Wheat Germ Agglutinin Blocks the Acrosome Reaction in *Strongylocentrotus purpuratus* Sperm by Binding a 210,000-mol-wt Membrane Protein

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ABSTRACT Wheat germ agglutinin (WGA) binds to the entire surface of *Strongylocentrotus purpuratus* sperm, and inhibits the egg jelly-induced acrosome reaction. The binding was found to be species dependent and was completely inhibited by 5 mM *N*-acetyl-D-glucosamine. Blockage of the acrosome reaction by WGA was bypassed by a combination of the ionophores A23187 and monensin, although neither ionophore was effective individually. These experiments suggest that WGA blocks both Ca^{2+} uptake and Na^+/H^+ exchange in these sperm, which was confirmed by direct measurements of $^{45}Ca^{2+}$ uptake and H^+ efflux. The target of WGA in *S. purpuratus* sperm appears to be a membrane glycoprotein of $M_r = 210{,}000$. Treatment of this protein with neuraminidase or endo-β-*N*-acetylglucosaminidase F abolished WGA binding.

The acrosome reaction of sea urchin sperm, induced by a high molecular weight glycoprotein component of the egg jelly coat (8, 23, 24), must occur before sperm can fertilize eggs. The reaction includes exocytosis of the acrosome granule, formation of an acrosomal process, and a series of ion movements across the sperm plasma membrane. Within seconds after contact with egg jelly, sea urchin sperm show a net influx of Ca^{2+} and Na^+, a net efflux of H^+, depolarization of the membrane potential, and, after a brief delay, a net efflux of K^+ (10, 21, 22). The ionic changes associated with the acrosome reaction have been studied with ionophores, with channel-blocking drugs, and by manipulation of external ion concentrations; however, the mechanism by which sperm membrane components effect these ion movements remains unclear.

The acrosome reaction can be blocked by antibodies directed against certain sperm membrane components (4, 11, 19, 20). Polyclonal antibodies produced against the externally disposed *Strongylocentrotus purpuratus* sperm membrane proteins of $M_r = 210{,}000$ and 80,000 appear to inhibit both the Ca^{2+} uptake and Na^+/H^+ exchange that normally occur in response to egg jelly (19). Antigenic cross-reactivity of these two proteins has thus far impeded determination of their individual functions in the acrosome reaction; however, a method to resolve this problem is presented in this paper. Here, we show that the lectin wheat germ agglutinin (WGA)[1] also blocks the acrosome reaction. We examine the nature of WGA binding to *S. purpuratus* sperm, explore the mechanism by which it inhibits the acrosome reaction, and identify a membrane glycoprotein which may be responsible for the lectin's effect on sperm. A preliminary report of this work has appeared in abstract form (18).

MATERIALS AND METHODS

Gamete Isolation and Handling: Gametes of *S. purpuratus*, *Strongylocentrotus franciscanus*, *Lytechinus pictus*, and *Arbacia punctulata* were obtained by injecting the animals with 0.5 M KCl. Gametes were collected with a pasteur pipette ("dry") or in 0.45-μm-pore filtered seawater (MFSW). Artificial seawater (ASW), consisting of 480 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 27 mM $MgCl_2$, 29 mM $MgSO_4$, and 2 mM $NaHCO_3$ at pH 8.0, was used in some experiments. Sperm concentrations were determined spectrophotometrically by comparing A_{340} with a standardized curve constructed using a hemocytometer (26).

Egg jelly (19) was used to induce the acrosome reaction at a concentration of 3.0 nmol hexose/ml, as determined by the phenol-sulfuric acid assay, using fucose as a standard (3). Acrosome reactions were also induced with ionophores, as described previously (19). The percentage of acrosome-reacted sperm was determined by phase-contrast microscopy of sperm fixed in 5% glutaraldehyde

[1] *Abbreviations used in this paper:* ASW, artificial seawater; MFSW, 0.45-μm-pore filtered seawater; WGA, wheat germ agglutinin; endo F, endo-β-*N*-acetylglucosaminidase F.

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in ASW at × 1250 magnification, with the identity of the samples unknown to the observer. All reagents were from Sigma Chemical Co. (St. Louis, MO) except where indicated otherwise.

Localization of Lectin Binding: WGA was purified from raw wheat germ, purchased from a local health food store, by the method of Vretblad (27), lyophilized from distilled water, and stored dry at 5°C until use. Some batches of WGA were modified by succinylation (13) and/or conjugated to tetramethylrhodamine isothiocyanate (6). Lectin solutions were made fresh each day and stored at 0°C.

