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Characterization of the Mouse Sperm Plasma Membrane Zona-Binding Site Sensitive to Trypsin Inhibitors[1]

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ABSTRACT

The first contact of mammalian gametes is the binding of the spermatozoon to the zona pellucida of the egg. Previous work has shown that binding of the spermatozoon to the zona in the mouse occurs prior to the acrosome reaction and that trypsin inhibitors block this initial binding. This suggests that the sperm surface contains a trypsinlike binding site that functions by an active site mechanism to effect initial zona binding. When suspensions of twice-washed spermatozoa were incubated with the serine protease active site titrant, 4-methylumbelliferyl p-guanidinobenzoate (MUGB), the titrant was hydrolyzed at a rate of 8 pmoles/min-10^6 cells. MUGB was found to inhibit the binding of spermatozoa to the zona pellucida. The degree of inhibition and the rate of hydrolysis of MUGB by washed spermatozoa depend on the concentration of titrant, with half maximal effects at 13 μM and a linear correlation with r=0.99. The analogous lysyl and arginyl trypsin substrates containing 7-amino-4-methylcoumarin as the fluorogenic leaving group were not hydrolyzed under the same conditions and did not inhibit zona binding. Both binding of sperm to zona-intact eggs and the hydrolysis of MUGB by sperm are inhibited by p-nitrophenyl guanidinobenzoate, soybean trypsin inhibitor, and acid-solubilized zonae. The linear correlation coefficients of the inhibition of sperm binding and MUGB hydrolysis by these three substances are greater than 0.92. This "trypsinlike" sperm site is essential for sperm binding to the zona: its stereospecificity is unique in that it reacts with trypsin inhibitors but not with trypsin substrates.

INTRODUCTION

The binding of the mammalian spermatozoon to the zona pellucida of its homologous egg is not a simple collisional process: a series of events in strict sequence involving specific reactions seems to be involved. In an early study of this process, Hartmann et al. (1972) resolved hamster sperm binding to the zona into an initial attachment step and a second binding step, which followed the first by 30 min. Attached sperm could be removed by washing, whereas bound sperm could not. Only the binding process was species-specific. Hamster sperm could participate in these processes only after capacitation (Hartmann et al., 1972).

Mouse sperm binding to its homologous zona pellucida has been characterized more recently in considerable detail. Fresh cauda epididymal sperm bind poorly to the zonae of cumulus-free, zona-intact eggs, but the ability to bind is rapidly acquired (Inoue and Wolf, 1975), even in the absence of bovine serum albumin (BSA) (Saling et al., 1978; Heffner and Storey, 1982). This process is too rapid to be associated with capacitation (Ward and Storey, 1984), but increased ability to bind appears to develop as capacitation proceeds (Lambert and Le, 1984). The binding occurs with the plasma membrane of the sperm still fully intact (Saling and Storey, 1979; Saling et al., 1979; Florman and Storey, 1982; Bleil and Wassarman, 1983; Florman et al., 1984; Storey et al., 1984; Lee and Storey, 1985; Saling and Lakoski, 1985; Bleil and Wassarman, 1986). Saling et al. (1978) demonstrated that Ca^{2+} is obligatory for binding and that ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) reverses the binding at early binding times. Closer examination of the kinetics of sperm binding has shown that two sequential reactions are involved in the early set of sperm-binding reactions (Florman et al., 1982). The first reaction is binding, which is reversible by EGTA with a half-time of 6 min. The second reaction is the

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acquired resistance to EGTA reversal of binding; it is effectively complete in 20 min. Saling (1981) made the important observation that inhibitors of the enzyme, trypsin, inhibited sperm binding to the zona, but that the inhibition is ineffective if the agents are added after 20 min. These inhibitors have long been known to prevent fertilizaton in vitro (Stambaugh et al., 1969; Gould, 1973; Bhattacharya et al., 1979) and in vivo (Zaneveld et al., 1971; Miyamoto and Chang, 1973), but the inhibition was thought to be caused by prevention of zona digestion due to the inactivation of acrosin (McRorie and Williams, 1974).

The implication of Saling's observation is that interaction between zona protein and the sperm plasma membrane resembles the interaction between substrate and enzyme (Florman et al., 1982). Shur et al. (1982a,b) presented evidence for another such interaction between zona protein and sperm plasma membrane in which the sperm binding site appears to act analogously to galactosyltransferase. Florman et al. (1982) pointed out that the binding reaction may resemble the first stage of trypsin-catalyzed esterolysis or amidolysis: acylation of the active site (Bender and Kezedy, 1965). Once this reaction occurs, a covalent bond forms between the sperm site and the functional group on the zona protein. The second stage of trypsin catalysis, in which the active site promotes hydrolysis of the bond, was postulated to be absent. The result of this reaction would be covalent bonds between the zona protein ZP3 and the sperm plasma membrane site.

This hypothetical mechanism for binding makes the following two predictions: the sperm-binding site should react with titrants for the active site of trypsin, such as the esters of p-guanidinobenzoic acid (Chase and Shaw, 1970), to give a single "burst" of activity; inhibition of binding by these compounds should correlate with the active site titration. The sperm-binding site should also react with arginyl and lysyl compounds, which are normally used as trypsin substrates, in a manner similar to the active site titrants; these compounds should also inhibit binding in a similar manner. In the present study, we further characterize the site reported by Saling (1981), and report that the first prediction is fulfilled while the second one is not.

