

Lewis X-Containing Neoglycoproteins Mimic the Intrinsic Ability of Zona Pellucida Glycoprotein ZP3 to Induce the Acrosome Reaction in Capacitated Mouse Sperm¹

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ABSTRACT

The binding of zona pellucida (ZP) glycoprotein ZP3 to mouse sperm surface receptors is mediated by protein-carbohydrate interactions. Subsequently, ZP3 induces sperm to undergo the acrosome reaction, an obligatory step in fertilization. We have previously identified Lewis X (Le^x; Galβ4[Fucα3]GlcNAc) as a potent inhibitor of *in vitro* sperm-ZP binding (Johnston et al. *J Biol Chem* 1998; 273:1888–1895). This glycan is recognized by ~70% of the ZP3 binding sites on capacitated, acrosome-intact mouse sperm, whereas Lewis A (Le^a; Galβ3[Fucα4]GlcNAc) is recognized by most of the remaining sites (Kerr et al. *Biol Reprod* 2004; 71:770–777). Herein, we test the hypothesis that Le^x- and Le^a-containing glycans, when clustered on a neoglycoprotein, bind ZP3 receptors on sperm and induce sperm to undergo the acrosome reaction via the same signaling pathways as ZP3. Results show that a Le^x-containing neoglycoprotein induced the acrosome reaction in a dose-dependent and capacitation-dependent manner. A Le^a-containing neoglycoprotein also induced sperm to undergo the acrosome reaction but was less potent than Le^x-containing neoglycoproteins. In contrast, neoglycoproteins containing β4-lactosamine (Galβ4GlcNAc), Lewis B (Fucα2Galβ3[Fucα4]GlcNAc), and sialyl-Le^x glycans were inactive, as were four other neoglycoproteins with different nonfucosylated glycans. Consistent with these results, unconjugated Le^x- and Le^a-capped glycans were dose-dependent inhibitors, which at saturation, reduced the ZP-induced acrosome reaction by about 60% and 30%, respectively. Experiments utilizing pharmacological inhibitors suggest that induction of the acrosome reaction by solubilized ZP and Le^x- and Le^a-containing neoglycoproteins require the same calcium-dependent pathway. However, only the ZP-induced acrosome reaction requires a functional G_i protein. Thus, Le^x-containing neoglycoproteins bind to a major class of ZP3 receptors on capacitated sperm. A Le^a-containing neoglycoprotein binds a second ZP3 receptor but is a less-potent inducer of the acrosome reaction.

acrosome reaction, fertilization, gamete biology, sperm

¹This work was supported by National Institute for Child Health and Human Development (NICHD) 1 R01 HD-35699 and by the Johns Hopkins University Population Center (P30-HD-06268). W.F.H. and C.L.K. were supported in part by NICHD 5T32 HD-07276.

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Received: 30 September 2003.

First decision: 21 October 2003.

Accepted: 9 April 2004.

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ISSN: 0006-3363. <http://www.biolreprod.org>

INTRODUCTION

In the mouse, fertilization is characterized by a sequence of steps initiated by adhesion of acrosome-intact sperm to the zona pellucida (ZP) and ends with sperm-egg fusion and egg activation [1]. The ZP is composed of three glycoproteins (ZP1, ZP2, and ZP3) that form the extracellular matrix surrounding the egg [2]. There is considerable evidence that the adhesion of sperm to the ZP involves the interaction of specific sperm surface molecules with ZP3. Current models posit that *O*-linked oligosaccharides are essential for this interaction [2]. Following adhesion, the binding of ZP3 to sperm-surface receptors stimulates a series of intracellular second messengers. In response, the plasma membrane and underlying outer acrosome membrane are vesiculated and shed along with the contents of the acrosome. This process, called the acrosome reaction, is required for sperm to penetrate the ZP and reach the egg plasma membrane. Currently, it is assumed but not proven that the *O*-linked glycans on ZP3 and complementary sperm surface molecules proposed to mediate sperm-ZP adhesion are also the functional ligands and receptors that mediate the acrosome reaction. Additionally, the structure of one or more sperm-binding, *O*-linked glycans, and the identity of their complementary sperm surface receptors remain open questions.

Several steps in the fertilization cascade can be replicated by *in vitro* assays. We and others have used a competitive, *in vitro* sperm-ZP binding assay to identify glycans that inhibit the binding of sperm to ZP-enclosed eggs [3, 4]. The prevailing interpretation is that glycans that effectively inhibit this sperm-ZP binding mimic the structures of authentic sperm-binding glycans on ZP3. Using this approach, we demonstrated that a Lewis X (Le^x; Galβ4[Fucα3]GlcNAc)-containing glycan was a high-affinity (IC₅₀ ~0.5 μM), competitive inhibitor of sperm-ZP binding. Consistent with those results, recent experiments indicated that Le^x-Lactose (Lac) and Le^x are recognized by ~70% of the ZP3 binding sites on capacitated, acrosome-intact mouse sperm [5]. Those experiments also identified a second class of ZP3 binding sites that recognize Lewis A (Le^a; Galβ3[Fucα4]GlcNAc), which is an isomer of Le^x. However, those experiments did not identify the biological functions of these two classes of sites. It is possible that Le^x, Le^a, or both bind to receptors that mediate the ZP3-induced acrosome reaction. Alternatively, these binding sites may act only as ZP adhesion molecules. ZP3 would, therefore, trigger the acrosome reaction by binding to a second set of sperm surface molecules. In this alternative scenario, sperm-ZP3 interactions would be reminiscent of the interactions of lymphocytes and the vascular endothel-

	Abbreviation	Structure	Glycans/ BSA	Manufacturer & Catalog No.
i	Glc ₄ -BSA	Glc _α 6Glc _α 4Glc _α 4Glc-3C-BSA	15	Sigma C-0539
ii	Le ^x -Lac-BSA	Galβ4[Fucα3]GlcNAcβ3Galβ4Glc-3C-BSA	19	Dextra NGP0502
iii	Le ^x -BSA	Galβ4[Fucα3]GlcNAc-3C-BSA	11	Dextra NGP0302
iv	αGal-Lac-BSA	Galα3Galβ4GlcNAcβ3Galβ4Glc-3C-BSA	8.8	Dextra NGP0537
v	GlcNAc-BSA	GlcNAc-14C-BSA	9.5	Dextra NGP1101
vi	βGal-BSA	Galβ4GlcNAc-3C-BSA	11	Dextra NGP0201
vii	Le ^a -Lac-BSA	Galβ3[Fucα4]GlcNAcβ3Galβ4Glc-3C-BSA	6.8	Dextra NGP0501
viii	Le ^b -Lac-BSA	Fucα2Galβ3[Fucα4]GlcNAcβ3Galβ4Glc-3C-BSA	7.5	Dextra NGP0601
ix	SLe ^x -BSA	Neu5Acα3Galβ4[Fucα3]GlcNAc-3C-BSA	7.6	Dextra NGP0403
x	SβGal-BSA	Neu5Acα3Galβ4GlcNAc-3C-BSA	10.5	Dextra NGP0301

FIG. 1. Neoglycoproteins used in this study and tested for their ability to induce the acrosome reaction. The oligosaccharides are coupled to lysine residues on the BSA via alkyl spacers. Abbreviations that include *Lac* refer to the presence of an extra lactose at the reducing termini of the neoglycoprotein's oligosaccharides. The average levels of substitution (moles of glycan per mole of BSA) are supplied for all neoglycoproteins tested and were determined by matrix-assisted laser/desorption ionization, time-of-flight (MALDI-TOF) mass spectrometry by the manufacturer.

lium. A series of distinct and specific cellular interactions are required for lymphocytes to bind and exit blood vessels. One interaction requires the binding of cell surface carbohydrates to complementary sites on the opposing cell surface (reviewed in [6]).

The primary purpose of the experiments described herein was to test the hypothesis that the two classes of ZP3 binding sites, which recognize Le^x or Le^a, are functional receptors. To this end, we examine whether Le^x- or Le^a-containing neoglycoproteins induce capacitated mouse sperm to undergo the acrosome reaction. Additionally, by use of neoglycoproteins derivatized with other glycans, we examined whether this response of sperm was specific for Le^x and Le^a. Neoglycoproteins, rather than unconjugated glycans, were used because the induction of the acrosome reaction requires multivalency of functional oligosaccharides on ZP [7]. Additionally, to further test whether Le^x-Lac and Le^a-Lac bound to ZP3 receptors, we examined whether these unconjugated glycans were dose-dependent inhibitors of the ZP-induced acrosome reaction. Finally, by use of specific pharmacological inhibitors, we examined whether the neoglycoprotein-induced acrosome reaction requires the same signal transduction pathways in sperm as the ZP-induced acrosome reaction [8].

MATERIALS AND METHODS

Culture Media

For most experiments, the culture medium used was Medium 199 (M199; Gibco, Gaithersburg, MD), containing 0.4% crystalline BSA (Gibco) and 273 μM sodium pyruvate (M199-CM). Incubations of sperm in this medium were conducted at 37°C in 5% CO₂. For experiments requiring the use of uncapacitated sperm, a modified Medium 199 (M199-NCM) was used. M199-NCM was prepared from sodium bicarbonate-free Medium 199, and contained 0.1% polyvinyl alcohol, 25 mM Hepes (pH 7.4), 26 mM sodium chloride, 0.01% BSA, and 273 μM sodium pyruvate. Incubations performed in M199-NCM were carried out at 37°C in air. In experiments that used pertussis toxin to block the acrosome reaction, M199-CM was supplemented with either 100 ng/ml pertussis toxin (Calbiochem, La Jolla, CA) or vehicle (50 mM sodium chloride, 10 mM sodium phosphate, pH 7.0).