Sperm to be used in localization studies were fixed in ASW containing 3% paraformaldehyde and 0.1% glutaraldehyde and then washed and incubated in a blocking solution, as described previously (19). Fluorescent WGA or succinylated WGA was added at various concentrations and mixed with sperm in 0.1 M glycine in ASW for 3 h. In some cases, 0.1 M *N*-acetyl-D-glucosamine (GlcNAc) was included as a control. Unbound lectins were removed by three washes with ASW, and then sperm were observed with a fluorescence microscope (19).

Sperm Agglutination: Lectins to be tested for sperm agglutination were serially diluted twofold in 12 × 100 mm borosilicate tubes, each containing 1.0 ml of MFSW. In some experiments, the MFSW contained 0.1 M GlcNAc. Each tube received an additional 1.0 ml of sperm in MFSW, so that the final sperm concentration was 2.3×10^7 cells/ml in a final volume of 2.0 ml. The tubes were agitated at room temperature on a Lab-Line Junior Orbital shaker (Lab-Line Instruments, Inc., Melrose Park, IL) at 200 rpm for 45 min, then centrifuged in a Sorvall GLC-2B centrifuge (E. I. DuPont de Nemours & Co., Newtown, CT) for 4 min at 600 rpm. 1 ml of the supernatant was removed, and its A_{340} was recorded exactly 10 s after it was pipetted into the cuvette, to measure the number of sperm remaining in suspension (26).

WGA Binding to Sperm: WGA binding to sperm was measured directly using ^{125}I-labeled lectin. Immediately before use, 2.0 mg of WGA was dissolved in 1.0 ml of 150 mM NaCl, 10 mM Tris, pH 8.2, or MFSW, and labeled for 10 min at room temperature with 500 μCi of ^{125}I (carrier free, New England Nuclear, Boston, MA) and one iodobead (Pierce Chemical Co., Rockford, IL). Free ^{125}I was removed by passing the solution (without the bead) over a 1.4 × 7 cm column of coarse Sephadex G-25 (Pharmacia Inc., Piscataway, NJ) equilibrated in the same buffer. Various amounts of ^{125}I-labeled WGA were added to 0.4 ml of MFSW containing approximately 10^8 sperm and 10 mM sodium azide. The azide, which does not affect WGA-induced sperm agglutination, reversibly inhibits sperm motility (V. D. Vacquier, unpublished observations). Inhibition of motility greatly reduces the loss of sperm cells during washing procedures. The sperm were agitated for 10 min at room temperature with the ^{125}I-WGA, then pelleted by centrifugation for 10 min at 2,000 *g* in a Sorvall GLC-2B centrifuge. The supernatant was removed and the sperm were resuspended in 1.0 ml of MFSW, 10 mM sodium azide. This washing procedure was performed a total of four times, then the ^{125}I remaining with the sperm was counted in a gamma counter. The number of sperm present was determined spectrophotometrically at the end of the experiment (26). In some experiments, 0.1 M GlcNAc was included to determine the level of nonspecific ^{125}I binding to sperm. In other experiments, the pH of the MFSW was raised to 9.1 using NH_4OH.

Enzyme Digestions: Whole sperm (5×10^9 cells/ml) were incubated at room temperature in 0.2 mg/ml neuraminidase (Sigma No. N2876) or heat-inactivated neuraminidase (boiled 20 min), in MFSW adjusted to pH 6.5 with 5 mM PIPES buffer. After 1 h, the pH was returned to 8.0 with 0.1 N NaOH, and the cells were assayed for WGA binding. Sperm membrane vesicles (17) were suspended at a concentration of 0.5 mg protein/ml in 5 mM benzamidine, 100 mM sodium acetate, pH 5.0, and incubated at room temperature with 0.2 mg/ml neuraminidase or heat-inactivated neuraminidase for 15 min to 24 h. Some sperm membrane preparations were treated instead at room temperature with endo-β-*N*-acetylglucosaminidase F (endo F) (reference 5; a generous gift from Drs. J. H. Elder and S. Alexander, Scripps Clinic and Research Foundation) in 50 mM EDTA, 0.5% (wt/vol) Triton X-100, 100 mM sodium phosphate, pH 6.1. Enzyme digestions of isolated sperm membrane proteins were stopped by adding an equal volume of two-times concentrated Laemmli electrophoresis sample buffer (9) and heating to 100°C for 10 min. The products were analyzed on 6% polyacrylamide slab gels (9).