MATERIALS AND METHODS

Reagents

4 - Methylumbelliferyl - p - guanidinobenzoate (MUGB), soybean trypsin inhibitor type 1S (SBTI), sodium pyruvate, and hyaluronidase (bovine testicular extract) were obtained from Sigma Chemical Co. (St. Louis, MO). Bovine pancreatic trypsin was obtained from Boehringer Mannheim (Indianapolis, IN). p-Nitrophenyl guanidinobenzoate (NPGB) was from ICN Biochemicals (Cleveland, OH). Nα-benzyloxy-carbonyl (Z) lysyl and arginyl amides derived from 7-amino-4-methylcoumarin (Z-lys-AMC and Z-arg-AMC) were obtained from Enzyme Systems Products (Livermore, CA). N, N-dimethylformamide (DMF), photorex spectrophotometric grade, was from J. T. Baker (Phillipsburg, NJ).

Collection of Gametes

Spermatozoa from the cauda epididymides of sexually mature Swiss Webster mice (Harlan Sprague-Dawley Inc., Walkersville, MD) were collected in an N- hydroxymethylpiperazine- N'- ethanesulfonate (HEPES) buffered noncapacitating medium (HMB) consisting of 120 mM NaCl, 4.8 mM KCl, 1.7 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5.6 mM glucose, 1mM Na-pyruvate, 10 mM $NaHCO_3$, 0.1% polyvinyl alcohol, and 25 mM HEPES, pH 7.4. The above medium without $NaHCO_3$ was designated HM. The spermatozoa were obtained by puncturing the excised caudae and allowing the sperm to swim out into HMB (300 μl/cauda) for 15 min. The crude suspension of spermatozoa was centrifuged in a 1.5-ml plastic centrifuge tube at 40 × *g* for 10 min over a 100 μl dextran pad (20% dextran w:v in HMB) to remove large debris. Sperm suspensions (1 ml) were washed by centrifugation at 600 × *g* for 15 min in 10 volumes of HMB in a 16-mm diameter round bottommed centrifuge tube. The maximum concentration of spermatozoa in the washing procedure was 10^8/10 ml. The round bottommed tube allowed the centrifuged spermatozoa to spread evenly in a thin layer about the bottom of the tube, which minimized cell damage and so maximized the recovery of motility after multiple washings. Washed samples were resuspended in HMB or HM as required. Concentrations of spermatozoa in the suspensions were determined by counting in a hemacytometer.

Eggs were obtained from mature (10- 13-wk-old), virgin Swiss Webster mice (Harlan Sprague-Dawley Inc.). The mice were superovulated with i.p. injections of 10 IU pregnant mare's serum gonadotropin (Gestyl; Diosynth, Chicago, IL), which were followed 36–56 h later by injections of 10 IU human chorionic gonadotropin (hCG, Sigma). The mice were killed

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13–16 h after injection of the hCG, and the oviducts were removed and submerged in sterile silicone oil (Dow Corning 200; Dow Corning Corp., Midland, MI) at 37°C. The ampullae were slit with sterile fine-point watchmaker's tweezers, and the cell mass containing the cumulus-oophorus and eggs was removed under oil and transferred into 2 ml HMB containing 10 mg/ml hyaluronidase to disperse the cumulus cells. The eggs were separated from the dispersed cumulus by pipetting with a narrow bore pipette (tip diameter approximately equal to egg diameter) and washed in HMB 3 times by pipette transfer to remove mechanically any remaining adhering cumulus cells. Cumulus-free, zona pellucida-intact eggs were then used in sperm-binding assays.

Sperm-Binding Assays

All binding studies were carried out in 500 μl HMB with not less than 30 eggs in each incubation vessel. The volume of the washed sperm suspension added to the incubation mixture gave a final sperm concentration of $1-1.4 \times 10^5$ sperm/ml. The binding reaction was allowed to proceed for 20 min. The eggs then were removed from the incubation vessel and transferred to fresh HMB to separate the eggs with bound and loosely associated spermatozoa from possible aggregations of unassociated spermatozoa. To separate the eggs with tightly bound spermatozoa from loosely associated spermatozoa, a "stop-fix" dextran step-gradient based on the procedure of Saling et al. (1978) was employed. The gradient was formed in 400-μl capacity lithium fluoride/heparin-coated tubes (Beckman Instruments, Palo Alto, CA). Since these tubes are conical and the shearing forces caused by rolling the eggs down the tapered sides of the tubes can lead to erratic results, the bottom layer of the gradient consisted of a 100-μl pad of 20% (w:v) dextran in HMB containing 0.5% (w:v) myoglobin to visualize the interface between this layer and the overlying one. The next layer consisted of 50 μl 2.55% (w:v) dextran in HMB containing 2.5% (v:v) gluteraldehyde to fix the sperm on the egg surface. The upper two layers of the gradient consisted of 100 μl 1.8% (w:v) dextran in HMB and 50 μl HMB, respectively. Eggs with associated spermatozoa were pipetted onto the top layer of the gradient with care taken to avoid having the eggs near the sides of the tube. The tubes were then centrifuged at room temperature at $90 \times g$ for 90 s, after which the tubes were sliced with a cigar cutter (D. La Follette Co., Culver City, CA) at the interface of the 1.8% and 2.55% dextran layers. Eggs with tightly bound spermatozoa were collected at the interface of the 2.55% dextran layer and the 20% dextran pad and transferred to microscope slides for counting the number of sperm bound per egg. The mean recovery of eggs from the stop-fix gradient was 90% (range 75–100%).