Neoglycoproteins and Oligosaccharides

All neoglycoproteins were BSA-based and were purchased from either Sigma (St. Louis, MO) or Dextra Labs (Reading, U.K.). The oligosaccharide structures, levels of substitution, and suppliers are summarized in Figure 1. Neoglycoproteins were solubilized in distilled water and stored as 0.1% stock solutions at -20°C prior to use. The neoglycoprotein Glc₄-BSA was used as a negative control in all experiments. The concentrations of neoglycoproteins are presented as micromoles of polypeptide backbone rather than micromoles of oligosaccharide. The unconjugated oligosaccharides βGal-Lac (Galβ4GlcNAcβ3Galβ4Glc; Calbiochem), Le^x-Lac (Galβ4[Fucα3]GlcNAcβ3Galβ4Glc; Dextra Labs) and Le^a-Lac (Galβ3[Fucα4]GlcNAcβ3Galβ4Glc; Dextra Labs) were used as competitive inhibitors of the ZP-induced acrosome reaction. These oligosaccharides were stored as stock solutions in distilled water at a concentration of 2.2 mM at -20°C.

Preparation of ZP

Zonae pellucidae were isolated from frozen ovaries of 6- to 7-wk-old ICR mice (Harlan BioProducts, Indianapolis, IN) on Percoll (Amersham-Pharmacia, Piscataway, NJ) density gradients as described previously [9]. ZP fragments were suspended in distilled water and solubilized by heating for 30 min at 60°C. The concentration of solubilized ZP was estimated using the QuantiGold protein assay (Diversified Biotech, Boston, MA) and is expressed in terms of ZP equivalents per microliter, based on the calculation that one ZP contains 3 ng of protein [10]. Samples of solubilized ZP were fractionated by SDS-PAGE and silver-stained to assess purity. Solubilized ZP were dispensed into single-use aliquots and stored at -80°C until used.

Isolation of Sperm

Sperm were isolated from CD-1 retired breeders with minor modifications to previously described methods [11]. Briefly, the caudae epididymides of three mice were placed in 0.5 ml of medium and minced. The sperm were allowed to swim out of the tissue for 15 min, at which time the tissue fragments were removed. The sperm suspension was then pipetted to the bottom of a polypropylene culture tube (12 × 75 mm) containing 1.25 ml of medium and incubated for 45 min. During this time, motile sperm swam into the upper portion of the tube. Once the incubation was complete, the top quarter (~440 μl) of the medium in the culture tube was removed, placed in a culture dish, and covered with medium-washed mineral oil (Sigma). The sperm that were contained in this fraction were counted on a hemacytometer and were incubated immediately with test substances. Sperm suspensions that contained fewer than 60 000 sperm per milliliter (after swim-up) were discarded. Typically, ideal sperm preparations isolated using this protocol yield 60 000–90 000 sperm per milliliter. The use of animals for these experiments was approved by the Animal Care and Use Committee of the Johns Hopkins University Bloomberg School of Public Health.

Incubations with ZP and Neoglycoproteins

All incubations were carried out in 25 μl in microcentrifuge tubes (0.5 ml). Test substances (dissolved in water) were added to an equal volume of 2× medium and diluted to a final volume of 15 μl with 1× medium. Reaction mixtures were warmed to 37°C and equilibrated with 5% CO₂ in air or with air alone prior to the addition of sperm. Immediately after swim-up, 10 μl of the sperm suspension (60 000–90 000 cells) was added to the reaction tubes and incubated with test substances for 90 min. Pilot studies demonstrated that 90-min incubations provided the highest percentage of acrosome-reacted sperm when treated with solubilized ZP without an increase in the percentage of negative control sperm (incubated without ZP) that underwent a spontaneous acrosome reaction. In experiments that employed the T-type calcium-channel inhibitor flunarizine (Calbiochem), sperm incubations were adjusted to either 2.2 μM flunarizine or 0.1% dimethyl sulfoxide (DMSO; vehicle) immediately following swim-up. It should be noted that flunarizine has been reported to interact with Na⁺ channels and dopamine D₂ receptors in neuronal cells, and inhibits catecholamine release from chromaffin cells [12, 13]. There is no evidence for roles for these activities in the ZP3-induced acrosome reaction. In experiments that required the use of pertussis toxin, the pertussis toxin (or vehicle) was present throughout sperm isolation (including swim-up) and incubation with agonists. Previous studies have demonstrated that pertussis toxin has no significant effect on capacitation [14].

Determination of Acrosomal Status

Once the incubation was complete, sperm were pelleted at $500 \times g$ for 5 min at room temperature, the supernatant was removed, and the sperm were fixed in 70% ethanol for 10 min. Fixed sperm were transferred to SuperFrost Plus microscope slides (Fisher Scientific, Suwanee, GA) and air-dried overnight. To visualize the acrosomes, cells were stained with a fluorescein isothiocyanate-conjugated *Arachis hypogea* (peanut) agglutinin (FITC-PNA; Vector Labs, Burlingame, CA), which has been used previously to discriminate between acrosome-intact and acrosome-reacted sperm. PNA has been demonstrated to bind both the outer acrosomal membrane and certain components of the acrosomal matrix [15, 16]. Slides were stained with 100 $\mu\text{g/ml}$ FITC-PNA (in PBS) for 10 min and rinsed in PBS for 5 min. Samples were mounted in Vectashield (Vector Labs) containing 0.5 $\mu\text{g/ml}$ propidium iodide as a counterstain. Slides were viewed immediately after staining on a Nikon Eclipse E800 microscope (Nikon, Tokyo, Japan) with a triple-band filter (DAPI/FITC/TRITC) at 400 \times magnification. Sperm that displayed continuous green staining along their acrosomal arcs were defined as acrosome-intact; those that displayed no green staining or punctate staining were defined as acrosome-reacted [9]. Prior to counting, all slides were coded, and slides were counted blindly. All experiments were conducted at least three times. Each time, three to five independent replicates of each test group were analyzed, and 200 sperm from each replicate were counted. In the event that treatment with a negative control (Glc₄-BSA or 0 μM test substance) resulted in $\geq 15\%$ acrosome-reacted sperm, data from the entire experiment were discarded. Typically, 1 out of 10 experiments was discarded because the background in the assay exceeded this cutoff value.

Competitive Acrosome Reaction Assays

For experiments in which unconjugated oligosaccharides were used to inhibit the acrosome reaction, sperm were isolated under capacitating conditions by swim-up. Immediately thereafter, aliquots of sperm were incubated for 15 min in the absence of free glycan or in the presence of a dose response (0.18 μM to 3.6 μM) of either Le^x-Lac or Le^a-Lac. Additionally, separate aliquots of sperm were incubated with 3.6 μM $\beta\text{Gal-Lac}$ as the negative control. Following this incubation, total heat-solubilized ZP sufficient to bring the ZP3 content of the incubation to ~ 90 nM was added (see [9] for estimation of the amount of ZP3 in a preparation of total, heat-solubilized ZP). Samples were incubated at 37°C for 90 min and then subjected to FITC-PNA staining, as described above.

Statistical Analysis

Dose-response curves were analyzed as previously described [4]. Briefly, data were fit to a rectangular hyperbola, and regression analysis was performed using the SigmaPlot program (SPSS, Chicago, IL). This program estimated ED₅₀ value (concentration of heat-solubilized ZP or of a neoglycoprotein causing a half-maximal induction of the acrosome reaction) and the IC₅₀ value (molar concentration of an unconjugated glycan producing a half-maximal inhibition in the ZP-induced acrosome reaction), as well as maximal stimulation or inhibition of the acrosome reaction. The program also determined how well the data fit the regression line (R^2 and P values). A linear dose-response was observed with one neoglycoprotein, and those data were subjected to linear regression analysis. Where appropriate, comparisons of individual means were accomplished by analysis of variance, with the Fisher protected least significant difference testing using StatView 4.5.1 (SAS, Cary, NC). In all analyses, data were defined as statistically significant at $P < 0.05$.

RESULTS

The Neoglycoprotein Le^x-Lac-BSA Induces the Acrosome Reaction in a Dose-Dependent Manner

Recent studies identify Le^x-containing oligosaccharides as high-affinity, competitive inhibitors for $\sim 70\%$ of ZP3 binding sites on capacitated mouse sperm [5]. Therefore, we reasoned that if these oligosaccharides bind a ZP3 receptor, a neoglycoprotein containing multiple copies of Le^x should induce sperm to undergo the acrosome reaction in a glycan structure-specific manner. To test this prediction, we first incubated capacitated mouse sperm with increasing doses of Le^x-Lac-BSA, which contains the pentasaccharide Gal β 4[Fuc α 3]GlcNAc β 3Gal β 4Glc (19 M glycan/M BSA;

Fig. 1-ii). In the presence of Le^x-Lac-BSA, sperm underwent the acrosome reaction in a dose-dependent manner with saturation reached at $\sim 40\%$ of acrosome-reacted sperm, a fourfold increase over untreated sperm (Fig. 2A). Regression analysis demonstrated that the data fit a rectangular hyperbola ($R^2 = 0.98$, $P < 0.05$) and estimated an EC₅₀ of 350 nM Le^x-Lac-BSA.