Ion Flux Measurements: Studies of H^+ efflux were performed in a 12°C room, using a Corning model 12 pH meter (Corning Medical and Scientific, Corning Glass Works, Medfield, MA) coupled to a Varian model 9176 chart recorder (Varian Associates, Inc., Instrument Group, Palo Alto, CA). All solutions were adjusted with 0.1 N NaOH or 0.1 N HCl to pH 8.0 at 12°C except for egg jelly, which was pH 7.9. The ASW used for pH experiments contained 1 mM 2-(*N*-morpholino)ethane sulfonic acid instead of $NaHCO_3$. 20 μl of "dry" sperm was mixed with 80 μl of ASW with or without acrosome reaction inhibitors, then incubated for 5 min at 0°C. This mixture was added to 4.7 ml of ASW in a10-ml beaker and dispersed with a 1 × 5 mm magnetic stir bar during pH measurement. 100 μl of egg jelly (final concentration; 3 nmol hexose/ml) was added when the pH reached 7.9. Sperm concentration ($1–3 \times 10^8$ sperm/ml) and the percentage of acrosome-reacted cells were determined at the end of each experiment. Moles of acid released were calculated by back-titrating the reaction mixture with 0.01 N NaOH. Calcium uptake was measured by filtration with $^{45}Ca^{2+}$ using the method of Schackmann and Shapiro (22).

Identification of WGA Binding Proteins: Sperm membrane vesicles were prepared from *S. purpuratus* sperm as described (17), then electrophoresed on 7% SDS polyacrylamide slab gels (9). Some lanes were cut out and stained with silver (14) or 1% Coomassie Blue-R in 50% methanol, 10% acetic acid. Other lanes were transferred to nitrocellulose paper (BA-85, Schleicher and Schuell, Keene, NH) with a Bio-Rad Trans-Blot apparatus (Bio-Rad Laboratories, Richmond, CA) for 14–16 h at 3.5 V/cm, in 20 mM Tris, 150 mM glycine, 20% methanol, pH 8.3. Nitrocellulose blots were either stained with Coomassie Blue for 10 s and destained in 50% methanol, and 10% acetic acid, or incubated for 2 h at room temperature in 1% periodate-treated BSA (7) dissolved in 150 mM NaCl, 10 mM Tris, pH 8.2.

WGA was labeled with ^{125}I as described above. 0.8 mg of ^{125}I-labeled WGA was added to each blot, along with 10 ml of 0.5 M NaCl, 10 mM Tris, pH 8.2, 1 mM EDTA, 0.5% (wt/vol) Polydet P-40 (Polysciences, Inc., Warrington, PA), 0.1% SDS, 0.05% (wt/vol) Tween 20, and 1.0 mg/ml periodate-treated BSA (buffer A). Some samples also contained 40 mM GlcNAc. The blots were allowed to react for 3 h at 5°C, then unbound WGA was removed by two 20-min washes in 50 ml of buffer A, followed by two 1-h washes in 1.0 liter of 0.5 M NaCl, 10 mM Tris, pH 8.2. The blots were dried and placed on Kodak XAR-5 x-ray film.

RESULTS

WGA Binding to Sperm

By fluorescence microscopy, WGA appeared to bind the entire surface of fixed *S. purpuratus* sperm, and this binding was completely inhibited by 0.1 M GlcNAc (Fig. 1). Agglutination of sea urchin sperm by WGA was species dependent (Fig. 2). Of the four species tested, *S. purpuratus* was most easily agglutinated (50% agglutination at 130 μg/ml) followed by close relative *S. franciscanus* (50% agglutination at 260 μg/ml). *A. punctulata* sperm are only partially agglutinated at much higher WGA concentrations, and *L. pictus* sperm failed to show any agglutination with WGA.

The agglutination of *S. purpuratus* sperm was completely inhibited by GlcNAc, a sugar that avidly binds to WGA (Fig. 3; 50% inhibition at 0.8 mM). The sugar derivatives *N*-acetyl-D-galactosamine (50% inhibition at 6 mM) and *N*-acetylneuraminic acid (50% inhibition at 5 mM) were six to seven times less effective at blocking agglutination. WGA did not inhibit sperm motility, observed by light microscopy, until the cells became so densely agglutinated that mobility was impossible.

WGA is known to react strongly with various sialic acid derivatives as well as GlcNAc (1, 15, 16, 28). Experiments were conducted to determine whether or not WGA binds to sialic acid residues on sea urchin sperm. Succinylated WGA, which does not bind sialic acids but retains GlcNAc affinity (13), does not agglutinate *S. purpuratus* sperm. Treatment of sperm with neuraminidase for 1 h at pH 6.5 and 23°C caused only a slight decrease in ^{125}I-WGA binding to sperm (Fig. 4); however, optimal conditions for neuraminidase activity (pH 5.0, 37°C) were radically different from those required to maintain healthy *S. purpuratus* sperm (pH 8.0, 5–15°C). The conditions chosen as a compromise (pH 6.5, 23°C) probably do not allow complete enzymic digestion to occur. The sperm did not remain viable under these conditions for >1 h. Standard chemical techniques for removing sialic acid cannot be used on intact sperm, because the cells die in the presence of 10 mM periodate or at pH levels <5.0 (Podell, S., unpublished data).