Fluorimetric and Spectrophotometric Assays

Fluorimetric measurements were made with a spectrofluorimeter modified for electronic compensation of light source fluctuations and intrinsic fluorescence of the sample (Chance et al., 1970; Storey, 1971). Spectrophotometric measurements were taken with a DW 2A dual wavelength spectrophotometer (American Instruments Co., Silver Springs, MD). The hydrolysis of MUGB, an active site titrant of trypsin (Jameson et al., 1973), was determined fluorimetrically at an excitation wavelength of 370 nm and an emission wavelength of 450 nm. Fluorescence emission was detected at 90° to the excitation beam. The fluorescence cuvette had a chamber 0.2 cm wide and 0.5 cm long; the sample volume required was 200 μl. Sperm suspensions in HM were added to the cuvette in 200-μl portions; the signal from the intrinsic fluorescence of the sample was supressed by adjusting the electronic compensation, and the gain of the fluorimeter was increased to 20–50 ×. One μl 2 mM MUGB in DMF was added, and the increase in fluorescence was monitored over a time course of 5–30 min as required. At the end of the observation period, the gain of the fluorimeter was reduced to 1×, and 0.2 μl 1 mM 4-methylumbelliferone (MU) in DMF was added to calibrate the system.

Incubations were also performed in the presence of benzyloxycarbonyl (Z) lysyl and arginyl trypsin substrates containing the fluorogenic group 7-amino-4-methylcoumarin (AMC) (Zimmerman et al., 1977). The fluorimeter was set at an excitation wavelength of 380 nM and an emission wavelength of 450 nm. The order of addition of the substrates, Z-lys-AMC and Z-arg-AMC, was the same as with MUGB assays, with 0.2 μl of 100 mM Z-lys or Z-arg AMC in DMF added to the incubation mixture. After obtaining the reaction rate, the system was calibrated with 0.2 μl 1 mM AMC.

The fluorimetric system using the coumarin titrant and substrates was checked with trypsin. In these determinations with the titrant, the enzyme dissolved

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in HM was added to the medium containing 10 μM MUGB, after compensation for the intrinsic fluorescence of the ester. MUGB gave a single "burst" of fluorescence but no detectable turnover with trypsin even after 10 min. The detectability limit for the enzyme was 10 pmol, about twofold lower than reported by Jameson et al. (1973). With the substrates, Z-lys-AMC and Z-arg-AMC, no burst was detected; the fluorescence increased linearly with time. With Z-arg-AMC, the detectability limit for the enzyme was 0.05 pmole, about fourfold lower than reported by Zimmerman et al. (1977).

Hydrolysis of NPGB by washed sperm suspensions in medium HMB was measured in a 200-μl, 0.5-cm lightpath cuvette in the DW 2A dual wavelength spectrophotometer set at wavelengths of 405 nm and 465 nm, with the sensitivity set at 0.05 absorbance for full scale. NPGB (0.2 μl) in DMF was added to start the reaction. After monitoring the hydrolysis of NPGB, the spectrophotometer was set to 1.0 absorbance full scale, and 0.2 μl 1 mM p-nitrophenol was added to calibrate the system. The molar extinction coefficient of p-nitrophenol in the dual wavelength mode (405–465 nm) was determined to be 15 mM^{-1} cm^{-1} in medium HM (pH 7.4); the extinction coefficient was not affected by spermatozoa suspended at the concentrations used in the experiments.

Acid Solubilization of Zonae Pellucidae

To obtain acid solubilized zonae pellucidae (ASZP), a minimum of 600 zona-intact eggs were placed in a 10-μl drop of HMB in a cell culture dish. To this was added 600 μl of a solution consisting of 130 mM NaCl, 4.8 mM KCl, 1.7 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5.6 mM glucose, 1 mM Na-pyruvate, 0.1% polyvinyl alcohol and 10 mM lactate, pH 3.2, to give a final concentration of 1 egg/μl. The mixture was incubated for 45 min at 37°C, after which the solution containing the solubilized zonae was pipetted away from the now zona-free eggs into a 500-μl centrifuge tube and centrifuged at 1000 × *g* for 5 min to remove any remaining debris. The supernatant was transferred to a Minicon concentrator (Amicon Corp., Danvers, MA) and allowed to concentrate twentyfold. The resulting solution was partially neutralized by adding 5 μl 0.1 N NaOH and used immediately for studies on the inhibition of sperm binding to zona-intact eggs or sperm hydrolysis of MUGB.

Inhibition Studies

For studies of zona-binding and MUGB hydrolysis inhibition using low molecular weight compounds (NPGB, QNB, Z-lys-AMC, Z-arg-AMC) the compounds dissolved in DMF were added in volumes less than 5 μl directly to the incubation mixtures. Control runs with 5 μl DMF showed no inhibition. In binding studies using high molecular weight inhibitors (SBTI, ASZP) the washed spermatozoa were preincubated with the test substance in HMB for 15 min, after which the zona-intact eggs in HMB containing the same concentration of test substance were added. For MUGB hydrolysis studies with high molecular weight inhibitors, the spermatozoa were preincubated with the test substance for 15 min, after which 200 μl of the suspension was placed in the cuvette for the MUGB hydrolysis assay. The percentage of spermatozoa that were uncapacitated, capacitated, or acrosome-reacted in the presence of NPGB, SBTI, and ASZP under the conditions used in the binding experiments was determined by the chlortetracycline (CTC) fluorescence assay described in detail by Ward and Storey (1984), and Lee and Storey, (1985). In brief, 5 μl CTC (500 μM in chilled buffer containing 20 mM Tris, 130 mM NaCl, 5 mM cysteine, pH 7.8) was added on a warm microscope slide to an equal volume of sperm suspension that had been incubated for 30 min in HMB (control) or HMB-containing NPGB (100 μM), SBTI (4 mg/ml), or ASZP (4 zonae/ μl) under conditions described for the binding assay. To this mixture, 12.5% glutaraldehyde in 1M Tris buffer, pH 7.8, was added to a final concentration of 0.1%. Sperm were scored for epifluorescent patterns by using a Nikon Optiphot microscope as described in Ward and Storey (1984). Uncapacitated sperm show a uniform fluorescence over the sperm head, capacitated sperm have a dark band over the equatorial and post-equatorial segment of the sperm head with a brightly fluorescent anterior portion, and acrosome-reacted sperm show no fluorescence on the head.