Incubation of sperm with heat-solubilized ZP, the positive control for these experiments, also produced a dose-dependent stimulation of the acrosome reaction. Regression analysis estimated an EC₅₀ of 3.2 ZP/ μl , which is equivalent to 39 nM ZP3 (based on [10]). Figure 2A shows that a maximally stimulating dose of ZP (7.5 ZP/ μl) induced $\sim 40\%$ of the sperm to undergo the acrosome reaction, comparable with previously published work [17]. Thus, in our hands, saturating doses of Le^x-Lac-BSA and ZP produced comparable results.

To ensure that the effects observed with Le^x-Lac-BSA were due specifically to the Le^x-Lac oligosaccharide, sperm were incubated with 3 μM of the negative control neoglycoprotein Glc₄-BSA, which contains a tetrasaccharide of glucose (Fig. 1-i). Glucose residues have not been reported on any mature ZP glycoproteins and therefore should neither bind the ZP3 receptor nor stimulate the acrosome reaction [18, 19]. The percentage of sperm that underwent the acrosome reaction in response to 3 μM Glc₄-BSA was statistically comparable to the percentage of acrosome-reacted sperm observed in the absence of neoglycoprotein (Fig. 2A).

We next compared the abilities of Le^x-Lac-BSA and Le^x-BSA (Fig. 1-iii) to induce the acrosome reaction. These neoglycoproteins differ with respect to the presence or absence of a lactose spacer. There were two reasons for this comparison. First, the glycan on Le^x-Lac-BSA is potentially twice as long as the glycan on Le^x-BSA (20 Å versus 10 Å from the reducing to the nonreducing terminus). It was possible that the distance of the Le^x trisaccharide from the surface of the BSA molecule influenced its accessibility to its corresponding sperm receptor and thus its biological activity. Second, we used commercially available neoglycoproteins for this study. A demonstration that this lactose spacer does not affect biological activity of a neoglycoprotein would allow us to test the largest repertoire of commercially available reagents. Figure 2B demonstrates that 2 μM Le^x-Lac-BSA and 2 μM Le^x-BSA were equally effective at induction of the acrosome reaction. Thus, the presence or absence of the lactose spacer at the reducing terminus of the glycan did not affect the ability of the neoglycoprotein to induce the acrosome reaction.

It should also be noted that the level of substitution of Le^x-Lac-BSA (19 M glycan/M BSA) and Le^x-BSA (11 M glycan/M BSA) differed, whereas their ability to induce the acrosome reaction was similar. These results were confirmed and expanded by the observation that 2 μM Le^x-Lac-BSA (containing 9 M glycan/M BSA) was equally effective at inducing the acrosome reaction (data not shown). Thus, the percentage of sperm that underwent the acrosome reaction was comparable, despite a twofold increase in the level of substitution (from 9 to 19 M glycan/M BSA).

Neoglycoproteins Containing Linear Oligosaccharides with a Nonreducing Terminal α -3-Galactosyl Residue Do Not Induce the Acrosome Reaction

There is considerable evidence for the presence of α -galactose-capped oligosaccharides on ZP3 and for the abil-

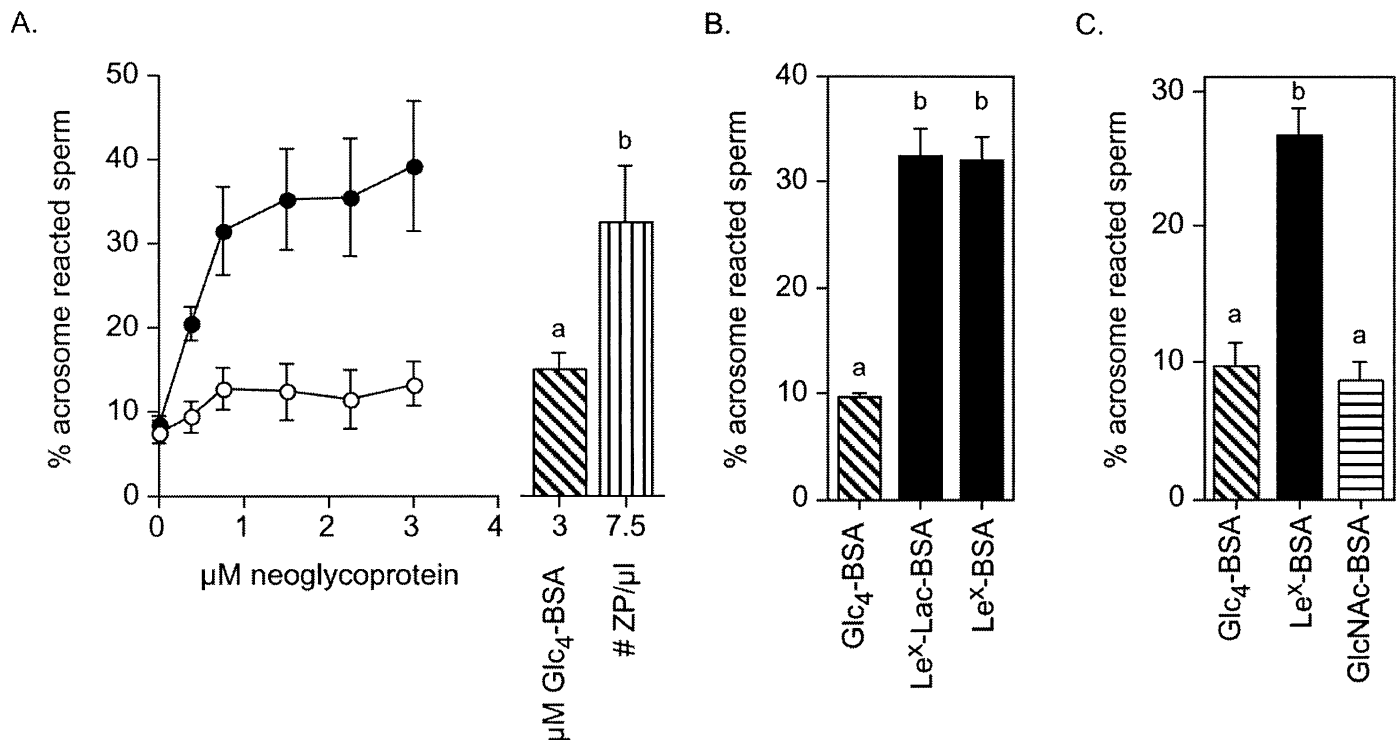


FIG. 2. A Le^x-Lac containing neoglycoprotein induces the acrosome reaction in a glycan structure-dependent manner. **A)** Dose-response analyses were conducted with the neoglycoproteins Le^x-Lac-BSA (closed circles) and αGal-Lac-BSA (open circles) that contain the oligosaccharides Galβ4[Fucα3]GlcNAcβ3Galβ4Glc and Galα3Galβ4GlcNAcβ3Galβ4Glc, respectively. Sperm were incubated with these neoglycoproteins at the following concentrations: 0, 0.375, 0.75, 1.5, 2.25, and 3 μM (based on neoglycoprotein concentration). The negative control neoglycoprotein Glc₄-BSA was tested at 3 μM (diagonally-stripped bar). Solubilized mouse ZP served as a positive control (vertically-stripped bar) and was tested at a concentration of 7.5 ZP/μl, which constitutes a saturating dose in our experiments (data not shown). Following the incubations, sperm were fixed and stained to determine whether they possessed intact acrosomes. These data (mean ± SEM; n ≥ 3) are expressed as the percentage of sperm that were acrosome-reacted after the incubation. Saturating doses of Le^x-Lac-BSA (3 μM) and ZP (7.5/μl) are not statistically different, whereas both are significantly higher than 3 μM Glc₄-BSA. There was no statistically significant response to αGal-Lac-BSA by sperm. **B)** A comparison of the activities of two Le^x-containing neoglycoproteins that differ both in the length of their glycans and their levels of substitution was conducted. Sperm were incubated with 2 μM Glc₄-BSA (30 μM Glc₄ glycan; diagonally-stripped bar), 2 μM Le^x-Lac-BSA (38 μM Le^x-Lac glycan), which contains a reducing terminus lactose spacer, or with 2 μM Le^x-BSA (22 μM Le^x glycan), which lacks this lactose spacer (black bars). All data (mean ± SEM; n = 4) are expressed as the percentage of sperm that are acrosome-reacted after the incubation. Bars with different superscripts are statistically different. **C)** To test whether an N-acetylglucosamine-containing neoglycoprotein could induce the acrosome reaction, sperm were incubated with 2 μM Glc₄-BSA (diagonally-stripped bar), 2 μM Le^x-BSA (black bar), or 2 μM GlcNAc-BSA (horizontally-stripped bar). These data (mean ± SEM; n = 4) are expressed as the percentage of sperm that are acrosome-reacted after incubation. Bars with different superscripts are statistically different (P < 0.05).