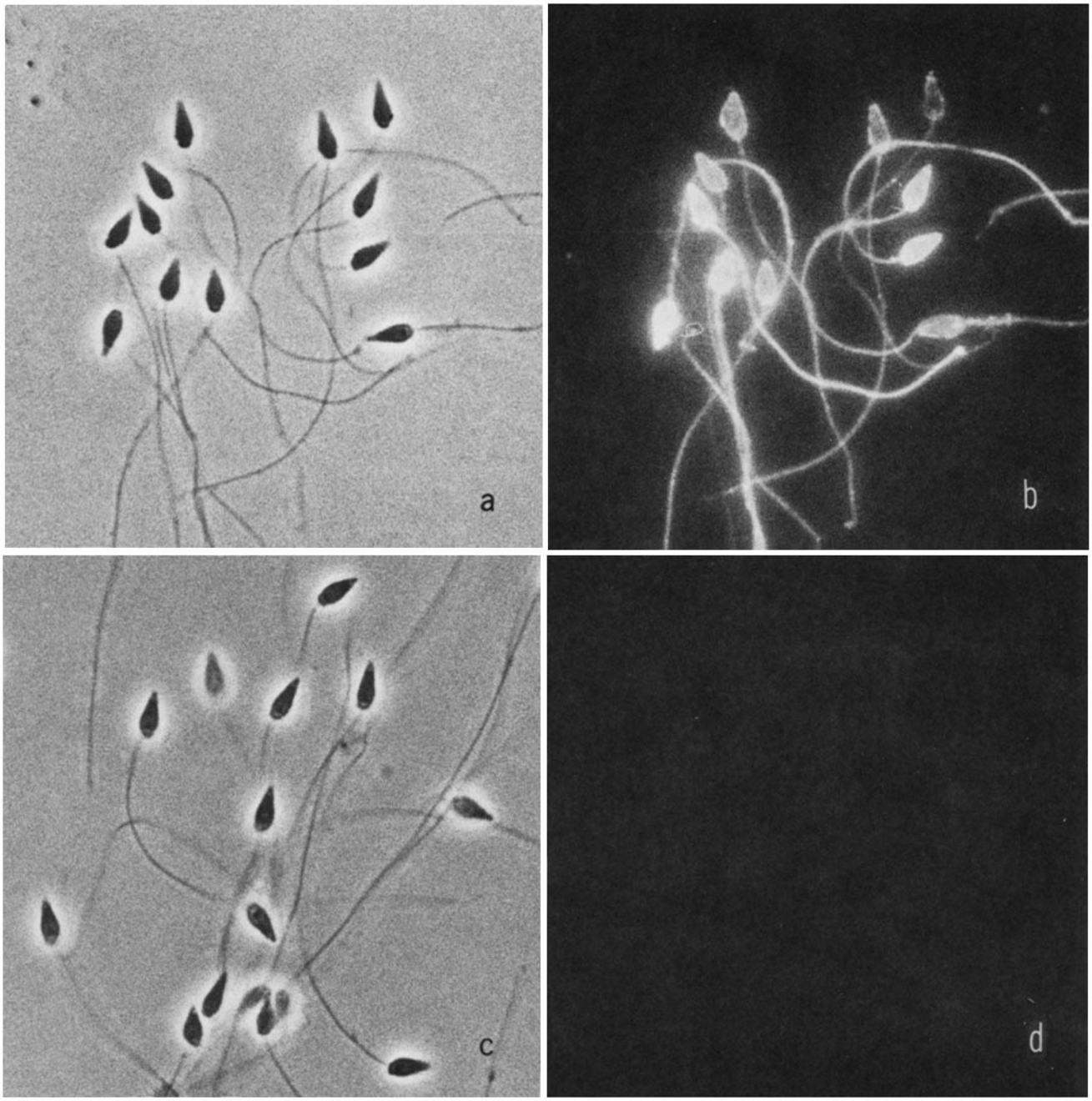

FIGURE 1 WGA binding to *S. purpuratus* sperm. Panels at left are phase-contrast, and those at right are corresponding fluorescence micrographs at approximately × 2,300 magnification. WGA is 32 μg/ml in *a* and *b*; 0.1 M GlcNAc is present along with WGA in *c* and *d*.