Statistical Analyses

Correlations of MUGB hydrolysis and inhibition of sperm binding to zona-intact eggs and correlations of the effects of NPGB, SBTI, and ASZP on the inhibition of MUGB hydrolysis and binding were calculated according to Mosteller et al. (1983). Multiple regression

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of the inhibitor data was performed by using a computer program (Statworks; Heyden and Sons, Inc., Philadelphia, PA). Statistical significance of data was determined by paired or unpaired Student's t-tests as indicated in the tables. A value of $p<0.05$ was taken as statistically significant.

RESULTS

Hydrolysis of MUGB by Sperm Suspensions

Three compounds with analogous fluorogenic leaving groups were used to test the hypothesis that the site would react in a manner to both trypsin-active site titrants and substrates. The active site titrant was MUGB (Jameson et al., 1973). The two trypsin substrates containing AMC, the amine analog of methylumbelliferone, were Z-lys-AMC and Z-arg-AMC (Zimmerman et al., 1977). Addition of MUGB at a final concentration of 10 μM to sperm suspensions gave the surprising result that, after an initial sudden increase in fluorescence, the MUGB was turned over in the manner of a substrate, showing a linear increase in fluorescence as 4-methylumbelliferone was liberated by hydrolysis (Fig. 1). The hydrolysis of MUGB was monitored in some experiments for over 30 min with no decrease in the rate. Autohydrolysis of MUGB was not detected. Unwashed sperm suspensions gave the highest and most variable rates of MUGB hydrolysis. Unwashed spermatozoa from the caput epididymis turned over the MUGB at a rate of 528 pmol/min-10^6 spermatozoa, and cauda sperm suspensions gave a rate of 385 pmol/min-10^6 spermatozoa (Table 1). Most of this activity was found to be in the supernatant. After 2 washes, the soluble MUGB hydrolyzing activity was removed: incubation of the supernatant with 10 μM MUGB gave no detectable hydrolytic activity. The hydrolysis rate of the sperm suspensions fell to 8 pmol/min-10^6 spermatozoa and remained constant during successive washes (Table 1). When the supernatants from suspensions (5×10^6 sperm/ml) of twice-washed sperm that had been allowed to stand at room temperature for times ranging from 15–60 min were assayed for MUGB hydrolytic activity, the hydrolysis rate was below the limit of detectability. Increasing the cell concentration in the suspension to 9×10^6 sperm/ml for a 60 min incubation resulted in an apparent MUGB hydrolysis rate in the supernatant of 0.02 pmol/min-10^6 sperm, which is the detectability limit of the rate determinations. This shows that dissociation of the MUGB hydrolysis activity from the washed sperm cells occurs at a negligible rate.

The initial rapid increase in fluorescence seen on the addition of MUGB occurred in the absence of sperm and was due to the fluorescence of MUGB itself (Fig. 1). The amplitude of the intrinsic MUGB fluorescence decreased slightly with increasing sperm concentration, as would be expected for shadowing effects from the suspended cells. The hydrolysis rate observed with twice-washed spermatozoa increased linearly with the sperm concentration (Fig. 2).

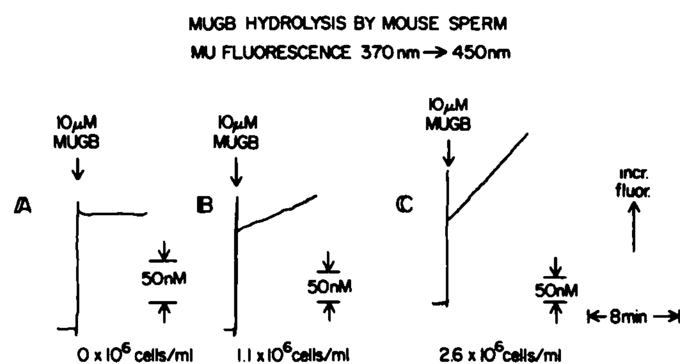

FIG. 1. MUGB hydrolysis by mouse sperm. Fluorimetric chart traces, at a gain of 50X, of the hydrolysis of MUGB by twice washed spermatozoa. Sperm were added to the incubation mixture, then 1 μl 2 mM MUGB in dimethyl formamide was added. After the rate of hydrolysis was measured, the gain was decreased to 1X, and 0.2 μl MU was added to calibrate the system. MU fluorescence was measured at an excitation wavelength of 370 nm with an emission wavelength of 450 nm.

TABLE 1. Turnover of 10 μM MUGB by unwashed epididymal spermatozoa and washed cauda epididymal spermatozoa.

No. of washes	MUGB turnover pmol/min-10^6 sperm	n[a]
Unwashed cauda sperm[b]	385 ± 177[c]	8
1	22.3 ± 7.9	8
2	8.1 ± 1.6	12 *
3	9.3 ± 3.3	3 *
4	12.3 ± 3.0	3 *
Unwashed caput sperm	528 ± 127	3

[a]Number of replicate determinations.

[b]Wash supernatants were also monitored for hydrolyzing activity; no detectable activity remained in the sperm-free supernatant after 2 washes.

[c]Means ± SD.

*$p>0.1$, standard two-tail t-test of independent means, df=2.

