

# Egg Cortical Granule N-Acetylglucosaminidase Is Required for the Mouse Zona Block to Polyspermy

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**Abstract.** The mammalian egg must be fertilized by only one sperm to prevent polyploidy. In most mammals studied to date, the primary block to polyspermy occurs at the zona pellucida, the mammalian egg coat, after exocytosis of the contents of the cortical granules into the perivitelline space. The exudate acts on the zona, causing it to lose its ability to bind sperm and to be penetrated by sperm previously bound to the zona. However, the cortical granule components responsible for the zona block have not been identified. Studies described herein demonstrate that *N*-acetylglucosaminidase is localized in cortical granules and is responsible for the loss in sperm-binding activity leading to the zona block to polyspermy.

Before fertilization, sperm initially bind to the zona by an interaction between sperm surface GalTase and terminal *N*-acetylglucosamine residues on specific oligosaccharides of the zona glycoprotein ZP3 (Miller, D. J., M. B. Macek, and B. D. Shur. 1992. *Nature (Lond.)*. 357:589–593). These GalTase-binding sites are lost from ZP3 after fertilization, an effect that can be duplicated by *N*-acetylglucosaminidase treatment.

Therefore, *N*-acetylglucosaminidase, or a related glycosidase, may be present in cortical granules and be responsible for ZP3's loss of sperm-binding activity at fertilization. Of eight glycosidases assayed in exudates of ionophore-activated eggs, *N*-acetylglucosaminidase was 10-fold higher than any other activity. The enzyme was localized to cortical granules using immunoelectron microscopy. Approximately 70 or 90% of the enzyme was released from cortical granules after ionophore activation or in vivo fertilization, respectively. The isoform of *N*-acetylglucosaminidase found in cortical granules was identified as  $\beta$ -hexosaminidase B, the  $\beta$ ,  $\beta$  homodimer. Inhibition of *N*-acetylglucosaminidase released from activated eggs, with either competitive inhibitors or with specific antibodies, resulted in polyspermic binding to the zona pellucida. Another glycosidase inhibitor or nonimmune antibodies had no effect on sperm binding to activated eggs. Therefore, egg cortical granule *N*-acetylglucosaminidase is released at fertilization, where it inactivates the sperm GalTase-binding site, accounting for the block in sperm binding to the zona pellucida.

**I**n monospermic species, blocks to polyspermy immediately after fertilization are necessary to prevent incorporation of more than one sperm nucleus into the fertilized egg, which would result in abnormal development (for review see Jaffe and Gould, 1985; Cran and Esper, 1990; Ducibella, 1991). In nonmammalian organisms such as sea urchins and *Xenopus laevis*, a "fast block" occurs seconds after the fertilizing sperm activates the egg. This block involves depolarization of the egg membrane, which prevents additional sperm from binding to the membrane (Nuccitelli and Grey, 1984; Webb and Nuccitelli, 1985). A second block to polyspermy, the "slow block," develops in ~30 s, resulting from the exocytic release of cortical granules, whose contents modify the egg coat irreversibly to prevent binding of

additional sperm as well as penetration by bound sperm (Allegro and Schuel, 1988; Carroll and Epel, 1975; Foltz et al., 1993; Lindsay and Hedrick, 1989; Shapiro et al., 1989; Somers et al., 1989; Wyrick et al., 1974).

Much less is known about the molecular basis of the block to polyspermy in mammals. The initial block to polyspermy is due to the retention of sperm by the lower parts of the female reproductive tract, eliminating the vast majority of sperm before they reach the fertilization site (Cummins and Yanagimachi, 1982). After the fertilizing sperm activates the egg, there is little evidence for an electrical "fast block" to polyspermy at the level of the egg plasma membrane, but there is evidence for some other poorly understood block at the egg plasma membrane (Wolf, 1978; Miyazaki and Igusa, 1981; Jaffe et al., 1983; McCulloch et al., 1987; Stewart-Savage and Bavister, 1988). Approximately 5–8 min after egg activation, cortical granules are released and a "slow block" is established at the mammalian egg coat, or zona pel-

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lucida. This block has been termed the zona reaction and, like the invertebrate block to polyspermy, results in an inability to bind sperm and an inability to be penetrated by sperm already bound to the zona. Although evidence has existed for many years that trypsin-sensitive and heat-sensitive components of cortical granule exudate are responsible for the zona block to polyspermy (Barros and Yanagimachi, 1971; Gwatkin et al., 1973; Gwatkin and Williams, 1974), the proteins responsible have not been identified.

In contrast to our limited understanding of the mammalian zona block to polyspermy, we have a better appreciation of the mechanisms underlying initial sperm binding to the zona pellucida, primarily from studies in the mouse.  $\beta$ 1,4-Galactosyltransferase (GalTase) is found on the plasma membrane overlying the acrosome of mouse sperm, where it binds to specific oligosaccharides terminating in *N*-acetylglucosamine on the zona pellucida glycoprotein, ZP3 (Bleil and Wassarman, 1980; Shur, 1991; Miller et al., 1992). Other candidate ZP3 receptors have also been identified, but their function is not as clearly understood (Leyton and Saling, 1989; Bleil and Wassarman, 1990). When ZP3 binds sperm, it induces exocytosis of the acro-some by aggregating its sperm surface receptor (Bleil and Wassarman, 1983; Leyton and Saling, 1989), possibly GalTase (Macek et al., 1991; Miller et al., 1992). Acrosome-reacted sperm penetrate through the zona pellucida using a released *N*-acetylglucosaminidase that is thought to remove potential GalTase-binding sites on zona glycoproteins in the vicinity of the penetrating sperm (Miller et al., 1993). The fertilizing sperm binds to the egg membrane and fuses with the egg, triggering egg activation. During the activation process, cortical granules are exocytosed and their contents are hypothesized to inactivate ZP3, resulting in a loss of ZP3's sperm-binding activity. Although there is proteolysis of another zona glycoprotein, ZP2, by an unidentified protease (Moller and Wassarman, 1989), there is not observable proteolysis of ZP3 at fertilization; in fact, ZP3 is indistinguishable by SDS-PAGE before and after fertilization. The loss of ZP3's sperm binding activity must, therefore, result from subtle, but critical, modifications in its structure.

It has been shown that ZP3 from fertilized eggs loses its GalTase-binding sites, which could result from release of an *N*-acetylglucosaminidase at egg activation (Miller et al., 1992). This enzyme would not markedly alter the electrophoretic behavior of ZP3 but account for its loss of sperm binding activity and is, therefore, a candidate for effecting the zona block to polyspermy. The objectives of this study were to determine whether *N*-acetylglucosaminidase activity is present in mouse egg cortical granules, is released at egg activation, and functions in the development of the zona block to polyspermy. We demonstrate that of those assayed the predominant glycosidase in mouse cortical granules is *N*-acetylglucosaminidase, which is exocytosed at egg activation into the perivitelline space. There, it acts on ZP3 to remove terminal *N*-acetylglucosamine residues, thereby inactivating ZP3's ability to serve as a sperm GalTase receptor and leading to a loss of ZP3's sperm-binding activity. This is the first identification of a mammalian egg cortical granule enzyme and elucidation of its role in the zona block to polyspermy.