Effects of WGA on the Acrosome Reaction

WGA was found to be a potent inhibitor of the egg jelly–induced sperm acrosome reaction in *S. purpuratus*, even at concentrations too low to cause agglutination (Fig. 5). The inhibition did not occur in the presence of 5 mM GlcNAc or 2.5 mg/ml sialopeptides derived from hog gut mucin (12). Sialic acid binding may play a role in this phenomenon, since succinylated WGA had very low inhibitory potency (Fig. 5). It is not possible to directly test the role of sialic acid binding on WGA-mediated inhibition of the acrosome reaction, because the low pH and high temperature required to completely remove sialic acid from the cell surface render *S. purpuratus* sperm unable to acrosome react.

To study the effects of WGA on ion movements associated with the acrosome reaction, we induced the reaction by various artificial means (Fig. 6 and Table I). The effects of WGA are partially bypassed by nigericin, and WGA does not significantly inhibit the acrosome reaction induced by NH_4OH (25), proving that the sperm are still capable of reacting. WGA binding to sperm was not substantially reduced by NH_4OH (Fig. 4). Adding monensin to egg jelly, to promote Na^+/H^+ exchange, did not restore the acrosome reaction in the presence of WGA (Table I). WGA also blocked the acrosome reactions normally induced by the ionophore A23187; however, when A23187 was combined with monensin, the acrosome reaction occurred. This result was the same whether the ionophores were added simultaneously or se-

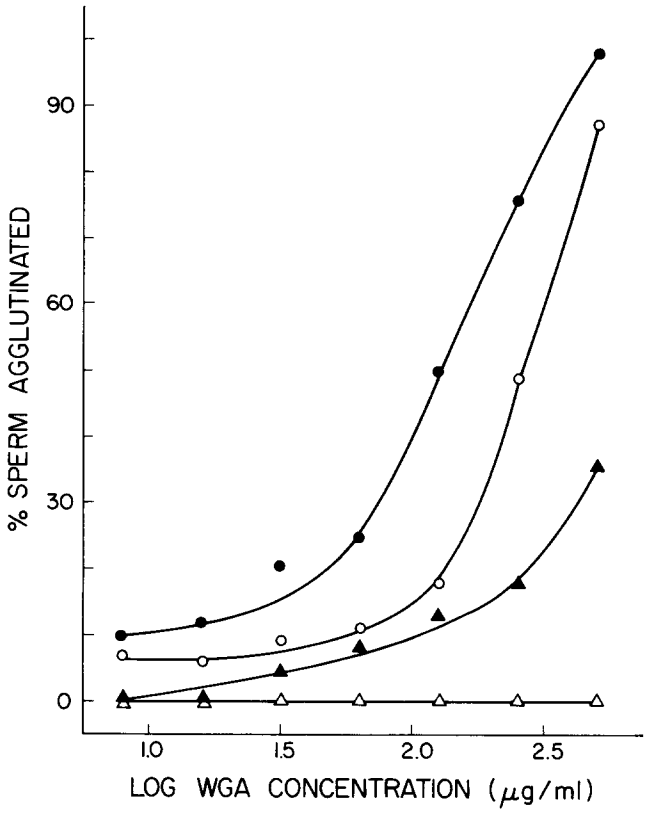

FIGURE 2 Comparison of WGA agglutination in the following sea urchin species: *S. purpuratus* (●); *S. franciscanus* (○); *A. punctulata* (▲); *L. pictus* (△).

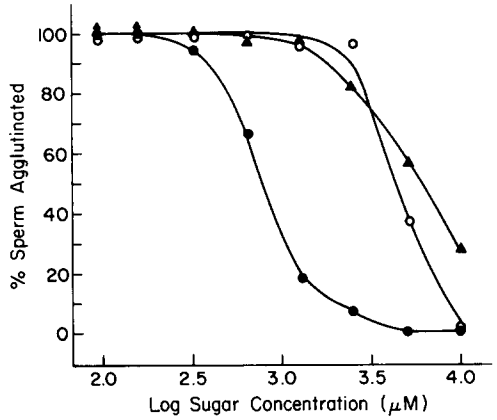

FIGURE 3 Inhibition of WGA-induced *S. purpuratus* sperm agglutination by (●) GlcNAc, (▲) *N*-acetyl-D-galactosamine, and (○) *N*-acetylneuraminic acid.

quentially, in any order, within a 2-min period. The requirement for both ionophores suggests that WGA either blocks both Ca^{2+} uptake and Na^+/H^+ exchange mechanisms individually or inhibits a common regulator of these ion fluxes. Only partial inhibition of the nigericin-induced acrosome reaction was observed.

Ion Flux Measurements

Direct measurements of egg jelly–induced H^+ efflux and Ca^{2+} influx in *S. purpuratus* sperm showed that both responses were blocked by the presence of 40 μg/ml WGA (Figs. 7 and 8). In each case, the block occurs quite rapidly (within 5 min at 0°C) at a WGA concentration that agglutinates <10% of the cells. The WGA-treated sperm showed normal motility when observed in the light microscope, and their baseline level of H^+ efflux did not differ significantly from untreated cells, suggesting normal metabolic activity. WGA had no effect on net sperm H^+ or Ca^{2+} ion fluxes in the presence of 0.1 M GlcNAc.