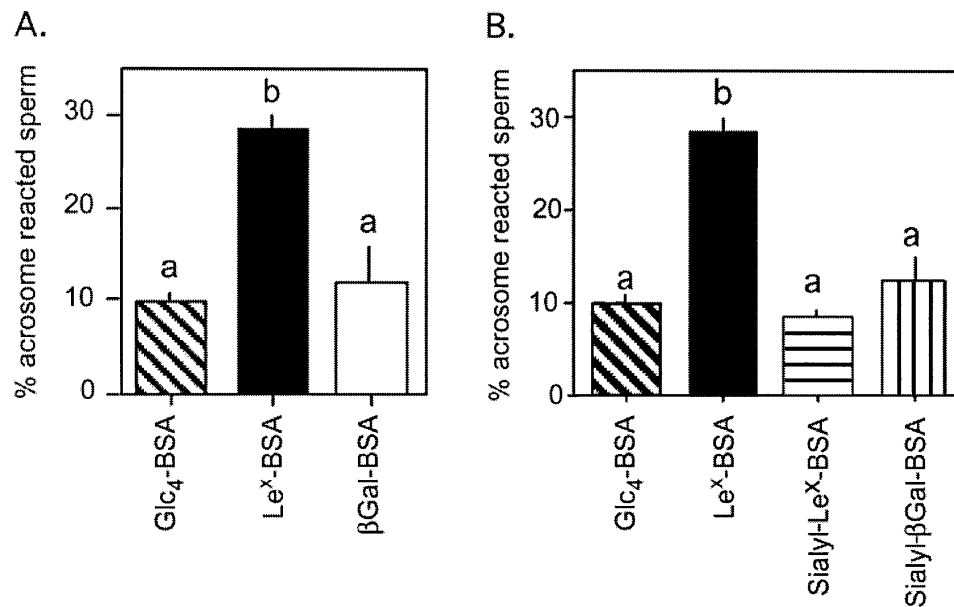
ity of oligosaccharides containing the structure Galα3Galβ4GlcNAc to inhibit both the binding of sperm to a ZP-enclosed egg and the binding of isolated ZP3 to capacitated mouse sperm [3–5]. Thus, α-galactose-capped glycans on ZP3 may be functional agonists for a ZP3 receptor. However, female mice that do not express the gene responsible for adding nonreducing terminal α3-galactosyl residues to glycoproteins are, nonetheless, fertile [20]. Thus, whether α3-galactose-capped glycans are effective agonists for a sperm surface ZP3 receptor is an open question. To address this, we incubated sperm with increasing doses of the neoglycoprotein αGal-Lac-BSA, which contains the pentasaccharide Galα3Galβ4GlcNAcβ3Galβ4Glc (Fig. 1-iv). Sperm did not respond to αGal-Lac-BSA at any concentration tested (0–3 μM; Fig. 2A). An additional neoglycoprotein that contains only the trisaccharide Galα3Galβ4GlcNAc was also tested with similar results observed (data not shown). The maximum dose of αGal-Lac-BSA (3 μM) is equivalent to 26.4 μM αGal-Lac oligosaccharide (taking into account there are an average of 8.8 moles of αGal-Lac oligosaccharide per mole of BSA). This concentration of oligosaccharide is fivefold higher than the IC₅₀ value obtained in competitive sperm-ZP ad-

hesion assays using free oligosaccharides containing non-reducing terminal α-galactosyl residues.

GlcNAc-BSA, βGal-BSA, and Sialyl-Le^x-BSA Do Not Induce the Acrosome Reaction

The data presented so far suggest that a major class of sperm surface ZP3 binding sites, which recognize Le^x-containing glycans, are functional receptors. Kerr et al. [5] indicate that these glycans are recognized by this class of sites in a structure-specific manner. Therefore, if these ZP3 binding sites are in fact functional receptors, then glycans that are not recognized by this class of sites should be incapable, when clustered on a neoglycoprotein, to induce the acrosome reaction. The next experiments tested this prediction. The first neoglycoprotein tested was GlcNAc-BSA (Fig. 1-v). GlcNAcβ4GlcNAcβ4GlcNAc is unable to competitively inhibit either the binding of sperm to an intact ZP-enclosed egg or the binding of fluorochrome-labeled ZP3 to sperm [4, 5]. However, Shur and colleagues [21] have generated data in support of the proposition that a sperm surface form of the enzyme β4-galactosyltransferase-1 (β4GalT-1) binds nonreducing terminal GlcNAc residues

FIG. 3. The fucosyl residue of the neoglycoprotein Le^x -BSA is critical for induction of the acrosome reaction; sialyl- Le^x -BSA is inactive. **A**) To test whether the fucosyl residue was required for the activity of Le^x -BSA, sperm were incubated with 2 μ M Glc_4 -BSA (diagonally-stripped bar), Le^x -BSA (black bar), or β Gal-BSA, which contains the oligosaccharide $Gal\beta 4GlcNAc$ (white bar). **B**) To test the activity of a sialyl- Le^x -containing neoglycoprotein on the acrosome reaction, sperm were incubated with the following neoglycoproteins at a concentration of 2 μ M: Glc_4 -BSA (diagonally-stripped bar), Le^x -BSA (black bar), and sialyl- Le^x -BSA and sialyl- β Gal-BSA (horizontally- and vertically-stripped bars). These data (mean \pm SEM; $n = 4$) are expressed as the percentage of sperm that are acrosome-reacted after incubation. Bars with different superscripts are statistically different ($P < 0.05$).



on ZP3, which leads to the acrosome reaction. In the current experiment, sperm were incubated with 2 μ M Glc_4 -BSA, Le^x -BSA, or the neoglycoprotein $GlcNAc$ -BSA. $GlcNAc$ -BSA did not increase the percentage of sperm that underwent the acrosome reaction when compared to the negative control, Glc_4 -BSA. In contrast, sperm incubated with an equivalent amount of Le^x -BSA did undergo the acrosome reaction (Fig. 2C).

Recent work from our laboratory also indicates that the fucosyl residue of Le^x is required for creation of a high-affinity ligand for a major class of ZP3 binding sites [5]. If this class of ZP3 binding sites is a functional receptor, a neoglycoprotein containing the nonfucosylated glycan, $Gal\beta 4GlcNAc$ (β Gal-BSA, Fig. 1-vi) should not induce the acrosome reaction. To test this prediction, we compared the activity of Le^x -BSA with that of the neoglycoprotein β Gal-BSA. At a concentration of 2 μ M, β Gal-BSA was completely inactive (Fig. 3A).

While Le^x -containing glycans are recognized by a major class of ZP3 binding sites on capacitated mouse sperm, these same sites do not recognize sialyl- Le^x . Therefore, sialyl- Le^x -BSA, which contains the glycan $Neu5Ac\alpha 3Gal\beta 4[Fuc\alpha 3]GlcNAc$ (Fig. 1-ix), should not induce the acrosome reaction. In this experiment, the neoglycoprotein sialyl- β Gal-BSA, which contains the nonfucosylated oligosaccharide $Neu5Ac\alpha 3Gal\beta 4GlcNAc$ (Fig. 1-x), was also tested. This second glycan was examined because P-selectin, which recognizes sialyl- Le^x has been localized to the heads of porcine sperm, and a monoclonal antibody that recognizes L-selectin binds the heads of human sperm [22, 23]. In the current experiment, sperm were incubated in 2 μ M Glc_4 -BSA, Le^x -BSA, sialyl- Le^x -BSA, or sialyl- β Gal-BSA. As expected, Le^x -BSA induced the acrosome reaction (Fig. 3B). In contrast, sperm did not respond to sialyl- Le^x -BSA, demonstrating that the addition of an $\alpha 3$ -sialyl residue to the Lewis X results in a glycan that is unable to induce the acrosome reaction. Sialyl- β Gal-BSA was also inactive. These results, combined with the observation that $GlcNAc$ -BSA and β Gal-BSA do not induce the acrosome reaction, further support the conclusion that the major class of ZP3 binding sites, which recognize Le^x and the sperm surface receptor activated by Le^x -Lac-BSA, are the same sperm surface molecules. Additionally, these data do not

implicate a role for sperm surface selectins in mouse sperm-ZP interactions.

The Neoglycoprotein Le^x -Lac-BSA Is More Active at Inducing the Acrosome Reaction than Le^a -Lac-BSA

Recent work from our laboratory has led to the proposal that Le^a -Lac is recognized by $\sim 30\%$ of the ZP3 binding sites on capacitated mouse sperm [5]. Furthermore, those sites do not recognize Le^x -containing glycans. This raises the question of whether the binding sites that recognize Le^a -Lac are also functional ZP3 receptors. To address this issue, sperm were incubated with increasing concentrations (0, 0.375, 0.75, 1.5, 2.25, and 3 μ M) of the neoglycoprotein Le^a -Lac-BSA, which contains the oligosaccharide $Gal\beta 3[Fuc\alpha 4]GlcNAc\beta 3Gal\beta 4Glc$ (Fig. 1-vii). As a positive control, an aliquot of sperm was also treated with 3 μ M Le^x -Lac-BSA. Results demonstrated that Le^a -Lac-BSA induced the acrosome reaction in a dose-dependent manner (Fig. 4A). However, there were two major differences between this dose response and the dose responses obtained with Le^x -Lac-BSA.

First, while the dose response to Le^x -Lac-BSA described a rectangular hyperbola (see Fig. 2), the response to Le^a -Lac-BSA was linear ($R^2 = 0.86$, $P < 0.05$). Second, Le^a -Lac-BSA was substantially less potent than Le^x -Lac-BSA in inducing the acrosome reaction. While saturation with Le^x -Lac-BSA was reached by 0.75 μ M (Fig. 2A), Le^a -Lac-BSA at that concentration did not induce the acrosome reaction. Additionally, at 3 μ M neoglycoprotein, the fourfold induction of the acrosome reaction by Le^a -Lac-BSA was significantly less than the sevenfold induction by Le^x -Lac-BSA (Fig. 4A).

