7	85
SBTI (2.5 mg/ml)	128	316	2.5	32
LBTI (2.5 mg/ml)	135	323	2.4	30
OMTI (2.5 mg/ml)	146	393	2.7	34

After incubation for 90 min in CM, 1–2 × 10^5 sperm were exposed to various reagents. After 20-min, cumulus-free eggs were added to the sperm suspensions. The gametes were recovered at 15 min after egg addition by the dextran gradient centrifugation method, and the number of sperm bound per egg was quantitated. The data represent 4–6 replicate experiments for each reagent.

Several trypsin inhibitors were examined for their ability to reduce sperm binding levels in a concentration-dependent fashion. Increasing concentrations of each inhibitor tested—NPGB, $PhMeSO_2F$, $TosLysCH_2Cl$, and benzamidine—led to decreasing binding levels (Fig. 1). Sperm motility was unaffected by these compounds except for NPGB at 1 mM, at which concentration 30–40% fewer spermatozoa displayed vigorous motility than seen in the control suspensions.

The effect of the protease inhibitors on sperm–zona interaction was apparently directed toward the spermatozoon and not toward the zona pellucida (Fig. 2). Cumulus-free zona-intact eggs were incubated for 15 min in either $TosLysCH_2Cl$ or SBTI, as well as in CM alone; washed eggs were then added to capacitated mouse spermatozoa. When assayed 15 min later, sperm binding levels remained high (the levels obtained, in fact, were somewhat, but not significantly, higher than the control level), indicating the absence of an effect of the inhibitors on zonae.

To investigate the possibility that the specific step in gamete interaction affected by protease inhibitors was sperm binding to zonae, and not sperm penetration through zonae, the following experiments were performed. Capacitated mouse sperm were permitted to bind to zonae under standard conditions in the absence of protease inhibitors. The gametes were recovered 15–20 min later by the dextran gradient centrifugation technique, but with glutaraldehyde omitted. The recovered eggs, with 1–3 highly motile sperm bound to the zona surface, were transferred to drops of fresh CM containing various reagents. Both zona penetration and fertilization were assessed when these gametes were recovered 4 hr later. The protease inhibitors did not affect zona penetration or fertilization significantly when the spermatozoon had already bound securely to the zona surface, indicated by the high levels obtained for penetration (65–130%) and fertilization (72–144%) in the presence of the protease inhibitors (Table 2). The inhibitor concentrations used in this series of experiments were the same as those used in the experiments described in Table 1. Thus, although the inhibitors substantially reduced the initial interaction between the spermatozoon and the zona pellucida, once that association was es-