Identification of the WGA Receptor

When sperm membrane proteins were separated electrophoretically, transferred to nitrocellulose paper, and exposed to ^{125}I-WGA, a single membrane protein of M_r = 210,000 became labeled (Fig. 9). Although this interaction was strong enough to occur in a high ionic strength, detergent-containing medium, it was completely inhibited by 40 mM GlcNAc. The same result is obtained when the interaction was analyzed by immunoprecipitation instead of protein blotting (data not shown). After treatment with neuraminidase at pH 5.0, the M_r = 210,000 protein showed increased electrophoretic mobility (Fig. 10), which suggests that sialic acid residues have been removed. A similar mobility shift occurred after diges-

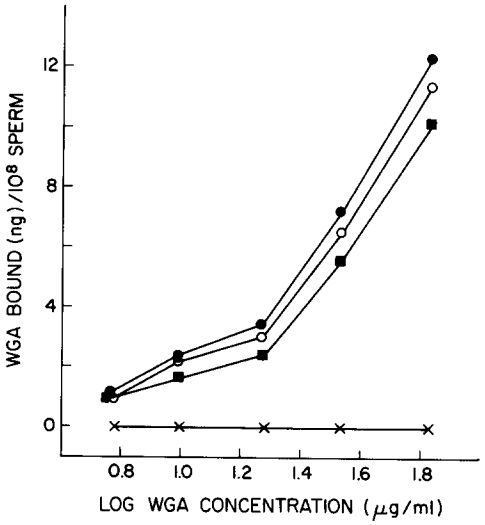

FIGURE 4 Binding of ^{125}I-labeled WGA to *S. purpuratus* sperm. Assay conditions were as follows: ●, control sperm; ×, control sperm in the presence of 0.1 M GlcNAc; ■, sperm assayed at pH 9.1; ○, sperm pretreated with 0.2 mg/ml neuraminidase at pH 6.5 for 1 h.

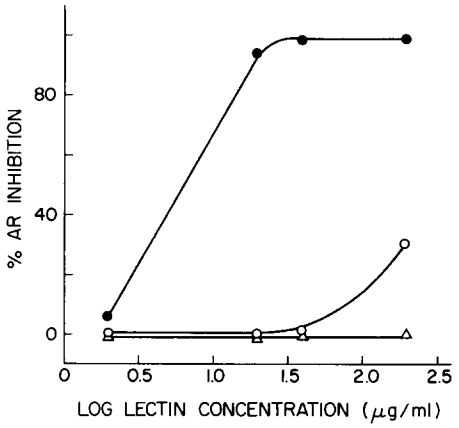

FIGURE 5 Effect of WGA (●); succinylated WGA (○) and WGA in the presence of 0.1 M GlcNAc (△) on the egg jelly-induced *S. purpuratus* acrosome reaction.

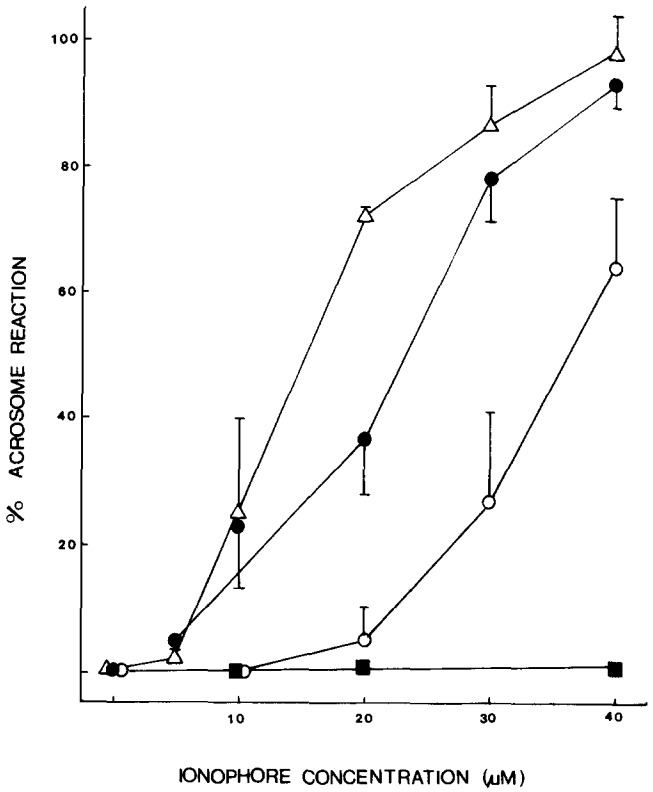

FIGURE 6 Ionophore-induced acrosome reactions in *S. purpuratus* sperm. Symbols indicate the following ionophores: nigericin (△); A23187 (○); monensin (■); A23187 plus monensin (●). Each pont represents the average of three experiments with different sperm batches.