## Materials and Methods

### Glycosidase Assays

Cumulus-free oviductal eggs were collected from female CD-1 mice superovulated with pregnant mare's serum gonadotropin (PMSG), and ovulated eggs were removed from oviducts 13 h after injection of human chorionic gonadotropin (hCG)<sup>1</sup> (Shur and Hall, 1982). Cumulus cells were removed by digestion for 5 min in 1 mg/ml of testicular hyaluronidase, and the eggs were washed by consecutive transfer through at least six droplets of dmKRBT (120 mM NaCl, 2 mM KCl, 2 mM CaCl<sub>2</sub>, 10 mM NaHCO<sub>3</sub>, 1.2 mM MgSO<sub>4</sub>, 5.6 mM glucose, 1.1 mM sodium pyruvate, 25 mM TAPSO (3-[N-tris (hydroxymethyl)methylamino]-2-hydroxy propane-sulfonic acid), 18.5 mM sucrose, 6 mg/ml BSA, pH 7.3; Neill and Olds-Clarke, 1988). This washing is sufficient to remove hyaluronidase and contaminating glycosidases from eggs.

To measure total activity, eggs were transferred to 25  $\mu$ l of dmKRBT containing a protease inhibitor cocktail (PIC; Miller et al., 1992) and 0.1% Triton X-100. After 20 min on ice, eggs were solubilized by repeated pipetting, and 25  $\mu$ l of 2 mM substrate was added to yield a final concentration of 1 mM substrate. Substrates were dissolved in 60 mM trisodium citrate, 40 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 1 mM KCl, 1 mg/ml BSA, with PIC at pH 5.0, yielding a final pH of 5.2. Substrates tested were 4-methylumbelliferyl (4-MU) derivatives of glycosides including 4-MU-*N*-acetyl- $\beta$ -D-glucosaminide, 4-MU-*N*-acetyl- $\beta$ -D-galactosaminide, 4-MU- $\alpha$ -D-fucoside, 4-MU- $\alpha$ -D-galactoside, 4-MU- $\beta$ -D-galactoside, 4-MU- $\alpha$ -D-glucoside, 4-MU- $\beta$ -D-glucoside, 4-MU- $\alpha$ -D-mannoside, and 4-MU-*N*-acetyl- $\beta$ -D-glucosaminide-6-sulfate (Research Products International, Mount Prospect, IL).

For measurements of calcium ionophore-released activity, eggs were incubated with 10  $\mu$ M A23187 in dmKRBT. After 30 min at 37°C, eggs were washed over 30 min through three drops of 10  $\mu$ l each of divalent cation-free dmKRBT (Cherr et al., 1988; Ducibella et al., 1988). All washes were pooled and used as a source of egg-released material. The material remaining associated with the eggs was homogenized in 0.1% Triton X-100 as above. From these pools, the equivalent of 50 eggs was brought up to 30  $\mu$ l and added to an equal volume (30  $\mu$ l) of divalent cation-free dmKRBT. Substrate (60  $\mu$ l of 2 mM 4-MU derivatives in citrate phosphate) was added and the assay stopped after 4 h by addition of 2 ml of stop buffer (133 mM glycine, 83 mM NaCO<sub>3</sub>, pH 10.7). Product was measured fluorometrically on a Hoescht fluorometer using an excitation wavelength of 380 nm and measuring emission at 460 nm (Johnson et al., 1972; Kaback, 1972). Similar results were obtained using *p*-nitrophenyl substrates (Sigma Chemical Co., St. Louis, MO), but with these substrates, 100–300 eggs and incubations of 16–20 h at 37°C were required for detection.

To measure *N*-acetylglucosaminidase in fertilized eggs, female CD-1 mice were superovulated by i.p. injections of 7.5 IU PMSG and then 48 h later with 7.5 IU hCG. Females were allowed to mate with males overnight, and 20 h after hCG administration, females were sacrificed and fertilized eggs collected from the oviducts. At this stage, no eggs had cleaved to two-cell embryos. Fertilized eggs and unfertilized eggs collected as above were transferred to 50  $\mu$ l of 0.1% Triton X-100 and 1 mM 4-MU *N*-acetyl- $\beta$ -D-glucosaminide in citrate-phosphate buffer pH 5 and assayed for *N*-acetyl- $\beta$ -D-glucosaminidase.

To determine the heat-sensitivity of *N*-acetylglucosaminidase released from cortical granules, eggs were activated with A23187 and released protein was heated to 52°C for 2 h. This treatment inactivates serum  $\beta$ -hexosaminidase B, but not  $\beta$ -hexosaminidase A (Johnson et al., 1972; Kaback, 1972). Controls were maintained at 4°C for 2 h. Enzyme assays were carried out as above.

To determine the pH optimum of the cortical granule *N*-acetylglucosaminidase, eggs were treated with A23187. Released enzyme was then incubated in a final concentration of 1 mM 4-MU-*N*-acetyl- $\beta$ -D-glucosaminide in citrate phosphate buffer at various pH's.

### Immunoprecipitation of $\beta$ -Hexosaminidase Activity

Cumulus-free oviductal eggs (50 per tube) were placed in 1.5-ml microfuge tubes and solubilized 60 min on ice in 100  $\mu$ l of lysis buffer (150 mM NaCl,

1. Abbreviations used in this paper: 4-MU, 4-methylumbelliferyl; hCG, human chorionic gonadotropin; PIC, protease inhibitor cocktail.

1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 7.5 plus PIC). Egg lysates were precleared by incubation with 25  $\mu$ l of normal goat serum for 4 h and then with 25  $\mu$ l of anti-goat IgG-agarose for 4 h at 4°C. The sample was centrifuged, and the supernatant placed into a microfuge tube. For immunoprecipitation, three goat antisera made against human  $\beta$ -hexosaminidase were used. Antisera made against  $\beta$ -hexosaminidase A (the  $\alpha$ ,  $\beta$  heterodimer), against  $\beta$ -hexosaminidase B (the  $\beta$ ,  $\beta$  homodimer), and against the purified  $\alpha$  subunit were obtained from Dr. Richard Proia (National Institutes of Health [NIH]). These antisera were previously characterized (Proia et al., 1984; Miller et al., 1993). A 1/10 dilution (12  $\mu$ l) of normal goat serum or antisera was incubated with lysates overnight at 4°C, and then 25  $\mu$ l anti-goat IgG-agarose was added. After 4 h, immune complexes were centrifuged and washed four times. The complexes were brought to 100  $\mu$ l with lysis buffer (as above), and 100  $\mu$ l of 2 mM 4-MU-N-acetylglucosaminide in citrate phosphate was added. Enzyme assays were allowed to incubate for 2–5 h, the reaction stopped by addition of stop buffer and the product measured fluorometrically, as described above (Johnson et al., 1972; Kaback, 1972).