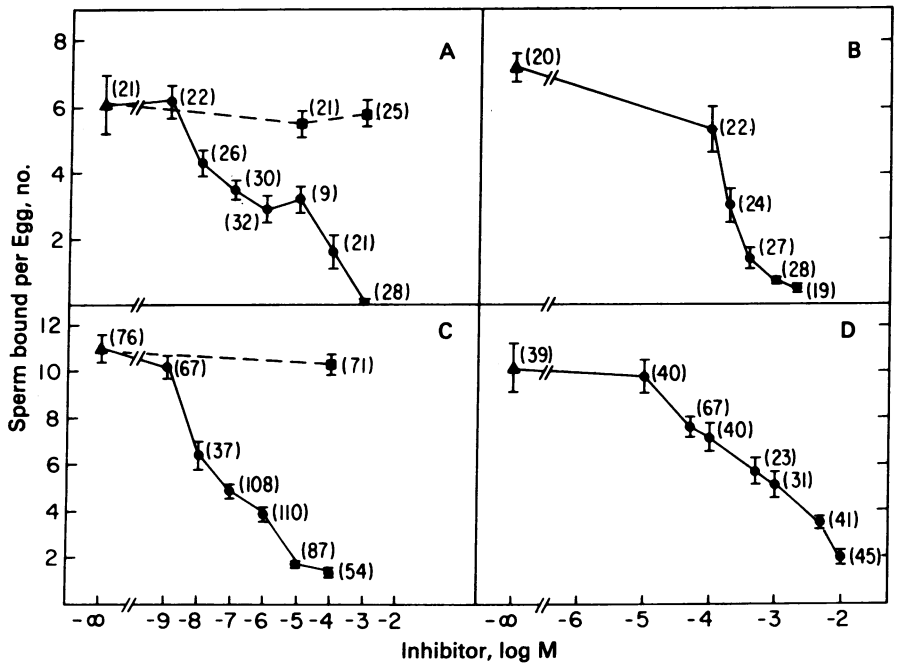

FIG. 1. Concentration-dependent reduction of sperm binding to zonae by protease inhibitors. (*A*) NPGB. (*B*) Tos-LysCH_2Cl. (*C*) Ph$MeSO_2F$. (*D*) Benzamidine. Sperm were incubated in CM for 90 min and then added to inhibitor to the concentrations indicated. After 20 min, cumulus-free zona-intact eggs were added to the sperm suspensions. Gametes were recovered at 20–30 min after egg addition by the stop-fix dextran gradient technique, and the number of sperm bound per egg was quantitated. ▲, Control (no inhibitor present); ●, test points; ■, solvent (Me_2CONH_2) controls. Results are mean ± SEM; numbers in parentheses represent numbers of eggs examined.

tablished firmly, the protease inhibitors no longer prevented zona penetration and gamete membrane fusion. To ensure that zona penetration had not occurred during the initial 15–20 min of gamete interaction in the absence of protease inhibitors, the following control experiments were performed. One aliquot of centrifuged unfixed eggs was reserved and recovered only after transferring the remainder of the centrifuged eggs into the inhibitor solutions. Examination of the 87 eggs treated in this manner showed that all of the associated sperm appeared to reside exclusively at the outer margin of the zonae. Penetration of the zona must have occurred, therefore, when the gametes were in the presence of the protease inhibitors.

To define more precisely the site of action of the trypsin inhibitors, sperm binding to zona-intact eggs was compared with sperm binding to zona-free eggs, both in the presence and in the absence of Tos-LysCH_2Cl (Fig. 3). Whereas sperm-to-zona binding decreased markedly as the concentration of Tos-LysCH_2Cl increased, sperm-to-egg plasma membrane binding remained consistently high. This result suggests that protease activity is required for interaction between the mouse spermatozoon and the surface of the zona pellucida specifically, not between the spermatozoon and the egg plasma membrane.

DISCUSSION

Three conclusions have emerged from the data presented here: (*i*) a trypsin-like, but not chymotrypsin-like, activity is apparently involved in the binding of mouse spermatozoa to zonae pellucidae; (*ii*) this trypsin-like activity appears to be borne by the sperm, not by the zona pellucida, and to exert its effect when the spermatozoon interacts with the zona pellucida specifically, not with the egg plasma membrane; and (*iii*) a trypsin-like activity may be unnecessary for mouse sperm penetration through the zona pellucida. Although the activity of the protease inhib-

Table 2. Penetration of zonae and fertilization in the presence of protease inhibitors

Treatment	Eggs examined, no.	Eggs penetrated		Eggs fertilized	
		No.	% of control	No.	% of control
None	141	52	100	35	100
Solvent alone (1.0% Me_2CONH_2)	25	7	76	7	112
1.5 mM *p*-Aminobenzamidine	95	23	65	17	72
1.5 mM Benzamidine	156	56	97	34	88
0.5 mM Tos-LysCH_2Cl	133	60	122	48	144
0.5 mM Tos-PheCH_2Cl (0.5% Me_2CONH_2)	78	24	81	21	108
SBTI (2.5 mg/ml)	129	40	84	30	92
LBTI (2.5 mg/ml)	146	47	86	31	84
OMTI (2.5 mg/ml)	146	70	130	38	104

Capacitated mouse sperm were added to cumulus-free zona-intact mouse eggs at 5–7 × 10^4 cells per ml in CM. Eggs having motile sperm attached were recovered by using the dextran gradient centrifugation technique in the absence of fixative and transferred to fresh CM containing the various reagents. After culture for 4 hr at 37°C in 5% CO_2/95% air, the gametes were examined for evidence of zona penetration and fertilization. None of the experimental values differed significantly from the control value ($0.01 < p < 0.005$). The corresponding values for nontransferred control eggs were 73% (67/92) eggs penetrated and 65% (60/92) eggs fertilized. As expected, centrifugation and transfer of eggs lead to a reduction in the overall fertilization level.