TABLE I

Effect of WGA on Artificially-induced Sperm Acrosome Reactions

AR inducer	AR		Inhibition of AR
	WGA	WGA + GlcNAc	
	%		%
Egg jelly	1	98	99
Monensin (40 μM) + jelly	0	91	100
A23187 (38 μM)	12	83	86
Monensin + A23187	96	96	0
Nigericin (40 μM)	59	100	41
NH_4OH (pH 9.1)	87	96	9
None	0	0	—

Data is average of two experiments with different sperm batches. Concentration of WGA is 40 μg/ml, and GlcNAc is 0.1 M. *AR*, acrosome reaction.

tion with endo F, indicating that the sialic acids may be attached to N-linked carbohydrate chains. When assayed by protein blotting, WGA had no detectable affinity for the M_r = 210,000 protein after treatment with either neuraminidase or endo F.

DISCUSSION

WGA adheres to the entire surface of *S. purpuratus* sperm, with higher affinity than to sperm of *S. franciscanus*, *A. punctulata*, or *L. pictus*. This variability in lectin binding among sea urchin sperm could be expected if cell surface glycoproteins are involved in a species-specific recognition process such as the egg jelly-induced acrosome reaction. Inhibition of the acrosome reaction by WGA suggests that the lectin binds at or near a site critical for the initiation of the reaction.

The sugar derivative on *S. purpuratus* sperm most probably responsible for WGA binding is sialic acid, as shown by agglutination and acrosome reaction studies with succinylated

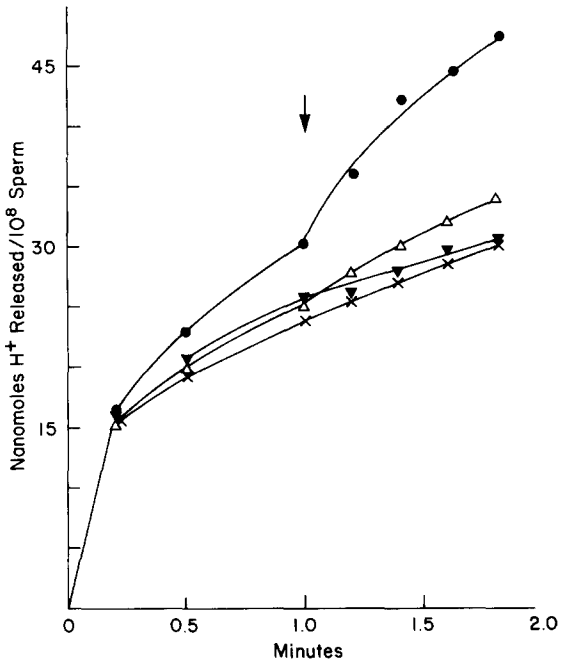

FIGURE 7 Effect of WGA on egg jelly-induced H^+ efflux. Symbols indicate *S. purpuratus* sperm treatment as follows: ●, addition of egg jelly at time indicated by arrow; ▼, 40 μg/ml WGA, then egg jelly at time indicated by arrow; △, 40 μg WGA (no jelly); ×, no treatment at all.

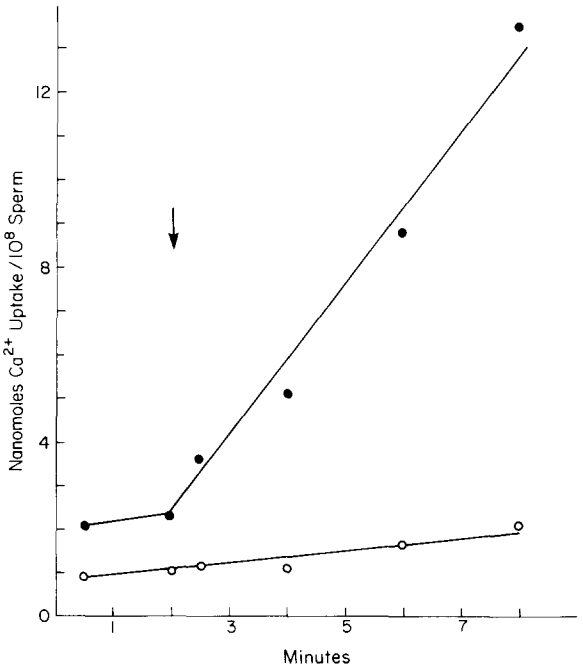

FIGURE 8 Effect of WGA on egg jelly-induced $^{45}Ca^{2+}$ uptake. Symbols indicate *S. purpuratus* sperm treatment with the following: ○, 40 μg/ml WGA; ●, 40 μg/ml WGA plus 0.1 M GlcNAc. Arrow indicates time at which egg jelly was added.