Based on the observation that Le^a -Lac-BSA is less effective at inducing the acrosome reaction than Le^x -Lac-BSA, we tested whether the activity of Le^a -Lac-BSA could be enhanced by adding an $\alpha 2$ -fucosyl residue to the Lewis A structure, yielding the Lewis B glycan structure. This was accomplished by incubating sperm with 2 μ M Glc_4 -BSA, Le^a -Lac-BSA, or Le^b -Lac-BSA, which contains the oligosaccharide $Fuc\alpha 2Gal\beta 3[Fuc\alpha 4]GlcNAc\beta 3Gal\beta 4Glc$ (Fig. 1-viii). Results demonstrated that the addition of the second fucosyl residue to the Le^a oligosaccharide resulted in a gly-

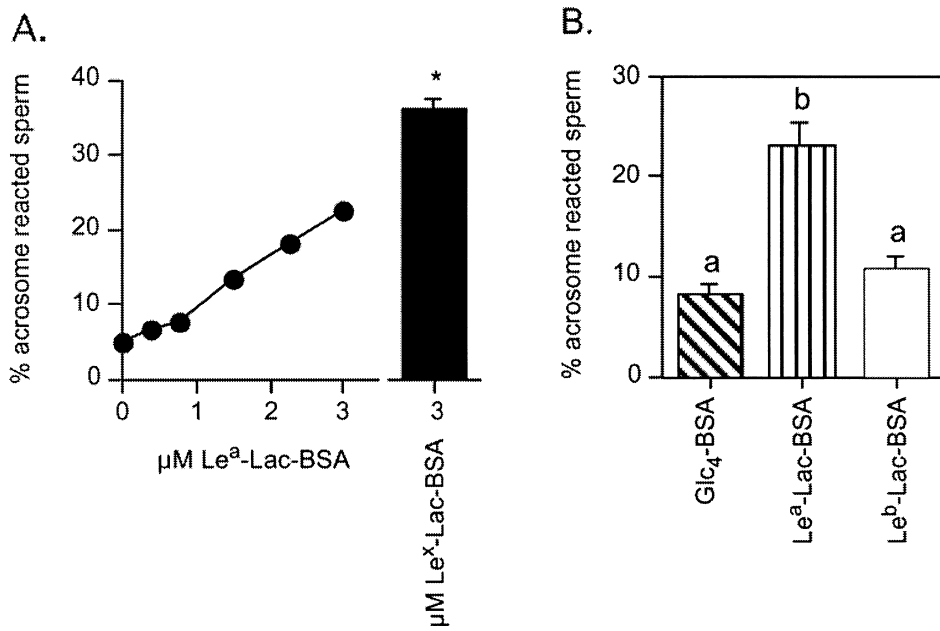


FIG. 4. Le^a-Lac-BSA is a less-potent inducer of the acrosome reaction than Le^x-Lac-BSA. **A**) To test whether Le^a-Lac-BSA (which contains an isomer of Le^x) is capable of inducing the acrosome reaction, sperm were incubated with increasing amounts (0, 0.375, 0.75, 1.5, 2.25, and 3 μM) of Le^a-Lac-BSA and assessed for the presence or absence of their acrosomes. Three-micromolar Le^x-Lac-BSA (black bar) served as a positive control. The effect of 3 μM Le^x-Lac-BSA is threefold higher than the effect of 3 μM Le^a-Lac-BSA. The error bars for these data are within the dimension of the circles. **B**) To determine whether the addition of an α2-fucosyl residue alters activity of Le^a-Lac-BSA, sperm were incubated with 2 μM Glc₄-BSA (diagonally-striped bar), Le^a-Lac-BSA (vertically-striped bar), or Le^b-Lac-BSA, which contains the oligosaccharide Fucα2Galβ3-[Fucα4]GlcNAcβ3Galβ4Glc (white bar). All data (mean ± SEM; n = 4) are expressed as the percentage of sperm that are acrosome-reacted after incubation. Bars with different superscripts are statistically different ($P < 0.05$).

can that, when clustered on a neoglycoprotein, was incapable of inducing sperm to undergo the acrosome reaction (Fig. 4B).

Le^x-Lac and Le^a-Lac are Dose-Dependent Inhibitors of the ZP-Induced Acrosome Reaction

The data presented thus far are consistent with the conclusion that Le^x-BSA and Le^x-Lac-BSA are able to bind a major class of sperm surface ZP3 receptors and, consequently, stimulate sperm to undergo the acrosome reaction. Additionally, our data suggest that Le^a-Lac-BSA induces the acrosome reaction via a second class of ZP3 receptors. However, results from those experiments do not exclude the possibility that these neoglycoproteins are acting via sites on the sperm surface, which are not authentic ZP3 receptors. However, if Le^x-Lac-BSA and Le^a-Lac-BSA are ligands for two distinct ZP3 receptors on capacitated sperm, then unconjugated Le^x-Lac and Le^a-Lac should act as competitive inhibitors for the ZP-induced acrosome reaction. Additionally, Le^x-Lac should be a more effective inhibitor

of the acrosome reaction than Le^a-Lac. To test these predictions, sperm were preincubated for 15 min with or without increasing concentrations (0.18 to 3.6 μM) of Le^x-Lac or Le^a-Lac, or with 3.6 μM βGal-Lac, the negative control oligosaccharide. Following this incubation, sperm were incubated for an additional 90 min with medium alone or with heat-solubilized ZP (equivalent to ~90 nM ZP3). Results demonstrate a dose-dependent inhibition of the ZP-induced acrosome reaction by Le^x-Lac (Fig. 5). Regression analysis demonstrated an excellent fit of the data to a rectangular hyperbola ($R^2 = 0.95$, $P < 0.05$). This analysis estimated an IC_{50} of 121 ± 16 nM Le^x-Lac with a saturating dose of glycan (≥ 0.9 μM) reducing the ZP-induced acrosome reaction to approximately 40% of control. In contrast, βGal-Lac did not inhibit the acrosome reaction. Le^a-Lac was also a dose-dependent inhibitor of the ZP-induced acrosome reaction, and the dose response to this glycan also described a rectangular hyperbola (Fig. 5; $R^2 = 0.92$, $P < 0.05$). However, while this glycan was a high-affinity, competitive inhibitor of the acrosome reaction, with an IC_{50} of

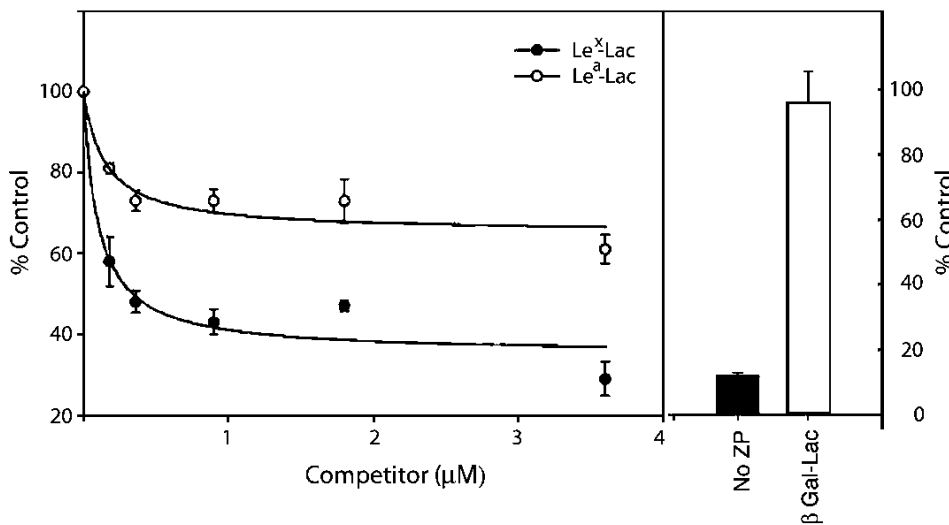


FIG. 5. Unconjugated Le^x-Lac and Le^a-Lac are dose-dependent competitive inhibitors of the ZP-induced acrosome reaction. To directly test the prediction that Le^x-Lac and Le^a-Lac bind to sperm surface ZP3 receptors, these glycans were assayed for their abilities to inhibit the ZP-induced acrosome reaction. Sperm were preincubated for 15 min at 37°C in 5% CO₂ with 0 to 3.6 μM Le^x-Lac or Le^a-Lac or 3.6 μM βGal-Lac, which served as the negative control glycan. Following this incubation, reaction mixtures were supplemented with either medium alone, or solubilized ZP estimated to generate a final ZP3 concentration of 90 nM. Sperm were then incubated for an additional 90 min at 37°C in 5% CO₂. These data (mean ± SEM; n = 3) are expressed as the percentage of sperm that are acrosome-reacted after incubation. Bars with different superscripts are statistically different ($P < 0.05$).