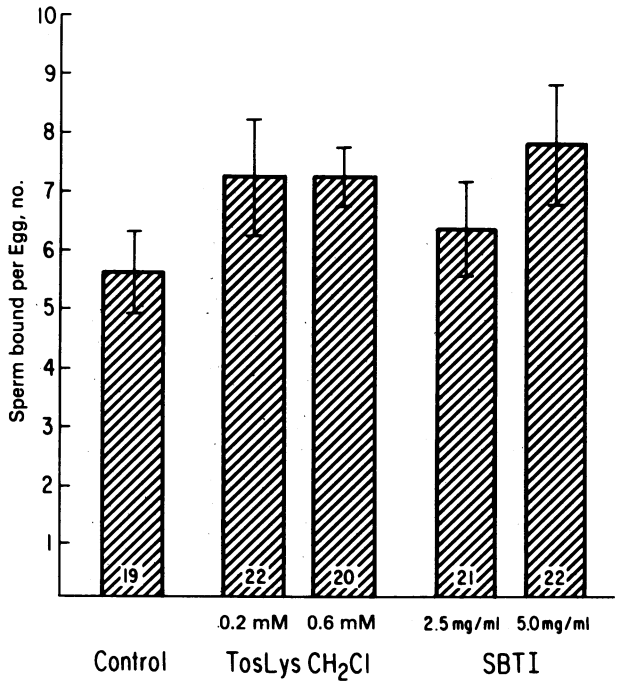

FIG. 2. Effect of previous incubation of eggs with $TosLysCH_2Cl$ or SBTI on sperm binding levels. Cumulus-free zona-intact eggs were incubated in CM, $TosLysCH_2CL$, or SBTI for 15 min. After washing in CM, the eggs were added to capacitated spermatozoa (2×10^5 cells per ml) and recovered 15 min later by the stop-fix technique. Numbers at the bases of the columns represent numbers of eggs examined. None of the experimental values are significantly different from control ($P < 0.01$).