### **Immunolocalization of $\beta$ -Hexosaminidase**

Cumulus-free oviductal eggs were collected and washed by consecutive transfer through droplets of divalent cation-free dmKRBT. The eggs were transferred to Beem capsules and fixed for 1–2 h in 4% paraformaldehyde in the presence of 0.1 or 1.0% glutaraldehyde in PBS or in 0.1 M sodium cacodylate, pH 7.2. To inhibit the breakdown of cortical granules that may occur during the initial stages of fixation, 10 mM EGTA was added to the fixatives in cacodylate buffer. Dehydration and embedding were performed without removing the eggs from Beem capsules by using a No. 22 gauge needle attached to a 1.5-ml plastic syringe to deliver the appropriate reagents into the capsules. The Beem capsules were rotated to allow easy removal of fluids. After primary fixation, all subsequent steps through embedding were performed at –20°C. The specimens were washed in 0.1 M sodium cacodylate or PBS and dehydrated in a graded series of dimethylformamide (50, 70, 95, and 100%). Lowicryl K4M was used for the final embedding, which was polymerized for 3 d in the presence of UV light. After immunolabeling (see below) and staining for 10 min with 2.0% uranyl acetate, thin sections of the specimens were examined in a Phillips 410 transmission electron microscope (Phillips Scientific, Mahwah, NJ) operated at 60 kV.

For immunolocalization, sections were blocked with 5% nonfat dry milk in PBS with 0.1% Tween 20. After several washes in PBS-Tween 20, 1/40 dilutions of immune or normal sera in PBS with 0.1% Tween 20 were applied to sections of eggs mounted on Formvar-reinforced grids (150 mesh). Additionally, goat IgG was purified from  $\beta$ -hexosaminidase A antiserum by Protein G affinity chromatography, and the resulting IgG, along with IgG purified from normal goat serum, were used. After incubation with primary antibody for 1–3 h, grids were washed through five aliquots (50  $\mu$ l each) of the PBS-Tween 20. The mounted sections were then incubated for 60 min at room temperature in the presence of a 1/20 dilution of rabbit anti-goat IgG coupled to 10 nm colloidal gold (Calbiochem Corp., La Jolla, CA). Excess secondary antibody conjugate was removed from the sections through several washes in PBS-Tween 20. The egg sections were washed in distilled H<sub>2</sub>O, stained with uranyl acetate, and quantitatively evaluated in the electron microscope to determine the relative number of gold particles bound to cortical granules after treatment with either serum or purified IgG.

As a positive control for cortical granule localization, biotinylated lens culinaris lectin (1/33 dilution in PBS-Tween 20; Sigma Chemical Co.) was applied to sections of eggs for 1–2 h (Cherr et al., 1988; Ducibella et al., 1988). Avidin-colloidal gold conjugate (15 nm particles) was used to localize the lectin. Secondary antibodies conjugated to colloidal gold were used as a reagent control and did not show significant binding to mouse eggs.

### **Inhibition of N-Acetylglucosaminidase Activity during Egg Activation**

Cumulus-free eggs were collected from the oviducts of superovulated mice as described above. The eggs were placed in droplets of dmKRBT covered with mineral oil. Eggs were activated by addition of 10  $\mu$ M (final concentration) A23187 for 30 min at 37°C and then transferred out of A23187 for 30 min (Cherr et al., 1988; Ducibella et al., 1988). To inhibit glycosidase activity, either PUGNAC [O-(2-acetamido-2-deoxy-D-glucopyranosylidene) amino N-phenylcarbamate], a competitive  $\beta$ -N-acetylglucosaminidase inhibitor, or the control PUGLU [O-(D-glucopyranosylidene) amino

N-phenylcarbamate], a  $\beta$ -glucosidase inhibitor (CarboGen, Zurich) was added to eggs to a final concentration of 500  $\mu$ M during activation with A23187 (Beer and Vasella, 1985). Previous experiments had shown that this concentration of PUGNAC yielded an effective concentration of  $\sim$ 25  $\mu$ M possibly because of PUGNAC binding to eggs or plastic (Miller et al., 1993); similar to those concentrations that inhibit zona penetration by mouse sperm and ascidian fertilization (Godknecht and Honegger, 1991; Miller et al., 1993). Alternatively, eggs were activated in a 1/10 dilution of anti- $\beta$ -hexosaminidase A or B antiserum or normal goat serum. After activation, the eggs were washed by consecutive transfer through five droplets of dmKRBT to remove glycosidase inhibitors or antibodies.

Eggs activated in the presence of inhibitors were assayed for their ability to bind sperm to determine if the zona block to polyspermy had been inhibited (Miller et al., 1992). Eggs (20–50 per droplet) were placed in 25- $\mu$ l droplets of dmKRBT under mineral oil. Sperm were collected from the cauda epididymides of CF-1 mice and capacitated for 60 min in dmKRBT at 37°C. Sperm were diluted to a concentration of  $2 \times 10^6$  sperm/ml, and 25  $\mu$ l was added to eggs yielding a final concentration of  $10^6$  sperm/ml. After 30 min of co-incubation, loosely adherent sperm were removed by pipetting the eggs through a narrow-bore mouth pipette, and eggs were fixed in 4% paraformaldehyde in PBS. Sperm bound to the zona were counted at 400 $\times$ . For statistical analysis, numbers of sperm bound to each egg were averaged in each droplet, log-transformed to normalize the data, and subjected to analysis of variance. Means were compared using Tukey's studentized range, and absolute means and standard errors are presented (Neter and Wasserman, 1974).

### **Analysis of ZP2 Conversion to ZP2<sub>f</sub>**

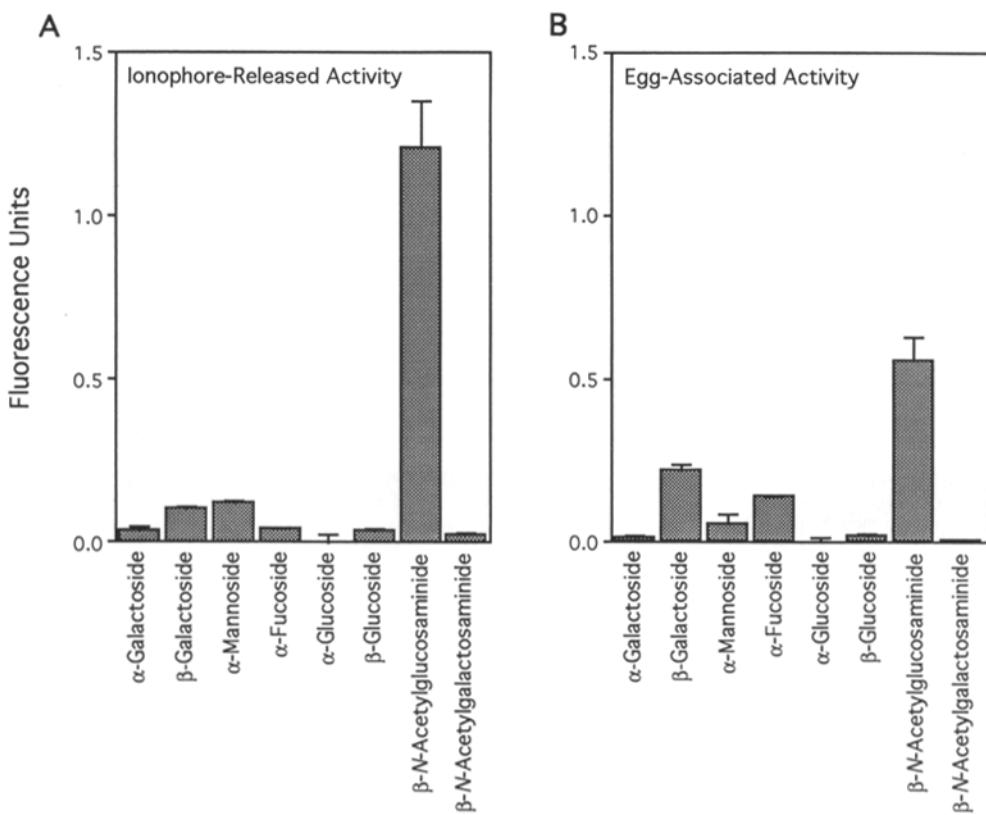
To determine if ZP2 was converted to ZP2<sub>f</sub> after egg activation, zonae from eggs activated with ionophore in the presence of 500  $\mu$ M PUGNAC and, as a control, PUGLU, were mechanically isolated. Zonae pellucidae from 200 eggs were radioiodinated with 0.1 mCi of <sup>125</sup>I-Bolton Hunter reagent using standard conditions, washed to remove unincorporated reagent, and solubilized in SDS-PAGE sample buffer containing  $\beta$ -mercaptoethanol. Aliquots of zona glycoproteins from 20 eggs were separated by SDS-PAGE and identified by autoradiography. Under these conditions, ZP2 from activated eggs, which has been cleaved by a cortical granule protease, migrates to nearly the same position as ZP3 (Moller and Wassarman, 1989).