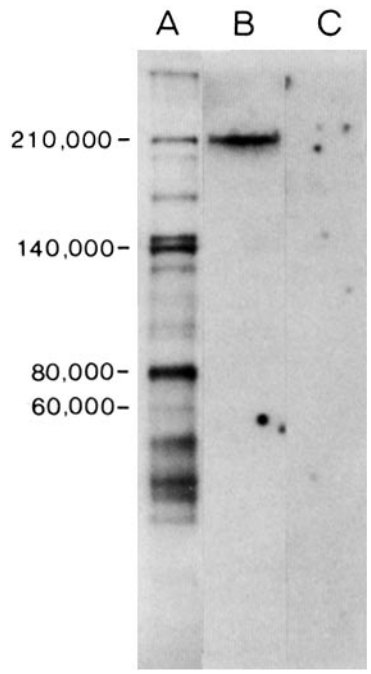

FIGURE 9 ^{125}I-WGA binding to *S. purpuratus* sperm membrane proteins. (*A*) Silver-stained 7% polyacrylamide gel with 2.5 μg of sperm membrane proteins loaded; (*B*) autoradiogram of duplicate lane transferred to nitrocellulose and incubated with ^{125}I-WGA. (*C*) autoradiogram of duplicate blot, incubated with 40 mM GlcNAc in addition to ^{125}I-WGA.

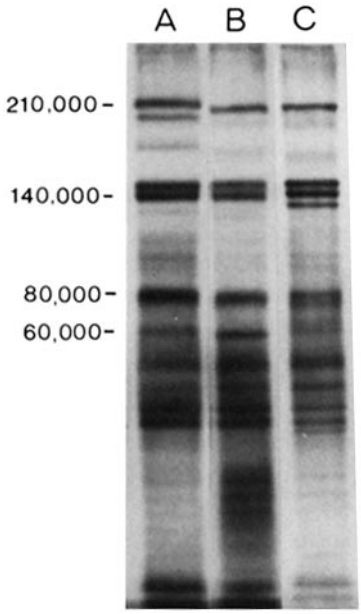

FIGURE 10 Silver-stained 7% polyacrylamide gel, showing *S. purpuratus* sperm membrane proteins treated for 24 h with (*A*) 100 mM sodium acetate; pH 5.0; (*B*) neuraminidase; (*C*) endo F. Protein loaded was 2.5 μg per lane.

WGA, as well as neuraminidase digestion of isolated sperm membrane proteins. Very low concentrations of GlcNAc (<5 mM) were observed to inhibit the effects of WGA on sperm. This result can be attributed to the strong affinity of the lectin for GlcNac, which is much greater than for sialic acid (16), rather than to similarity between the competing sugar and carbohydrates at the sperm surface.

How does WGA block the acrosome reaction? Studies with monensin and A23187 (Table I) suggest that the lectin interferes with both Na^+/H^+ exchange and Ca^{2+} influx. Direct measurements of external pH and $^{45}Ca^{2+}$ uptake support this conclusion, although the $^{45}Ca^{2+}$ filtration assay used could not detect very small, rapid, reversible ion fluxes. To block ionophore-induced acrosome reactions, the lectin could sterically impede or cross-link membrane proteins that normally move laterally, or allosterically inhibit a necessary conformational change in a regulatory glycoprotein. WGA could also prevent jelly binding to sperm, but this is difficult to test because of nonspecific WGA-jelly interaction. On nitrocellulose blots, WGA appeared to bind egg jelly (data not shown), but this interaction was not inhibited by 0.1 M GlcNAc, twenty times the sugar concentration that abolishes WGA inhibition of the acrosome reaction.

We have identified an M_r = 210,000 glycoprotein as the *S. purpuratus* sperm membrane component that most avidly binds WGA. We cannot rule out the possibility of minor, low-affinity interactions of the lectin with other membrane components under the mild conditions necessary to maintain sperm viability; however, the 210,000-mol-wt protein represents an attractive subject for further study. Previous reports have identified this protein as one of four *S. purpuratus* sperm proteins accessible to ^{125}I labeling in intact sperm (2, 17), and it has been shown that antibodies directed against it block the *S. purpuratus* acrosome reaction (19). Further characterization of the protein should yield new insights into the mechanisms that regulate ionic fluxes associated with the sperm acrosome reaction.

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