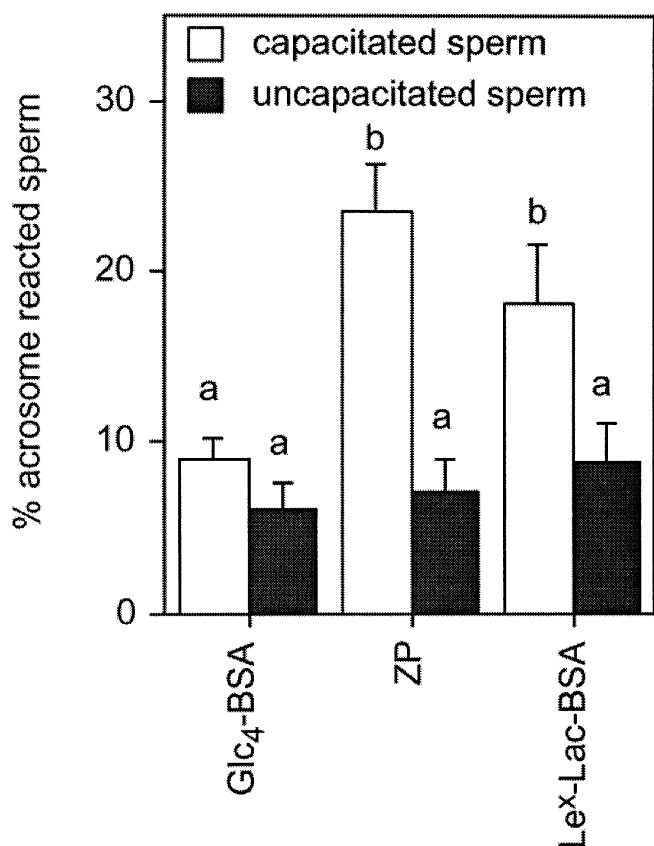


FIG. 6. Capacitation of sperm is a prerequisite for the Le^x-Lac-BSA-induced acrosome reaction. Sperm incubated in either M199-CM (white bars) or M199-NCM (black bars) were treated with Glc₄-BSA (2 μ M), solubilized ZP (7.5/ μ l), or Le^x-Lac-BSA (2 μ M), and tested for their ability to undergo the acrosome reaction. These data (mean \pm SEM; n = 6) are expressed as the percentage of sperm that are acrosome-reacted after incubation. Bars with different superscripts are statistically different ($P < 0.05$).

189 \pm 35 nM, a saturating dose (≥ 0.9 μ M) reduced the ZP-induced acrosome-reacted sperm to only about 70% of control. Two-way analysis of variance verified that Le^x-Lac was a more potent than Le^a-Lac as a competitive inhibitor of the ZP-induced acrosome reaction. It is noteworthy that the dose-dependent effects of Le^x-Lac and Le^a-Lac on the ZP-induced acrosome reaction are almost identical to their dose-dependent inhibition of the binding of ZP3 to capacitated, acrosome-intact mouse sperm [5]. Thus, the data presented herein support the hypothesis that both unconjugated Le^x- and Le^x-containing neoglycoproteins bind a major class of ZP3 receptors and capacitated mouse sperm, in response to the neoglycoprotein, undergo the acrosome reaction. These data also support the conclusion that less-abundant ZP3 binding sites, which differentially recognize Le^a-Lac, are also functional receptors. However, Le^x-Lac-BSA is a more potent inducer of the acrosome reaction than Le^a-Lac-BSA.

Capacitation Is a Prerequisite for the Sperm to Undergo the Acrosome Reaction in Response to Le^x-Lac-BSA

The ability of sperm to undergo the ZP-induced acrosome reaction is regulated by a maturation-like phenomenon called capacitation [24]. In order for sperm to undergo the acrosome reaction in response to the ZP, they must first become capacitated [25]. While the molecular mechanisms underlying capacitation remain under investigation, it is

clear that this process requires the activation of a tyrosine kinase and changes in membrane fluidity, intracellular ions (Ca²⁺, HCO₃⁻), and second messengers (cAMP and protein kinase A) [26].

The data presented thus far demonstrate that a Le^x-containing neoglycoprotein is the most effective mimic of ZP3 that we tested. Thus, the Le^x-Lac-BSA-induced acrosome reaction should also be capacitation-dependent. To test this, sperm were cultured in medium that supports capacitation (M199-CM) or medium that does not (M199-NCM) to obtain populations of either capacitated or uncapacitated sperm. We used two independent criteria to characterize the capacitation status of sperm. First, crude protein extracts prepared from sperm incubated in either M199-CM or M199-NCM were subjected to Western blot analysis with a monoclonal antibody against phosphotyrosine. It has been demonstrated that a subset of sperm proteins become tyrosine phosphorylated during capacitation [27]. Western blot analysis of proteins from sperm cultured in capacitating (M199-CM) and noncapacitating (M199-NCM) media displayed banding patterns that were consistent with previously published results (data not shown). Additionally, aliquots of these sperm were incubated with solubilized ZP and assayed for their ability to undergo the ZP-induced acrosome reaction. As expected, sperm cultured in M199-CM acrosome-reacted in response to ZP, whereas sperm cultured in M199-NCM did not (Fig. 6). Based on these criteria, we defined sperm isolated in M199-CM as capacitated and sperm from M199-NCM as uncapacitated.

To test the effect of capacitation on the neoglycoprotein-induced acrosome reaction, capacitated and uncapacitated sperm were incubated with 2 μ M Le^x-Lac-BSA or 2 μ M of the negative control neoglycoprotein Glc₄-BSA. Reminiscent of the activity of solubilized ZP, Le^x-Lac-BSA was able to induce the acrosome reaction only in capacitated sperm (Fig. 6). This demonstrates that both the ZP-induced and Le^x-Lac-BSA-induced acrosome reactions are capacitation-dependent, supporting the hypothesis that Le^x-containing neoglycoproteins can bind to ZP3 receptors on sperm and can mimic the biological activity of ZP3.

Evidence that Le^x-Lac-BSA and Le^a-Lac-BSA Induce Sperm to Undergo the Acrosome Reaction via the Same Calcium-Dependent Pathway as ZP3

While the binding of ZP3 to its receptor results in the acrosome reaction, it is simply the first in a series of events that occur in the sperm that lead to acrosomal exocytosis. Presently, at least two independent signaling pathways have been identified in sperm upon binding ZP3 that lead to the acrosome reaction. One pathway involves an increase in intracellular calcium levels, which is posited to occur via the sequential action of sperm surface T-type calcium channels, IP₃-mediated release of Ca²⁺ from the acrosome, and a sustained influx of calcium into sperm via the sperm surface, to store-operated Trp-2 channels [28–31]. Thus, if Le^x-Lac-BSA or Le^a-Lac-BSA binds to sperm surface ZP3 receptors, inhibitors of specific parts of the pathway downstream from these receptors should block the induction of the acrosome reaction by these neoglycoproteins.

To test the requirement of a functional T-type calcium channel, sperm were incubated with 2 μ M Le^x-Lac-BSA, 3 μ M Le^a-Lac-BSA, or seven heat-solubilized ZP per milliliter and in the presence or absence of flunarizine, a well-characterized inhibitor of such channels. The dose of flunarizine that was used, 2.2 μ M, is the reported IC₅₀ dose

for this compound [32]. As a control, sperm were treated with an equal volume of the solvent for flunarazine, DMSO. Figure 7, A and B show that flunarazine completely blocked induction of the acrosome reaction by heat-solubilized ZP, by Le^x-Lac-BSA, and by Le^a-Lac-BSA. In contrast, flunarazine did not reduce the low percentage of sperm that underwent a spontaneous acrosome reaction in the presence of the negative control neoglycoprotein, Glc₄-BSA. Thus, flunarazine inhibited the Le^x-Lac-BSA-, Le^a-Lac-BSA-, and ZP-acrosome reaction stimulations, but not the spontaneous acrosome reactions. To confirm that flunarazine was acting via T-type calcium channels in mouse sperm, we also tested the effect of a second T-type calcium-channel inhibitor, amiloride, on the Le^x-Lac-BSA-induced acrosome reaction. This inhibitor has previously been shown to block T-type calcium currents in dispersed spermatogenic cells [33]. Fifty-micromolar amiloride completely blocked the ability of Le^x-Lac-BSA to induce the acrosome reaction (Table 1). Because T-type calcium channels allow the influx of Ca²⁺ from the surrounding medium into sperm, we further tested the requirement of the Le^x-Lac-BSA-induced acrosome reaction for extracellular calcium. Sperm were incubated with 3 μM Le^x-Lac-BSA, 7 ZP/μl, or 3 μM Glc₄-BSA in the presence or absence of 3.75 mM EGTA, a calcium chelator. Results showed that EGTA completely blocked both the Le^x-Lac-BSA and ZP-induced acrosome reaction (Table 1).

Current models of the acrosome reaction posit that the influx of calcium into sperm via T-type calcium channels is followed by the IP₃-mediated release of intracellular stores of calcium and the sustained influx of calcium into sperm through a store-operated, Trp-2 channel [29, 31, 34]. To test whether the later part of the calcium-dependent pathway is activated by Le^x-Lac-BSA, sperm were incubated with 3 μM Le^x-Lac-BSA or 7 ZP/μl, and with 50 μM 2-APB or vehicle (DMSO) for this inhibitor. 2-APB blocks IP₃-stimulated release of intracellular calcium stores and inhibits Trp-1 channels [35, 36]. It is unknown whether 2-APB also inhibits Trp-2 channels. Results of this experiment show that incubation of sperm with 50 μM 2-APB blocked the ability of both solubilized ZP and Le^x-Lac-BSA to induce the acrosome reaction (Table 1). However, it had no effect on the spontaneous acrosome reaction of sperm incubated with the negative control, Glc₄-BSA. Thus, taken together, the effects of flunarazine, EGTA, and 2-APB described above support the hypothesis that Le^x-Lac-BSA and Le^a-Lac-BSA induce the acrosome reaction via the same calcium-dependent pathway as ZP3.