## **Results**

The zona block to polyspermy in the mouse is a consequence of two changes that occur upon fertilization. The zona pellucida loses its ability to bind sperm as well as its ability to be penetrated by bound sperm. Previous results have shown that ZP3, a zona pellucida glycoprotein that binds GalTase on mouse sperm to mediate gamete binding, loses its GalTase-binding site after egg activation. N-acetylglucosaminidase is an exoglycosidase capable of removing the GalTase-binding site from ZP3. Therefore, it was of interest to determine if N-acetylglucosaminidase or other glycosidases were released during the cortical reaction.

### **Mouse Egg Cortical Granules Contain High Levels of N-Acetylglucosaminidase**

The cortical reaction can be induced by treatment of eggs with the calcium ionophore A23187 (Cherr et al., 1988; Ducibella et al., 1988). Eggs were treated with A23187 and the released material, or detergent extracts of the remaining egg-associated material, were assayed for several glycosidases. Preliminary experiments using several substrates indicated that the enzyme assays were linear during the time of incubation (data not shown). Of those tested, the major glycosidase released from eggs was N-acetylglucosaminidase (Fig. 1A). This enzyme was tenfold more active than other glycosidases assayed in exudates. Of the total egg



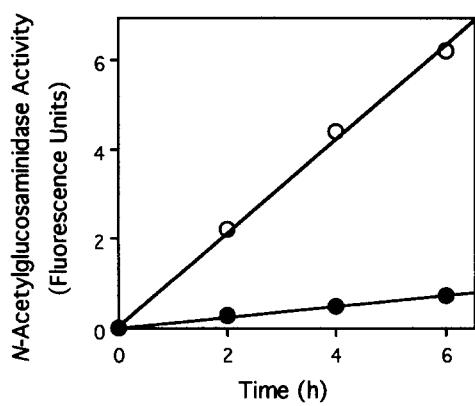
**Figure 1.** *N*-Acetylglucosaminidase is the most active glycosidase released during the egg cortical reaction. Cumulus-free oviductal mouse eggs were activated with calcium ionophore A23187. After egg activation, the material released from eggs was pooled and the eggs were solubilized in Triton X-100. Exudates (*A*) or remaining activated egg lysates (*B*) of 50 eggs were incubated with 1 mM 4-MU-*N*-acetylglucosaminide (final concentration) for 4 h at 37°C in citrate-phosphate buffer pH 5. Product was measured fluorimetrically. Data are representative of four assays performed in triplicate. Bars represent standard errors.

*N*-acetylglucosaminidase activity, 70% was released upon ionophore-induced activation (Fig. 1, *A* and *B*). Low amounts of other glycosidases were also detected, including  $\beta$ -galactosidase,  $\alpha$ -mannosidase, and  $\alpha$ -fucosidase, but the majority of  $\beta$ -galactosidase and  $\alpha$ -fucosidase remained associated with eggs after activation. Interestingly, although *N*-acetylglucosaminidases generally have some *N*-acetylgalactosaminidase activity (Beely, 1985), it appears that cortical granule *N*-acetylglucosaminidase had high specificity for *N*-acetylglucosamine; *N*-acetylgalactosaminidase activity was 2% of *N*-acetylglucosaminidase activity.

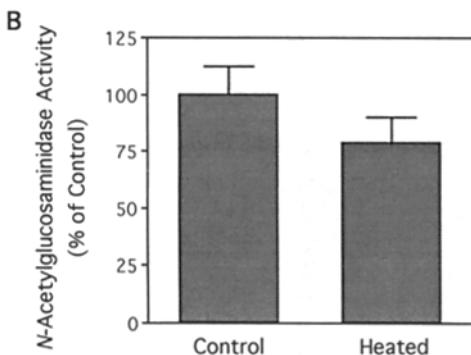
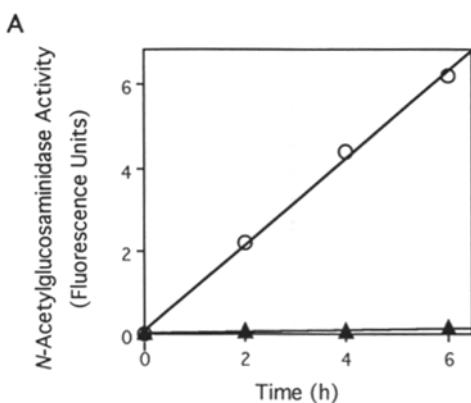
To verify that *N*-acetylglucosaminidase activity was lost during the normal egg activation process, total *N*-acetylglucosaminidase activity was compared between unfertilized eggs and eggs fertilized *in vivo*. Detergent-solubilized fertilized eggs retained only ~10% of the total *N*-acetylglucosaminidase activity of unfertilized eggs (Fig. 2). These results demonstrate that the majority of *N*-acetylglucosaminidase activity in unfertilized eggs was lost during the physiological cortical reaction that accompanies fertilization.

*N*-acetylglucosaminidase activity in most normal tissues is accounted for by two isoforms of the dimeric  $\beta$ -hexosaminidase enzyme.  $\beta$ -hexosaminidase A is a heterodimer composed of  $\alpha$  and  $\beta$  subunits, whereas  $\beta$ -hexosaminidase B is a homodimer of two  $\beta$  subunits (Proia et al., 1984; Mahuran, 1991). The two enzymes may be distinguished by their substrate specificity. The substrate 4-MU-*N*-acetylglucosamine-6-sulfate is cleaved specifically by the  $\alpha$  subunit found on  $\beta$ -hexosaminidase A, whereas the unsulfated substrate is cleaved by the  $\beta$  subunit present in both  $\beta$ -hexosaminidase isoforms (Kytzia and Sandhoff, 1985). Eggs were activated by addition of A23187 and the released material was incu-

bated with either the unsulfated or sulfated *N*-acetylglucosaminidase substrate. Low activity (~3% of the total activity) was detected towards the sulfated substrate, suggesting that little  $\beta$ -hexosaminidase A enzyme was present (Fig. 3 *A*). A second method of discriminating between the two isoforms is that, in most cells,  $\beta$ -hexosaminidase B is more heat stable than is  $\beta$ -hexosaminidase A (Johnson et al., 1972; Kaback, 1972). Therefore, material released from activated eggs was heated to 52°C for 2 h, conditions that inactivate serum  $\beta$ -hexosaminidase A. The released enzyme retained ~80% of its activity under these conditions; thus, most of the  $\beta$ -hex-



**Figure 2.** *N*-Acetylglucosaminidase activity is released as eggs undergo fertilization *in vivo*. Lysates of fifty unfertilized (○) or fertilized one-cell eggs (●) were assayed for *N*-acetylglucosaminidase activity. Data are representative of three experiments performed in triplicate.