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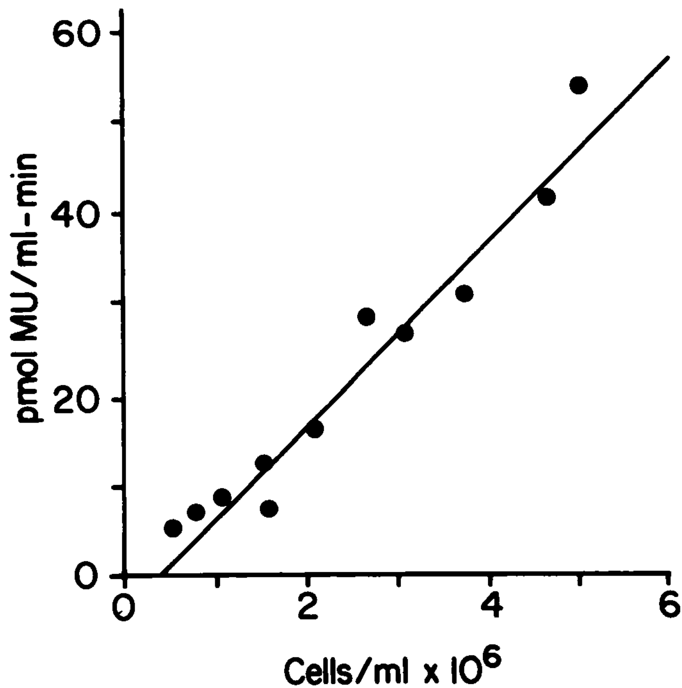

FIG. 2. Correlation of MUGB hydrolysis with concentration of spermatozoa. Concentrations of twice-washed spermatozoa ranging from 0.5–5.2 × 10^6 sperm/ml were incubated with 10 μM MUGB. Least squares regression, r=0.96, p<0.001, n=13.

Incubation of either washed or unwashed suspensions of cauda epididymal spermatozoa with 100 μM Z-arg-AMC gave no detectable reaction. Unwashed suspensions of spermatozoa caused barely detectable hydrolysis of Z-lys-AMC, but the reaction rate was below the range of detection when washed sperm were incubated with this substrate. The presence of 100 μM Z-arg-AMC or 100 μM Z-arg p-nitrophenylanilide did not inhibit the hydrolysis of 10 μM MUGB by washed sperm suspensions. MUGB hydrolysis was also unaffected by 3-quinuclidinyl-benzilate (QNB), a specific inhibitor of the zona-induced acrosome reaction (Florman and Storey, 1982).

When the twice-washed sperm suspensions were incubated with MUGB in the range of 3–10 μM MUGB, the rate of hydrolysis was dependent in a hyperbolic manner on the concentration of MUGB. Concentrations of MUGB higher than 10 μM were not feasible because of the limited solubility of the compound in aqueous media. The apparent K_m for MUGB was calculated to be 13 μM (SD ± 3, n=4) with a V_{max} of 16 pmol/min–10^6 cells (SD ± 3, n=4).

Twice-washed sperm also hydrolyzed NPGB (Fig. 3) the nitrophenyl ester analog of MUGB found by Saling (1981) to be an inhibitor of mouse sperm binding to zona. The apparent K_m was 16 μM (SD ± 3, range 12–20, n=4) with a V_{max} of 3 pmol/min–10^6 sperm (SD ± 1, range 2–4, n=4). The slower hydrolysis rate and less sensitive absorbance method available to monitor NPGB makes this compound a less useful probe for the binding site than MUGB.

To validate MUGB as a probe for the binding site, it was necessary to show that this compound inhibits the binding of sperm to zonae. Incubations of twice-washed sperm with eggs in the presence of concentrations of MUGB ranging from 0–10 μM indicated that the zona binding was inhibited in a concentration-dependent manner that was hyperbolic (Table 2). The degree of inhibition of binding gave an excellent linear correlation with the rate of hydrolysis of MUGB at the concentrations used in the fluorimetric assay mixtures (Fig. 4). The results shown in Figure 4 imply that MUGB hydrolysis should be inhibited by agents known to interfere with zona binding through a "trypsin-like" mechanism and by zona protein that must interact with the site. Two known inhibitors, NPGB and SBTI (Saling, 1981), and acid-solubilized zonae pellucidae (ASZP) (Bleil and Wassarman, 1980b) were therefore examined. All three agents inhibited sperm binding to zonae and MUGB hydrolysis in a concentration-dependent manner

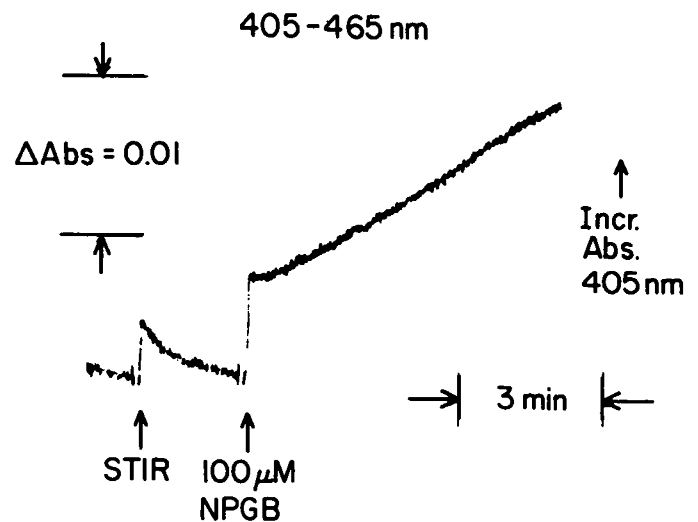

FIG. 3. NPGB hydrolysis by mouse sperm. Dual wavelength spectrophotometric chart trace of the hydrolysis of NPGB by twice-washed mouse spermatozoa. Absorbance wavelength = 405 nm; compensation wavelength = 465 nm. Sperm concentration = 7.3 × 10^7 spermatozoa/ml. Two hundred μl sperm suspension in HMB was placed in a cuvette and 2 μl 100 mM NPGB was added at the point indicated by the *arrow*. The extinction coefficient for p-nitrophenolate was 15 mM^{-1} cm^{-1} at pH 7.4. Calculated hydrolysis rate for this experiment was 4 pmol/min–10^6 spermatozoa.