The second intracellular pathway activated in sperm by ZP3 involves a G_i protein, which can be inhibited by pertussis toxin, a compound that inactivates G_i proteins via ADP-ribosylation of the alpha subunit [14]. To determine whether Le^x-Lac-BSA and Le^a-Lac-BSA also activate this second pathway, sperm were incubated with these neoglycoproteins, with heat-solubilized ZP or the negative control, Glc₄-BSA, in the presence or absence of 100 ng/ml pertussis toxin. This concentration of pertussis toxin is 10 times the amount required to inhibit the ZP-induced acrosome

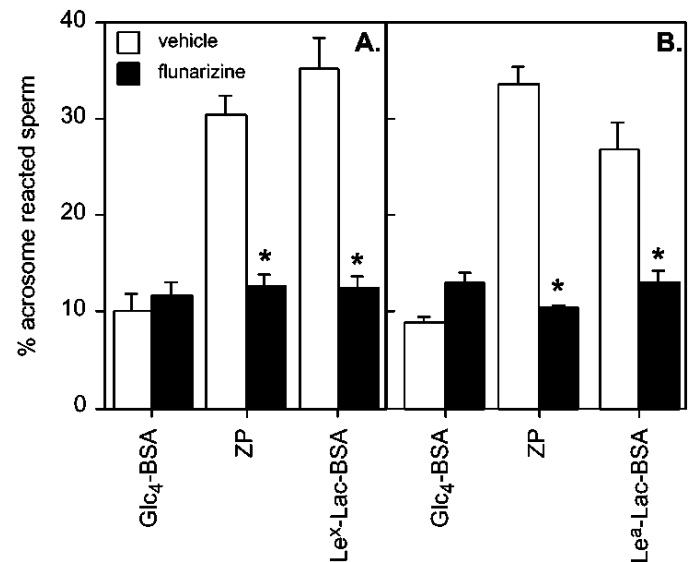


FIG. 7. The ZP-acrosome reaction and the Le^x-Lac-BSA and Le^a-Lac-BSA acrosome reactions require a functional T-type calcium channel. **A**) Capacitated sperm were incubated with 2 μM Glc₄-BSA, 7.5 ZP/μl, or 2 μM Le^x-Lac-BSA in 2.2 μM flunarazine (black bars) or vehicle (white bars) for 90 min. **B**) Capacitated sperm were incubated with 3 μM Glc₄-BSA, 7.5 ZP/μl, or 3 μM Le^a-Lac-BSA in 2.2 μM flunarazine (black bars) or vehicle (white bars) for 90 min. Data (mean ± SEM; n ≥ 3) are expressed as the percentage of sperm that are acrosome-reacted after incubation. Asterisks indicate where results of inhibitor treatment are significantly lower than vehicle treatment.

reaction [14]. In accordance with previous reports, sperm treated with pertussis toxin did not undergo the ZP-induced acrosome reaction. In contrast, pertussis toxin did not inhibit the ability of either 2 μM Le^x-Lac-BSA (Fig. 8A) or 3 μM Le^a-Lac-BSA to induce the acrosome reaction (Fig. 8B). We also conducted preliminary experiments with the muscarinic receptor antagonist quinuclidinyl benzilate (QNB), which also blocks the ZP-induced acrosome reaction [37]. In our experiments, 50 μM QNB inhibited the ZP-induced acrosome reaction, but not the Le^x-Lac-BSA-induced acrosome reaction (data not shown). These data demonstrate that the neoglycoproteins Le^x-Lac-BSA and Le^a-Lac-BSA induce the acrosome reaction in a G_i protein-independent manner.

DISCUSSION

The Neoglycoproteins Le^x-Lac-BSA and Le^a-Lac-BSA Bind to and Activate a Major Class of ZP3 Receptors on Sperm in a Glycan Structure-Specific Manner

The current paradigm for fertilization postulates that the interaction between the sperm and the ZP is a complex series of events. One of the first events is chiefly adhesive in nature and may be mediated by O-linked glycans on ZP3 and corresponding sperm surface proteins [2]. A subsequent

TABLE 1. Effect of amiloride, EGTA, and 2-APB on neoglycoprotein and ZP-induced acrosome reactions.*

Agonist	Without amiloride	With amiloride	Without EGTA	With EGTA	Without 2-APB	With 2-APB
ZP (7 μl)			46 ± 9.6	8.9 ± 1.8	32.9 ± 1.9	9.8 ± 0.7
Glc ₄ -BSA (2 μM)	22 ± 2	25 ± 1	6.3 ± 0.8		9.7 ± 0.7	10.1 ± 1.5
Le ^x -Lac-BSA (2 μM)	41 ± 2	22 ± 1	30.2 ± 3.5	7.6 ± 1.4	38.0 ± 1.9	11.75 ± 1.5

* Data are presented as the mean ± SEM of the percentage of sperm that underwent the acrosome reaction in response to ZP, Glc₄ BSA, and Le^x-Lac-BSA.

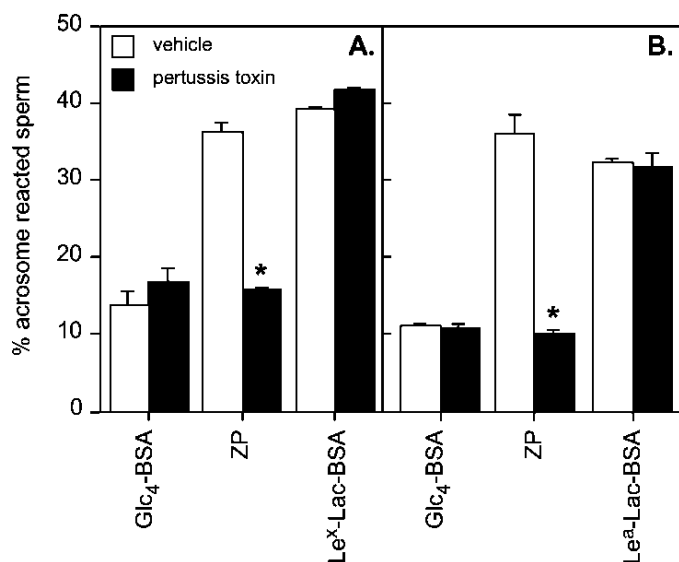


FIG. 8. The neoglycoprotein-induced acrosome reaction is pertussis toxin-insensitive. **A)** Sperm were incubated with 2 μ M Glc₄-BSA, 7.5 ZP/ μ l, or 2 μ M Le^x-Lac-BSA in 100 ng/ml of pertussis toxin (black bars) or vehicle (white bars) for 90 min. **B)** Sperm were incubated with 3 μ M Glc₄-BSA, 7.5 ZP/ μ l, or 3 μ M Le^a-Lac-BSA in 100 ng/ml of pertussis toxin (black bars) or vehicle (white bars) for 90 min. Data (mean \pm SEM; $n \geq 3$) are expressed as the percentage of sperm that are acrosome-reacted after incubation. Asterisks indicate where inhibitor blocked the acrosome reaction (as compared to vehicle).

step involves the activation of at least two signaling pathways in sperm that lead to the induction of the acrosome reaction. Experiments presented herein have addressed the issue of whether the sperm surface ZP3 binding sites that recognize Le^x are, in fact, functional receptors. Pharmacologically, sperm surface sites involved in adhesion to the ZP, but not in the acrosome reaction, would be characterized as acceptors because they would not trigger a biological event in the sperm. On the other hand, a true receptor is defined as a molecule that triggers an intracellular signal in the cell upon binding its ligand. Our previous studies demonstrated that Le^x-containing glycans were high-affinity inhibitors of sperm-ZP binding [4]. Additionally, these glycans are ligands for $\sim 70\%$ of the ZP3 binding sites detectable on capacitated mouse sperm [5]. However, neither of these studies could discriminate whether these glycans bound a sperm surface receptor or acceptor. This current study indicates that BSA-based neoglycoproteins containing Le^x mimic the effect of solubilized ZP on the acrosome

reaction by binding and activating a major class of ZP3 receptors. Both ZP3 and Le^x-Lac-BSA act in a dose-dependent and saturable manner and require prior capacitation of sperm. Seven other neoglycoproteins containing other, related glycan structures were inactive at the doses tested, as summarized in Table 2. Finally, unconjugated Le^x-Lac, but not the related nonfucosylated tetrasaccharide (β Gal-Lac), was a dose-dependent inhibitor of the ZP-induced acrosome reaction. As described by the IC₅₀ and percent maximal inhibition, Le^x-Lac had almost identical inhibitory effects on this process and on the saturable and specific binding of fluorescently labeled ZP3 to sperm [5]. Taken together, these data support the conclusion that Le^x-containing oligosaccharides bind in a glycan structure-specific manner to a major class of ZP3 receptors on the sperm surface, and when clustered on a polypeptide backbone, initiate the acrosome reaction.

The finding that Le^x-containing neoglycoproteins mimic the ability of ZP3 to induce the acrosome reaction leads to the issue of whether the native glycans on ZP3 contain Le^x structures. A complicating factor in the structural analysis of the mouse ZP's oligosaccharides is the scarcity of biological material that is available for complete and systematic study. Recent structural analysis of the major O-linked glycans from total mouse ZP3 did not detect Le^x structures [38]. Furthermore, our laboratory and others [39] have attempted to identify Le^x structures on the mouse ZP by immunocytochemistry or Western blotting with Le^x-specific monoclonal antibodies; these efforts have proved inconclusive. While these studies are certainly important, one must take into account two important aspects of protein glycosylation.