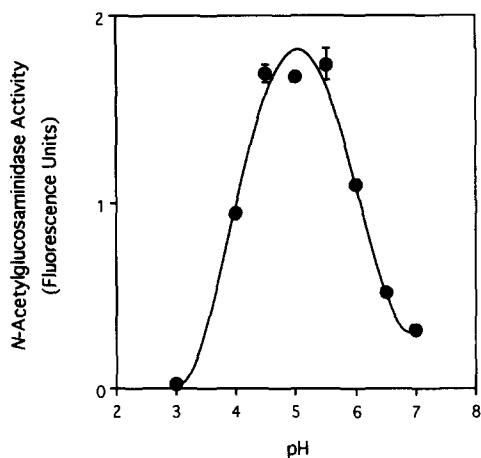


**Figure 3.** *N*-Acetylglucosaminidase activity released during the cortical reaction is due primarily to the  $\beta$ -hexosaminidase B isoform. (A) Exudate released from 50 A23187-activated eggs was incubated with either 1 mM 4-MU-*N*-acetylglucosaminide (○), which is cleaved by both  $\beta$ -hexosaminidases, or with 1 mM 4-MU-*N*-acetylglucosaminide-6-sulfate (▲), which is cleaved only by  $\beta$ -hexosaminidase A. Low activity (~5% of the total activity) towards the sulfated substrate was found, demonstrating low amounts of  $\beta$ -hexosaminidase A. Data are representative of four experiments performed in triplicate. (B) Exudate released from 50 A23187-activated eggs was heated to 52°C for 2 h, a treatment that selectively inactivates serum  $\beta$ -hexosaminidase A, and then incubated with 1 mM 4-MU-*N*-acetylglucosaminide. Controls were maintained at 4°C for 2 h. Enzyme activity was reduced only by ~20% after heating, demonstrating that the egg cortical granule  $\beta$ -hexosaminidase is predominantly the B isoform. Data are representative of four experiments performed in triplicate. Bars represent standard errors.

osaminidase released from eggs during the cortical reaction had characteristics of  $\beta$ -hexosaminidase B (Fig. 3 B).

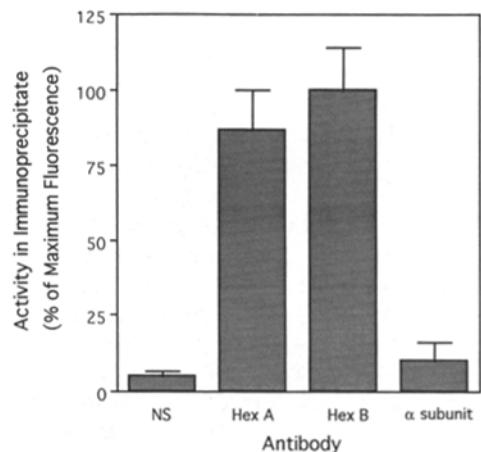
Lysosomal  $\beta$ -hexosaminidases have pH optima near pH 5 (Mahuran, 1991). Since  $\beta$ -hexosaminidase B in the cortical granule exudate is released into the extracellular fluid to reach its target, the zona pellucida, it was of interest to determine the pH requirements of egg cortical granule  $\beta$ -hexosaminidase B.  $\beta$ -Hexosaminidase released from activated eggs was incubated with 4-MU-*N*-acetylglucosaminide at several pH's. The enzyme retained maximal activity at pH 4.5–5.5 and had ~20% of maximal activity at pH 7.0 (Fig. 4).

Antibodies to  $\beta$ -hexosaminidase were used to confirm the enzyme isoform as well as assess its localization and function. Antisera made in goats against human  $\beta$ -hexosaminidase A ( $\alpha, \beta$  heterodimer),  $\beta$ -hexosaminidase B ( $\beta, \beta$  homodimer), and to the  $\alpha$  subunit (Proia et al., 1984) were tested in immunoprecipitation assays for reactivity with the