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TABLE 2. Inhibition by MUGB of sperm binding to zona-intact eggs.

MUGB (μM)	Sperm bound/egg[a]	% Inhibition	n[c]
0	1.8		7
2.5	1.4	15 ± 7[deg]	3
5	1.3	27 ± 6[ef]	6
10	1.1	41 ± 6[fg]	6

[a]Means, each point was determined with a minimum of 30 eggs and a sperm concentration of 1.2 X 10^5 ml.; mean interassay coefficient of variation was 13.8%.

[b]% Inhibition was calculated from % of control for each run.

[c]Number of replicate determinations.

[d]Means ± SD.

[e,f,g]p<0.03, p<0.01, p<0.02, respectively; two-tailed t-test for correlated means.

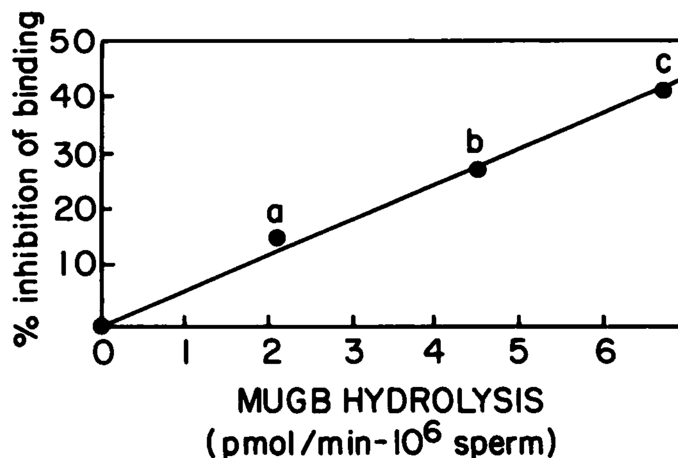

FIG. 4. Correlation of the rate of MUGB hydrolysis with the inhibition of sperm binding to eggs. MUGB hydrolysis and inhibition of binding to zona-intact eggs by twice-washed sperm were correlated at 3 concentrations of MUGB: *a*, 2.5 μM; *b*, 5 μM; *c*, 10 μM. Each *point* is the mean of 3 experiments. Least squares regression, r=0.998, p<0.001.

(Tables 3, 4 and 5). The inhibition of binding and MUGB hydrolysis gave excellent linear correlations (Fig. 5). Multiple regression analysis of the data for all three inhibitors showed no statistically significant difference between these lines and the idealized line of slope 1 going through the origin. Consistent with these results is the observation that the trypsin substrates, Z-lys-AMC and Z-arg-AMC, which do not inhibit MUGB hydrolysis, also do not inhibit sperm binding to zonae.

The experiments described above were all carried out in medium containing no BSA, conditions under which capacitation occurs slowly if at all (Wolf, 1979; Ward and Storey, 1984). Uncapacitated sperm do not undergo the acrosome reaction spontaneously or by induction with zona protein (Ward and Storey, 1984); noncapacitating conditions were used to avoid complications due to partial or complete acrosome reaction in the binding studies and to avoid contamination of the incubation mixtures by proacrosin or acrosin in the fluorimetric assays. The sperm, when treated with NPGB and SBTI as well as with ASZP under the above conditions, remained uncapacitated as determined by the CTC fluorescence assay. There were no sperm detected with fluorescence patterns associated with either the capacitated or acrosome-reacted state.

TABLE 3. Inhibition of sperm binding to zona-intact eggs and sperm hydrolysis of MUGB by NPGB.

NPGB* (μM)	Sperm bound/egg[b]	% Inhibition[cf]	n[d]	MUGB hydrolysis[a] pmol/min–10^6 sperm	% Inhibition[cf]	n[d]
0	1.6 ± 0.2[e]		4	9.5 ± 1.6[e]		4
10	1.0 ± 0.3	38	3	6.1 ± 1.2	28	3
20	0.5 ± 0.1	67	3	3.9 ± 0.8	54	3
50	0.3 ± 0.01	81	3	1.1 ± 0.3	75	3
100	0.2 ± 0.01	88	3	0.4 ± 0.2	94	4

[a]Concentration of MUGB was 10 μM; hydrolysis was monitored for at least 5 min before addition of NPGB.

[b]Each point was determined with a minimum of 30 eggs and a sperm concentration of 1.2 X 10^5/ml.

[c]% Inhibition was calculated by dividing the mean sperm/egg or hydrolysis rate at the given concentration of inhibitor by the mean control value.

[d]Number of replicate determinations.

[e]Means ± SD.

[f]Linear correlation between inhibition of sperm binding to zonae and inhibition of MUGB hydrolysis: r=0.979, p<0.003.

*p-Nitrophenyl p-guanidinobenzoate.

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TABLE 4. Inhibition of sperm binding to zona-intact eggs and sperm hydrolysis of MUGB by soybean trypsin inhibitor.