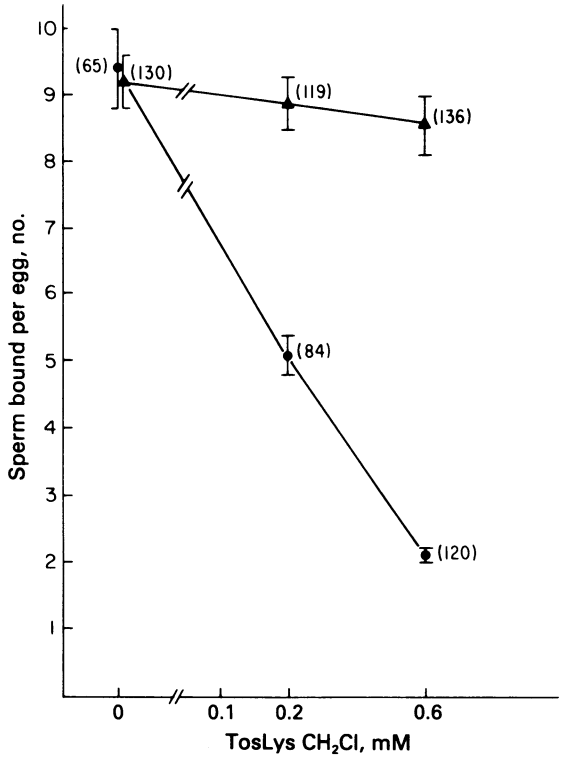

FIG. 3. Differential effect of $TosLysCH_2Cl$ on sperm binding to zona-intact vs. zona-free mouse eggs. Mouse sperm capacitated by incubation in CM for 120 min were exposed to CM or to $TosLysCH_2Cl$ at 0.2 or 0.6 mM. The sperm concentration was maintained at 3–5 $\times$ 10^5 cells per ml. After 20 min of exposure to the inhibitor, either cumulus-free zona-intact (●) or mechanically isolated zona-free (▲) eggs were added to the sperm suspensions. The gametes were recovered at 20 min after mixing by the stop-fix dextran gradient technique, and the number of sperm bound per egg was quantitated. The values obtained for zona-intact eggs exposed to $TosLysCH_2Cl$-incubated sperm at both concentrations tested differed significantly ($0.005 < P < 0.001$) from each other and from the remaining values. Results are mean ± SEM; numbers in parentheses represent numbers of eggs examined.

itors throughout the zona has not been demonstrated here, the permeability of these molecules through this envelope is not considered a problem on the basis of size, since large molecules, such as IgM, pass readily through the mouse zona pellucida (20, 21).

The general view concerning sperm penetration through the zona pellucida has, for well over a decade, included enzymatic digestion by the protease acrosin. This idea is based, primarily, on two types of experiment: inhibition of fertilization in the presence of trypsin inhibitors (4–8) and dissolution of zonae pellucidae *in vitro* by acrosomal extracts (12, 13, 22). The results given here are not inconsistent with those data, since neither the consideration of zona binding as a possible explanation nor the identification of the specific site of trypsin inhibitor activity was undertaken previously. Inhibition of fertilization by trypsin inhibitors is verified here, because zona binding rather than zona penetration, is prevented. It is interesting to note that in sea urchins, in which initial gamete association is mediated by the sperm-borne protein, bindin (23), a protease activity associated with bindin has been reported recently (24).

The finding that trypsin inhibitors do not affect zona penetration should not be surprising, particularly as data have been accruing that suggest that the role of acrosin in fertilization should be reconsidered. Bedford and Cross (9) demonstrated that rabbit zonae pellucidae, made highly resistant to both trypsin and acrosin digestion by exposure to wheat germ agglutinin, were penetrated by rabbit sperm as readily as control zonae. In addition, when the ability of acrosomal extracts to dissolve zonae was tested *in vitro*, crude preparations were much more effective than purified acrosin (13); moreover, some nontryptic enzymes and disulfide reducing agents dissolved zonae as effectively as trypsin-like enzymes (12). Furthermore, the concentration of Ca^{2+} required for optimal activity of extracted acrosin is far in excess of that found at the site of fertilization. Mouse acrosin is reported to require 50 mM Ca^{2+} for full activity (8), while the ram enzyme requires 200 mM Ca^{2+} (10). The mammalian oviduct, however, appears in general to maintain a Ca^{2+} concentration of <5 mM; in the mouse, the measured Ca^{2+} concentration is 2 mM or less (25). Consistent with the lack of acrosin participation in zona dissolution is the finding of Srivastava *et al.* (26) that crude rabbit acrosomal extracts were more effective in zona dissolution in the absence than in the presence of Ca^{2+}.

Recent work has shown that an intact plasma membrane on the sperm cell is involved in zona recognition and binding (27, 28); once bound to the zona, a functional acrosome reaction can occur (29). These findings suggest that the protease inhibitors that affect zona binding exert their action on the sperm cell surface rather than on enzymes located within the acrosome.

The identity of the activity mediating mouse sperm binding to zonae presented here is unresolved. Although there is a striking parallel between the levels of inhibition obtained in this study and those obtained by Bhattacharyya *et al.* (8) with extracted mouse acrosin and synthetic inhibitors, no conclusion is possible. Indeed, the activity here is termed trypsin-like due to its inhibition by a variety of protease inhibitors, all of which have trypsin inhibition in common. Mammalian spermatozoa are well known to contain a wide variety of proteolytic enzymes

(see refs. 11 and 12); thus, many condidates other than acrosin are available for assignment of the activity described here.

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