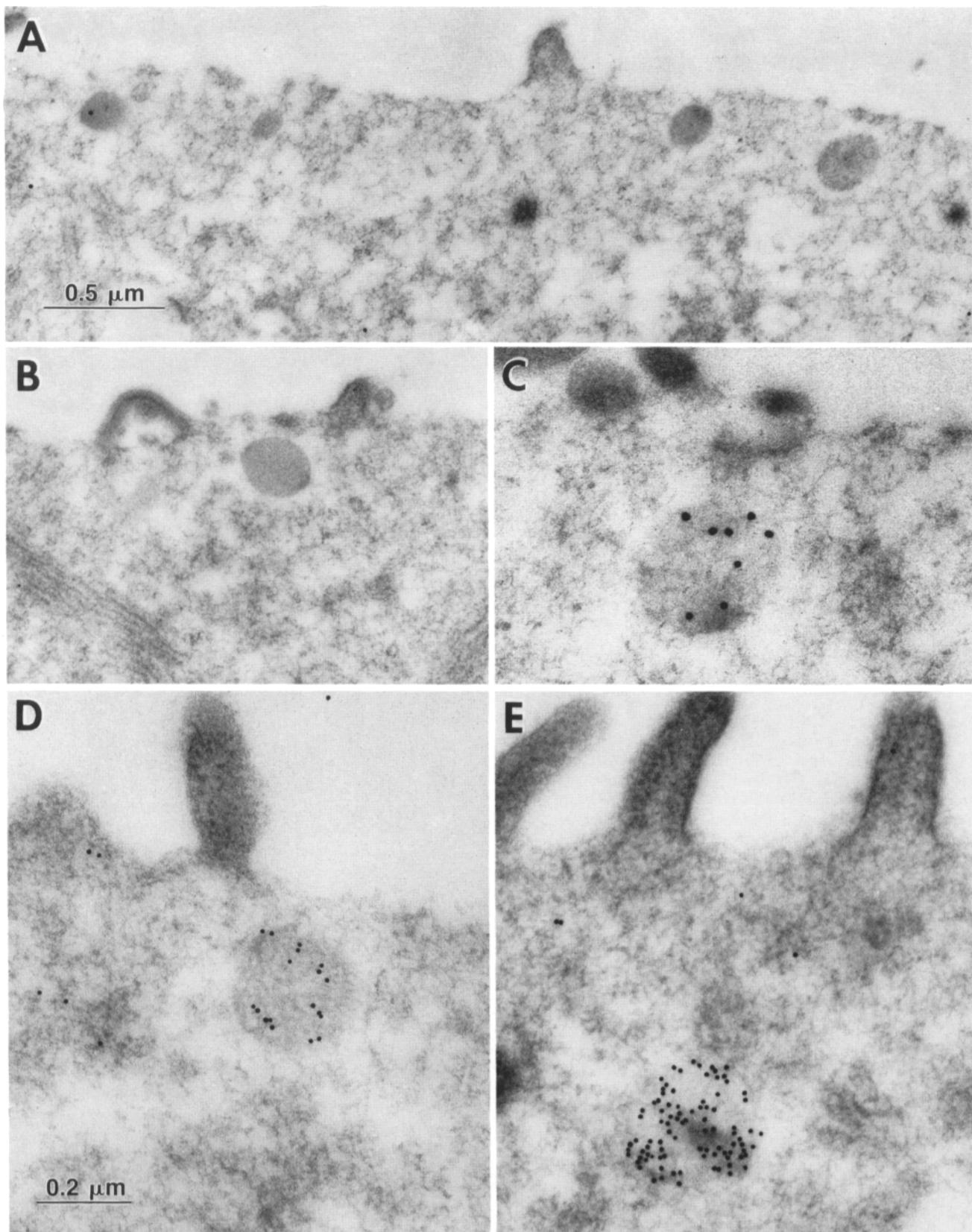


**Figure 4.** The optimum pH of egg *N*-acetylglucosaminidase activity is near pH 5.0. Eggs were activated by A23187 and released *N*-acetylglucosaminidase activity was assayed at various pH's using the substrate 4-MU-*N*-acetylglucosaminide. Data are representative of two experiments performed in triplicate. Bars represent standard errors and are too small to be visible at some points.

mouse egg enzyme. Detergent extracts of mouse eggs were incubated with all three antisera individually, or normal serum, and immune complexes precipitated with anti-goat IgG-agarose. *N*-Acetylglucosaminidase activity was assayed in the precipitated pellets. Normal serum or antiserum to  $\alpha$



**Figure 5.** Antibodies to human  $\beta$ -hexosaminidase immunoprecipitate mouse egg *N*-acetylglucosaminidase activity. Detergent extracts of eggs were incubated at 4°C with 1/10 dilution of normal goat serum (NS) or goat antiserum to human  $\beta$ -hexosaminidase A ( $\alpha, \beta$  heterodimer), to  $\beta$ -hexosaminidase B ( $\beta, \beta$  homodimer) or to the purified  $\alpha$  subunit. The first two antisera recognize both forms of  $\beta$ -hexosaminidase, but antisera to  $\alpha$ -subunit recognizes only  $\beta$ -hexosaminidase A. Immune complexes were precipitated with anti-goat IgG coupled to agarose. Immunoprecipitates were assayed for *N*-acetylglucosaminidase activity by incubation with 1 mM 4-MU-*N*-acetylglucosaminide in citrate-phosphate buffer, pH 5. Results are expressed as a percentage of the activity immunoprecipitated by antisera to  $\beta$ -hexosaminidase B. Antisera to  $\beta$ -hexosaminidase A or B precipitated activity, but none was precipitated by normal sera or antisera to  $\alpha$  subunit. Therefore, antisera to human  $\beta$ -hexosaminidase cross-react with the mouse egg enzyme, which is primarily  $\beta$ -hexosaminidase B. Results are averages and bars indicate standard errors of three experiments.



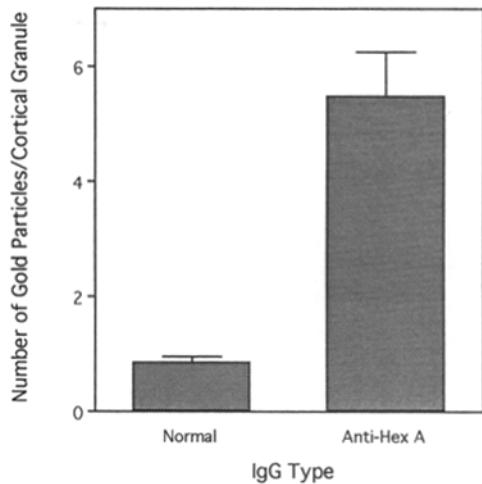
**Figure 6.**  $\beta$ -Hexosaminidase B is found in egg cortical granules. Sections of fixed and Lowicryl-embedded mouse eggs were incubated with normal goat serum or antisera to  $\beta$ -hexosaminidase A or B. Immune complexes were labeled by addition of anti-goat IgG coupled to colloidal gold (10 nm). **A** and **B** are at the same magnification; as are **C-E**. (**A**) Cortical region of unfertilized mouse egg immunostained with normal serum, representing the control for **D**. Four cortical granules are shown in the periphery of the egg. No specific staining is observed. (**B**) Cortical region of unfertilized mouse egg immunostained with normal IgG, representing the control for **E**. No specific stain-

subunit, which has been shown to precipitate  $\alpha$  subunit in other cells (Proia et al., 1984), did not precipitate activity (Fig. 5), corroborating the above observation that mouse eggs contain primarily  $\beta$ -hexosaminidase B (Fig. 3). However, antisera to  $\beta$ -hexosaminidase B and to  $\beta$ -hexosaminidase A, both of which recognize  $\beta$ -hexosaminidase A and B, precipitated mouse egg  $\beta$ -hexosaminidase activity (Fig. 5). This demonstrated that the antibodies to human  $\beta$ -hexosaminidase recognized the enzyme found in mouse eggs. Furthermore, since antibodies to the  $\alpha$  subunit (specific to  $\beta$ -hexosaminidase A) did not precipitate activity, these results verify that the primary egg isoform was  $\beta$ -hexosaminidase B.

These antibodies were used to localize  $\beta$ -hexosaminidase B in sections of mouse eggs using EM. Egg sections were stained with either normal serum, immune serum, or purified IgG made against  $\beta$ -hexosaminidase A or  $\beta$ -hexosaminidase B, both of which recognize both  $\beta$ -hexosaminidases because they share a common subunit. Antigen-antibody complexes were labeled by addition of anti-goat IgG coupled to colloidal gold. Gold particles were found in cortical vesicles in the cortex of the eggs stained with antibodies to  $\beta$ -hexosaminidase (Fig. 6, D and E). An average of ~6 particles per cortical granule was obtained using immune IgG (Fig. 7). Controls using normal serum or normal IgG showed no specific staining pattern (Figs. 6, A and B, and 7). Vesicles stained with antisera to  $\beta$ -hexosaminidase were identified as cortical granules since they were of similar diameter to cortical granules (200–400 nm), they varied in electron density, a characteristic of cortical granules, and they were located in the periphery of the eggs, as are cortical granules (Cran and Esper, 1990; Ducibella, 1991). Finally, these vesicles also stained with lens culinaris lectin (Fig. 6 C), which is indicative of cortical granules (Cherr et al., 1988; Ducibella et al., 1988).