SBTI* mg/ml	Sperm bound/egg[b]	% Inhibition[cf]	n[d]	MUGB hydrolysis[a] pmol/min−10^6 sperm	% Inhibition[cf]	n[d]
0	1.8 ± 0.3[e]		7	8.0 ± 2.2[e]		7
0.5	1.6 ± 0.3	12	4	6.1 ± 2.6	23	6
1	1.2 ± 0.3	34	5	5.1 ± 1.8	40	7
2	0.8 ± 0.3	52	5	4.0 ± 1.3	50	5
4	0.4 ± 0.2	78	5	2.5 ± 0.6	69	5

[a]Concentration of MUGB was 10 μM; samples were preincubated with SBTI for 15 min prior to MUGB hydrolysis or sperm-binding assay.

[b]Each point was determined with a minimum of 30 eggs and a sperm concentration of 1.2 × 10^5/ml.

[c]% Inhibition was calculated by dividing the mean sperm/egg or hydrolysis rate at the given concentration of inhibitor by the mean control value.

[d]Number of replicate determinations.

[e]Means ± SD.

[f]Linear correlation between inhibition of sperm binding to zonae and inhibition of MUGB hydrolysis: $r=0.967$, $p<0.001$.

*Soybean trypsin inhibitor.

DISCUSSION

The close correlation of the rate of hydrolysis of MUGB by washed spermatozoa with MUGB inhibition of sperm binding to zona-intact eggs is an indication that MUGB is reacting at the "trypsin-like" binding site. This is reinforced by the close correlation of the inhibition by NPGB and SBTI of sperm binding to eggs and the turnover of MUGB by washed sperm suspensions. Inhibition of MUGB hydrolysis activity by the protein inhibitor SBTI and by ASZP indicates that the activity is on the sperm plasma membrane surface rather than in the cell. The close correlation of inhibition of binding with inhibition of MUGB hydrolysis by ASZP provides further evidence that MUGB is interacting with a sperm site for zona binding.

The level of binding of twice-washed spermatozoa to zonae pellucidae of cumulus-free eggs in HMB is consistent with the findings of other workers for other media (Saling et al., 1978; Heffner et al., 1980). However, the concentration of NPGB required to produce 50% inhibition of binding is lower than was found by Saling (1981). The greater sensitivity of the sperm-zona system to guanidinobenzoate inhibition in this study is probably caused by the removal of the soluble guanidinobenzoate hydrolyzing activity of the epididymal fluid from the assay system by washing the sperm. The soluble activity appears to have the same substrate specificity as the activity bound to the sperm surface. Two washes suffice to remove the former activity completely from the sperm, while the latter activity is highly resistant to removal by washing

TABLE 5. Inhibition of sperm binding to zona-intact eggs and sperm hydrolysis of MUGB by acid-solubilized zonae pellucidae.

Zonae/μl	Sperm bound/egg[b]	% Inhibition[cf]	n[d]	MUGB hydrolysis[a] pmol/min−10^6 sperm	% Inhibition[cf]	n[d]
0	1.8 ± 0.4[e]		5	8.6 ± 0.8[e]		4
0.5	1.6 ± 0.4	11	3	7.3 ± 1.5	15	3
1.5	1.1 ± 0.3	39	3	4.1 ± 0.5	52	3
3.0	0.4 ± 0.1	78	3	2.3 ± 0.2	73	3
4.0	0.2	89	2	1.3	85	2

[a]Concentration of MUGB was 10 μM; samples were preincubated with SBTI for 15 min prior to MUGB hydrolysis or sperm-binding assay.

[b]Each point was determined with a minimum of 30 eggs and a sperm concentration of 1.2 × 10^5/ml.

[c]% Inhibition was calculated by dividing the mean sperm/egg or hydrolysis rate at the given concentration of inhibitor by the mean control value.

[d]Number of replicate determinations.

[e]Means ± SD.

[f]Linear correlation between inhibition of sperm binding to zonae and inhibition of MUGB hydrolysis: $r=0.969$, $p<0.002$.

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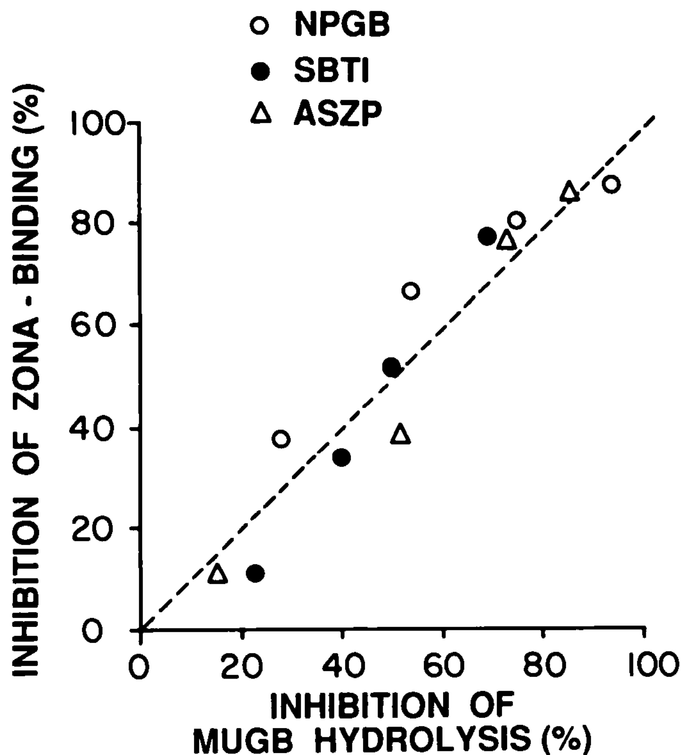

FIG. 5. Correlation of inhibition of MUGB hydrolysis and inhibition of sperm binding to zona-intact eggs by NPGB, SBTI and acid-solubilized zonae pellucidae. Inhibition of MUGB hydrolysis and inhibition of binding to zona-intact eggs by twice-washed sperm were correlated at different concentrations of 3 inhibitors: NPGB, SBTI, and ASZP. *Points* are means of at least 3 experiments. Concentrations in ascending order of the inhibitors were: NPGB; 10, 20, 50, and 100 μM. SBTI; 0.5, 1, 2, and 4 mg/ml. ASZP; 0.5, 1.5, 3, and 4 zonae/μl. *Broken line* represents an idealized plot of y=mx, r=1. Equations for the regressed lines are: NPGB y=0.948x + 7.2, r=.979; SBTI y=1.17x − 7.2, r=0.978; ASZP y=1.06x − 4.4, r=0.984.