#### Cortical Granule N-Acetylglucosaminidase Is Required for the Block to Sperm Binding

If N-acetylglucosaminidase activity is released at egg activation and is involved in the zona block to polyspermy, inhibiting this enzyme should result in polyspermic binding, thus preventing the block to polyspermy. To test this hypothesis, eggs were first treated with A23187 in the presence of PUGNAC, a competitive N-acetylglucosaminidase inhibitor or, as a control, PUGLU, a chemically related  $\beta$ -glucosidase inhibitor (Beer and Vasella, 1985). Control eggs treated with ionophore alone lost 85% of their ability to bind sperm, representing the zona block to sperm binding. However, this loss was significantly reduced if eggs were activated in the presence of PUGNAC (Fig. 8 A). This inhibition was not likely due to a contaminant or a nonspecific effect, since PUGLU, a structural homologue, had no effect on sperm binding activity. If eggs were activated in the presence of antiserum to  $\beta$ -hexosaminidase A or B, the loss in sperm bind-

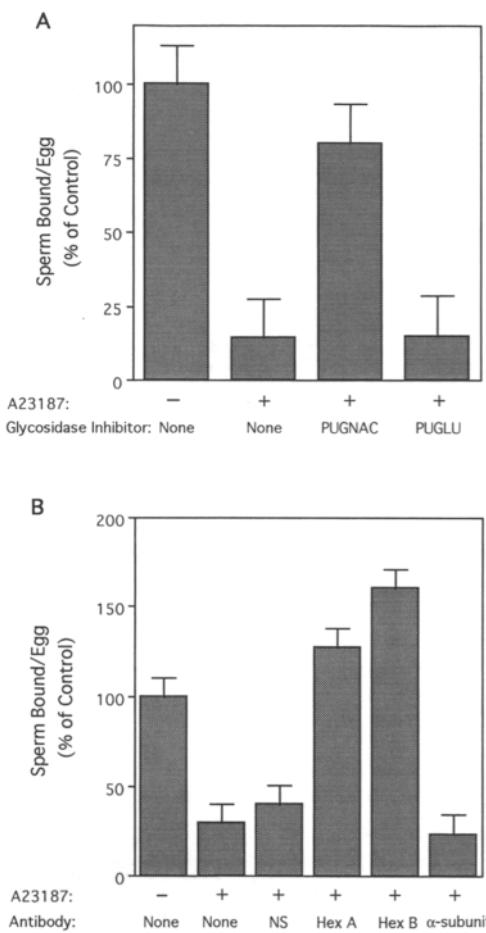


**Figure 7.** Quantitation of cortical granule labeling demonstrates specific immunostaining by  $\beta$ -hexosaminidase antibodies. Gold particles overlying cortical granules were counted in sections of eggs stained with anti- $\beta$ -hexosaminidase A IgG or normal IgG. Results are summaries of cortical granules from ~20 eggs for each IgG type. Approximately 120 cortical granules were counted for each IgG. Bars represent standard errors.

ing activity was completely inhibited, whereas normal serum or antiserum to  $\alpha$  subunit had no effect (Fig. 8 B). In fact, anti- $\beta$ -hexosaminidase allowed greater sperm binding than controls, which may result from inhibiting spontaneously released cortical granule N-acetylglucosaminidase. Regardless, these two experiments demonstrate that N-acetylglucosaminidase activity was required for formation of the zona block to polyspermic binding.

Because ZP3 is responsible for primary binding of acrosome-intact sperm,  $\beta$ -hexosaminidase was likely acting directly on ZP3. To determine if, in fact, PUGNAC was acting specifically to inhibit conversion of ZP3 to the inactive form, and that the eggs were being activated by ionophore in the presence of PUGNAC, we determined if ZP2 was converted to ZP2<sub>f</sub> (the form found in activated eggs) under the same conditions. At fertilization, ZP2 is cleaved by a cortical granule protease so that when its disulfide bonds are reduced, it migrates faster by SDS-PAGE, to about the same position as ZP3 (Moller and Wassarman, 1989). When eggs were activated by ionophore in the presence of PUGNAC, nearly all of ZP2 was converted to ZP2<sub>f</sub>, similar to the conversion in the presence of the control PUGLU (Fig. 9). Therefore, PUGNAC was specifically affecting only ZP3's conversion and did not affect the activation of eggs. PUGNAC's effect was to inhibit  $\beta$ -hexosaminidase which normally removes the GalTase-binding site from ZP3 (Miller et al., 1992). When released, cortical granule  $\beta$ -hexosaminidase B hydrolyzes the terminal N-acetylglucosamines on ZP3 recognized by sperm GalTase that are responsible for ini-

ting of any organelle is observed. (C) Cortical granules stained with biotinylated lens culinaris lectin, which binds to oligosaccharides present in cortical granules, and then with avidin-gold (15 nm particles). This lectin identifies cortical granules. (D) Cortical region of unfertilized mouse egg immunostained with antiserum to  $\beta$ -hexosaminidase B. Antiserum specifically stained 200–400 nm vesicles in the periphery of the egg. (E) Cortical region of unfertilized mouse egg immunostained with IgG to  $\beta$ -hexosaminidase A. Immunostaining was specific to cortical granules. Differences in immunostaining between D and E are insignificant and reflect normal variation within these preparations. Bars: (A and B) 500 nm; (C-E) 200 nm.

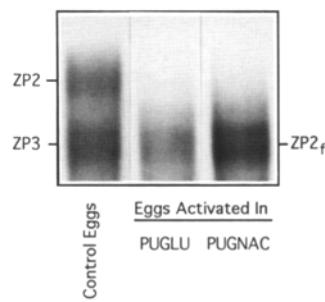


**Figure 8.** Inhibiting cortical granule *N*-acetylglucosaminidase prevents formation of the zona block to polyspermy. (A) Mouse eggs were activated by A23187 in the presence of the competitive *N*-acetylglucosaminidase inhibitor, PUGNAC, or the structurally homologous  $\beta$ -glucosidase inhibitor, PUGLU. Eggs were washed to remove the inhibitors and then incubated with  $10^6$  sperm/ml for 30 min in sperm-zona binding assays. After removing loosely adherent sperm, sperm remaining bound to the zona were counted. Data are summarized from four experiments performed in triplicate drops using a total of  $\sim 500$  eggs per treatment. Control eggs averaged 27.2 sperm bound/egg. Bars represent standard errors. (B) Mouse eggs were activated by A23187 in the presence of antisera to  $\beta$ -hexosaminidase A, B,  $\alpha$ -subunit or normal serum (NS). Eggs were washed to remove the antibodies and then incubated with sperm in sperm-zona binding assays, as above. Sperm bound to the zona were counted. Data are summarized from four experiments performed in triplicate drops using a total of about 500 eggs per treatment. Control eggs averaged 13.6 sperm bound/egg. Bars represent standard errors.

tial gamete binding, resulting in a zona pellucida that is unable to bind additional sperm.

## Discussion

These results demonstrate the presence of  $\beta$ -hexosaminidase B in cortical granules of mouse eggs and its requirement for the zona block to polyspermy at the level of sperm binding. This is the first identification of an enzyme localized to mammalian cortical granules that has been shown to affect the ability of the zona pellucida receptor, ZP3, to bind sperm.



**Figure 9.** *N*-Acetylglucosaminidase inhibitors do not affect egg activation or ZP2 proteolysis. Eggs were activated by ionophore in the presence of the *N*-acetylglucosaminidase inhibitor, PUGNAC, or the control  $\beta$ -glucosidase inhibitor, PUGLU. Control eggs were not activated by ionophore. Zonae pellucidae were mechanically isolated from these eggs,  $^{125}$ I-labeled, and separated by reducing SDS-PAGE. Under these conditions, ZP2 from activated eggs, which has been cleaved by a released protease, migrates to the same position as ZP3. These results show that glycosidase inhibitors did not nonspecifically inhibit conversion of zona pellucida glycoproteins during egg activation.

The enzyme was localized to cortical granules based on several observations. Immunoelectron microscopy demonstrated the presence of  $\beta$ -hexosaminidase in cortical granules of typical size, location, staining, and lectin-binding properties. The enzyme was released upon ionophore-induced or fertilization-induced egg activation, when cortical granules are exocytosed.  $\beta$ -Hexosaminidase B was shown to be responsible, at least in part, for the zona block to polyspermy because inhibiting this enzyme by antibodies or by competitive inhibitors prevented the loss of sperm binding activity normally seen in activated eggs.

The loss of sperm binding activity from the zona pellucida after fertilization is due to some subtle structural modification of ZP3, since ZP3 no longer has sperm binding activity although its migration is unaltered on SDS-PAGE (Wasserman, 1988; Miller et al., 1992); thus, gross proteolytic or glycosidase digestion seems unlikely. On the other hand, the inability of ZP3 to bind sperm after fertilization can be accounted for by the loss of its binding site for sperm GalTase (Miller et al., 1992). Since  $\beta$ -hexosaminidase digestion of ZP3 removes its GalTase-binding site (i.e., *N*-acetylglucosamine) and its sperm receptor activity, without altering its migration on SDS-PAGE (Miller et al., 1992), this enzyme fulfills several predictions of an enzyme participating in the zona block to polyspermy. It is found in cortical granules, it is released upon egg activation, it removes the sperm GalTase-binding site without significantly altering the molecular weight of ZP3, and its activity is required for the zona block to sperm binding.

Although the present study demonstrates the necessity for  $\beta$ -hexosaminidase B activity for the loss in sperm binding activity of the zona pellucida, there are other modifications in the zona after fertilization that likely contribute to the block to polyspermy. For example, a 21–34-kD protease activity released from eggs by A23187 (potentially in the cortical granules) has been shown to cleave ZP2 (Moller and Wasserman, 1989). Because ZP2 may serve as a secondary receptor for acrosome-reacted sperm (Bleil et al., 1988), this enzyme may be responsible for stopping penetration of zona-bound, acrosome-reacted sperm. However, the functional significance of ZP2 proteolysis remains unknown. The zona also becomes less susceptible to proteases, pH changes, and heat after fertilization (Shapiro et al., 1989), suggesting that other

proteases and cross-linking enzymes may be released or activated during the zona reaction (Gulyas and Schmell, 1980; Schmell and Gulyas, 1980; Moller and Wassarman, 1989). In this regard, there are several reports of protease and peroxidase activity released during the cortical reaction, but the function, location, zona substrate, and the identity of the enzymes have not been determined (Gwatkin et al., 1973; Gwatkin and Williams, 1974; Wolf and Hamada, 1977; Gulyas and Schmell, 1980). One function of these enzymes may be to prevent penetration of acrosome-reacted sperm through the zona, or they may harden the zona to make it more durable for later events in development (Kapur and Johnson, 1986; Moller and Wassarman, 1989). Finally, other workers have identified a 75-kD protein found in the cortical granules of mouse eggs that could conceivably be  $\beta$ -hexosaminidase B (Pierce et al., 1990), since  $\beta$ -hexosaminidase B is of similar molecular weight in mouse sperm (Miller et al., 1993). However, the identity and function of the 75-kD protein remain unknown.

$\beta$ -Hexosaminidase B found in mouse egg cortical granules seems to have a fairly specific substrate specificity. First, it has much greater activity towards terminal *N*-acetylglucosamine residues, compared to terminal *N*-acetylgalactosamine (Fig. 1). Most  $\beta$ -hexosaminidases have activity towards both substrates (Beeley, 1985). Second, it selectively removes terminal *N*-acetylglucosamine residues from ZP3 oligosaccharides that are recognized by sperm GalTase; it does not remove *N*-acetylglucosamine from other zona glycoproteins (Miller et al., 1992). The major isoform of  $\beta$ -hexosaminidase found in egg cortical granules is the homodimer B form, as determined by both substrate specificity and antibody reactivity. Apparently, eggs express very low levels of the  $\alpha$ -subunit before fertilization, since anti- $\alpha$  subunit antibodies were unable to immunoprecipitate activity from egg lysates, nor did egg lysates have any significant activity towards the sulfated substrate specific for  $\beta$ -hexosaminidase A. Sperm acrosomes also contain primarily  $\beta$ -hexosaminidase B (Miller et al., 1993). It is not apparent why both mouse gametes express primarily one isozymic form.

Like lysosomal  $\beta$ -hexosaminidases, cortical granule  $\beta$ -hexosaminidase B had a pH optimum near pH 5. Activity towards simple monosaccharide substrates was reduced (by ~80%) at pH 7.0, but still may be biologically significant. The pH optimum towards native ZP3 substrate is unknown, but interestingly, a rat sperm glycosidase has optimal activity towards glycoprotein substrates at pH 6.8 (Skudlarek et al., 1993). Thus, cortical granule  $\beta$ -hexosaminidase B may have sufficient affinity for, and activity towards, ZP3 at neutral pH. Alternatively, the pH of the perivitelline space may be sufficiently acidified during cortical granule exocytosis to enable  $\beta$ -hexosaminidase to retain high levels of activity.

Some evidence suggests that *N*-acetylglucosaminidase activity is found in eggs of other species including *Xenopus*. A cortical granule exudate from *Xenopus* eggs contains a 37–40-kD *N*-acetylglucosaminidase with different characteristics than an egg lysate *N*-acetylglucosaminidase activity (Greve et al., 1985). If *Xenopus* eggs are treated with *N*-acetylglucosaminidase, fertilization is blocked (Prody et al., 1985). Therefore *N*-acetylglucosaminidase may perform a function in *Xenopus* similar to its function in the mouse egg. It has been proposed that terminal *N*-acetylglucosamine residues are required for sperm binding to eggs of several

other animals including ascidians and swine (Berger et al., 1989; Lambert, 1989; Godknecht and Honegger, 1991), and an egg *N*-acetylglucosaminidase could also function to remove the sperm-binding site in those species. In ascidians, an unusual glycoprophatidylinositol-linked 180-kD *N*-acetylglucosaminidase is released from the surface of eggs (ascidians lack cortical granules), where it is thought to facilitate the block to polyspermy (Lambert, 1989; Lambert and Goode, 1992). It seems that *N*-acetylglucosaminidase may function across many species to generate the block to polyspermy, even though its substrate in the respective egg coats may differ significantly. That *N*-acetylglucosaminidase is the principal glycosidase in both mouse acrosomes (Miller et al., 1993) and mouse cortical granules (the present study) illustrates the need to carefully regulate the availability of terminal *N*-acetylglucosamine residues during mouse gamete interactions.

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