(Table 1). Further, there is essentially no dissociation of MUGB hydrolyzing activity into the medium from twice-washed sperm during 60 min, indicating that the sperm activity is tightly bound. The question of how the two activities, soluble and sperm-bound, are related, if indeed they are, is an interesting one, but one that is beyond the scope of this study.

The substrate specificity of the membrane-bound site shows that it is not acrosin. Trypsin and acrosin are known to cause an initial burst of fluorescence with MUGB, which correlates positively with the concentration of the enzyme (Jameson et al., 1973; Brown et al., 1975). Furthermore, there is little or no subsequent turnover of the titrant by either of these enzymes. Even the alpha form of trypsin does not give continued turnover beyond 10 min (Jameson et al., 1973), while the sperm-mediated MUGB hydrolysis continues for over 30 min with no indication of a plateau. Mouse acrosin has been reported to have characteristics similar to the acrosin found in ram, bull, and boar (Brown, 1983). Trypsin and acrosin readily catalyze the hydrolysis of Z-lys and Z-arg amides and esters (Brown and Hartree, 1978; Green et al., 1979). Neither the epididymal fluid nor the washed sperm suspensions give detectable hydrolysis of Z-lys-AMC or Z-arg-AMC. These fluorogenic substrates were chosen because they have been shown to have a detectable linear range of hydrolysis by trypsin from 0.2 to 20 ng/ml enzyme under conditions similar to those used in this assay system (Zimmerman et al., 1977). This is more sensitive than the detection range for trypsin using MUGB as active site titrant (Jameson et al., 1973; Brown et al., 1975). We conclude that any acrosin activity expressed by mouse sperm is below this low level of detectability under our assay conditions.

To check the possibility that NPGB, SBTI, or ASZP might alter the noncapacitated state or induce the acrosome reaction, the status of twice-washed spermatozoa was determined by the CTC microscopic fluorescence assay (Ward and Storey, 1984) after incubation with each of the three agents under conditions of the binding assay. In duplicate experiments, only membrane fluorescence patterns associated with uncapacitated sperm were found. There was complete absence of sperm showing the CTC fluorescence patterns corresponding to capacitated intact sperm or sperm that had undergone the first or second stages of the acrosome reaction (Lee and Storey, 1985). This result rules out interference from acrosome-reacted sperm in the binding studies or fluorimetric determinations and also provides direct evidence that capacitation of mouse sperm is not necessary for binding of sperm to zona. This characteristic of the binding process had been previously inferred from studies in media lacking BSA (Saling et al., 1978; Heffner et al., 1980).

The sperm surface site and soluble MUGB-hydrolyzing activity show properties similar to an enzyme that has been termed guanidinobenzoatase and has been found in ascites fluid, on ascites cells, and in mouse blood plasma (Steven and Al-Ahmad, 1983). The enzyme hydrolyzes MUGB and NPGB but does not hydrolyze benzoyl arginine ethyl ester. This enzyme was also reported to be on mouse spermatozoa and in seminal plasma (Steven and Al-Ahmad, 1983). The findings in the present study show that there is one important difference between the sperm activity and that found for ascites cells: the

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hydrolyzing activity on the sperm surface and in epididymal fluid extracts is not inhibited by arginyl esters and amides that do inhibit the guanidinobenzoatase found by Steven and Al-Ahmad (1983). It is interesting to note that guanidinobenzoatases are also found on the surface of normal cells that are capable of migrating as well as on tumor cells (Steven et al., 1985). To date, no physiological function has been found for this class of enzymes. Our studies indicate that the sperm surface activity of this type has binding-site properties, and so may represent a more general mechanism of cell-to-cell interaction than just a recognition factor for sperm and zona. This supposition awaits experimental testing.

The first prediction of Florman et al. (1982), that the "trypsin-like" site should react with trypsin-active site titrants, was fulfilled in an unexpected manner: these titrants do not bind irreversibly to the site, but instead act as substrates for hydrolysis. The second prediction, that trypsin substrates should act as active site titrants and inhibit sperm binding to the zona, was not fulfilled. The two compounds, Z-lys-AMC and Z-arg-AMC, which are excellent substrates for trypsin and acrosin, are not hydrolyzed by washed spermatozoa, do not inhibit sperm binding to zonae, and do not inhibit MUGB turnover. The trypsin inhibitors NPGB and SBTI do inhibit both activities. The stereospecificity of the site is therefore different from the reactive sites of trypsin and acrosin. The term "trypsin-like" may therefore not be a particularly accurate description of this site. A more accurate term would by "trypsin-inhibitor-sensitive site," which also distinguishes this binding site from the one resembling galactosyl transferase (Shur and Hall, 1982a,b; Lopez et al., 1985). These two sites would appear to be distinct, but must interact in such a manner that blockage of one of them inhibits sperm binding to the zona. This interaction is currently under active investigation.

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