First, glycoproteins exhibit a substantial degree of heterogeneity in their oligosaccharide structures, and our understanding of glycoform diversity is limited by our technology. For example, P0, a major glycoprotein component of myelin, contains one N-linked glycosylation site that was originally demonstrated by nuclear magnetic resonance (NMR) and fast atom bombardment-mass spectrometry (FAB-MS) to carry a distinct sulfated glycan structure, human natural killer cell carbohydrate epitope (HNK-1) (3-O-SO₃H-GlcUA β 3Gal β 4GlcNAc-R) [40]. However, more recent NMR and MS techniques have established that this one glycosylation site can carry at least 13 different oligosaccharide structures, of which only 40% contain the HNK-1 epitope [41]. By extrapolation to the ZP glycoproteins, the majority of the ZP3 oligosaccharides may not contain Le^x structures. There may be numerous glycan

TABLE 2. Summary of the acrosome reaction-inducing activities of the neoglycoproteins tested in this report.

Abbreviation	Structure	Activity*
Le ^x -Lac-BSA	Gal β 4[Fuca3]GlcNAc β 3Gal β 4Glc-3C-BSA	+++
Le ^x -BSA	Gal β 4[Fuca3]GlcNAc-3C-BSA	+++
Le ^a -Lac-BSA	Gal β 3[Fuca4]GlcNAc β 3Gal β 4Glc-3C-BSA	++
Glc ₄ -BSA	Glc α 6Glc α 4Glc α 4Glc-3C-BSA	-
α Gal-Lac-BSA	Gal α 3Gal β 4GlcNAc β 3Gal β 4Glc-3C-BSA	-
GlcNAc-BSA	GlcNAc-14C-BSA	-
β Gal-BSA	Gal β 4GlcNAc-3C-BSA	-
Le ^b -Lac-BSA	Fuca2Gal β 3[Fuca4]GlcNAc β 3Gal β 4Glc-3C-BSA	-
Sialyl-Le ^x -BSA	Neu5Ac α 3Gal β 4[Fuca3]GlcNAc-3C-BSA	-
Sialyl- β Gal-BSA	Neu5Ac α 3Gal β 4GlcNAc-3C-BSA	-

* Based on the ability of the neoglycoprotein to induce the acrosome reaction at a concentration of 2 μ M. The "+" indicates that treatment with neoglycoprotein results in an increase in the percentage of acrosome-reacted sperm that is significantly higher ($P \leq 0.05$) than treatment with the negative control, whereas the "-" indicates that treatment with neoglycoprotein does not significantly increase the percentage of acrosome-reacted sperm. The relative activities of neoglycoproteins that induced the acrosome reaction are indicated by the number of "+" signs (see Fig. 3, B and C).

structures on the ZP glycoproteins that have not been identified to date due to their low abundance in the ZP.

Second, while the issue of whether Le^x-containing glycans are present on ZP3 remains unresolved, data presented here and elsewhere [5] provide important information regarding the glycan structure specificity of the major class of ZP3 receptors. Clearly, this receptor contains a binding site for a fucosyl residue or a saccharide that closely mimics fucose, and the presentation of this residue is evidently important.

Le^a-Lac-BSA Binds to and Activates a Second Class of ZP3 Receptors on Sperm

Le^a-Lac binds to a second class of ZP3 binding sites, which have low affinity, if any, for Le^x-Lac [5]. The experiments described herein examined whether these less-abundant ZP3 binding sites are functional receptors. Our data demonstrate that Le^a-Lac-BSA stimulates sperm to undergo the acrosome reaction. Additional experiments document that unconjugated Le^a-Lac is a saturable and specific inhibitor of the ZP-induced acrosome reaction. As described by the IC₅₀ and percent maximal inhibition, Le^a-Lac had almost identical inhibitory effects on this process and on the saturable and specific binding of fluorescently labeled ZP3 to sperm [5]. Therefore, the most direct interpretation of these data is that the sperm surface sites, which bind Le^a-Lac, represent a second class of ZP3 receptors. To our knowledge, the data presented herein and in [5] provide the direct first evidence that mouse sperm express multiple ZP3 receptors that are distinguishable by their glycan binding specificity.

While Le^a-Lac-BSA induces the acrosome reaction by capacitated mouse sperm, it is clearly less potent than Le^x-Lac-BSA. The difference in the response to these two neoglycoproteins has a number of potential causes. The potency of these two neoglycoproteins may be dependent on the numbers of their respective sperm surface ZP3 receptors. The class of receptors, which recognize Le^x-Lac, clearly predominates. Alternatively, while Le^a-Lac is a ligand for a class of ZP3 receptors on sperm, Le^a-Lac-BSA may be less effective than Le^x-containing neoglycoproteins at inducing the structural changes in a ZP3 receptor that are needed to activate intracellular signal transduction pathways.

Le^x-Lac-BSA and Le^a-Lac-BSA Activate a Calcium-Dependent Pathway, but Not a G_i Protein-Dependent Pathway

The data presented in this report support the conclusion that Le^x-Lac-BSA and Le^a-Lac-BSA bind to distinct classes of ZP3 receptors on sperm, and that this binding activates the same calcium-dependent pathways in sperm as ZP3. Similar to the response of capacitated mouse sperm to heat-solubilized ZP, the response to these two neoglycoproteins requires a functional T-type calcium channel. Consistent with current models of the ZP-induced acrosome reaction, the Le^x-Lac-BSA-induced acrosome reaction is also inhibited by chelation of extracellular calcium with EGTA and by blocking release of intracellular calcium stores with 2-APB.

While the ZP-induced acrosome reaction was sensitive to pertussis toxin, an inhibitor of G_i proteins, it did not block the neoglycoprotein-induced acrosome reaction. Our data do not address the issue of whether Le^x-Lac-BSA or Le^a-Lac-BSA activate a G_i protein but do indicate that such

activation, if it occurs, is unnecessary for induction of the acrosome reaction by these two neoglycoproteins. Additionally, our data suggest that activation of the calcium-dependent pathway is sufficient for the induction of the acrosome reaction by these two neoglycoproteins. Why is activation of the calcium-dependent pathway sufficient for Le^x-Lac-BSA or Le^a-Lac-BSA to induce the acrosome reaction while a G_i protein-dependent pathway is also required for the ZP-induced acrosome reaction?

First, treatment of capacitated sperm with a calcium ionophore will induce the acrosome reaction [29, 42–45]. Thus, a rise in free intracellular calcium levels of sufficient magnitude is adequate to induce capacitated sperm to undergo the acrosome reaction. Second, studies in other cell types (e.g., lymphocytes) indicate that an increase in the numbers of cell surface receptors that are cross-linked by a single ligand can potentiate the resulting elevation in free intracellular calcium levels (see for example [46, 47]). By extrapolation to sperm, it is possible that Le^x-Lac-BSA and Le^a-Lac-BSA cross-link a larger number of ZP3 receptors than ZP3 itself. In our studies, there was an average of nine or more potential sperm binding glycans on each molecule of Le^x-Lac-BSA and Le^a-Lac-BSA. Thus, a single molecule of these two neoglycoproteins might cross-link multiple ZP3 receptors on a given capacitated sperm. In contrast, recent data indicate that there is an average of 11 O-linked glycans on each molecule of ZP3 [48]. However, as available evidence suggests that these glycans are structurally diverse, only a small subset of the 11 glycans may bind ZP3 receptors [19]. Thus, Le^x-Lac-BSA and Le^a-Lac-BSA may be able to cross-link a larger number of ZP3 receptors, and consequently cause a larger increase in intracellular free calcium levels than the natural ligand, ZP3. This might be sufficient to make the G_i protein-mediated pathway dispensable for the induction of the acrosome reaction.

Contrasting Data Concerning Glycan Structures Recognized by Mouse ZP3 Receptors

Using three different experimental approaches [4, 5], our laboratory has identified the Le^x trisaccharide as a high-affinity ligand for a major class of ZP3 receptors on mouse sperm. Additionally, our data indicate that the structural isomer of Le^x, Le^a, recognizes a second, less-abundant class of ZP3 receptors. Thus, while the specific glycans on ZP3 that bind sperm surface receptors have yet to be defined, our data support the hypothesis that there are, at minimum, two functional glycans on the ZP that bind distinct receptors on the surface of capacitated mouse sperm [4, 49, 50]. However, our conclusion about characteristics of sperm-binding glycans differs from conclusions of other laboratories. Consideration of the studies by these other laboratories is, therefore, warranted.

Tulsiani and colleagues [49, 51, 52] have proposed that a single class of ZP3 receptors on sperm recognizes non-reducing terminal mannose, GlcNAc and GalNAc residues. This proposal comes from studies of the binding of mouse sperm to mannose-BSA, GlcNAc-BSA, and GalNAc-BSA, and induction of the acrosome reaction by these neoglycoproteins. Sperm bound to GlcNAc-BSA, GalNAc-BSA, and Mannose-BSA immobilized on Sepharose beads, but not to Glc-BSA or Gal-BSA [53]. A single class of sperm surface molecules was shown to be responsible for this binding activity because unconjugated mannose, GlcNAc, and GalNAc inhibited the binding of sperm to neoglycoproteins containing any one of these three monosaccha-

rides. This apparent binding of a single class of sperm surface molecules to three different monosaccharides is unexpected given the theory that sperm bind to the ZP in a glycan-structure-specific manner [54].

Tulsiani and colleagues also demonstrated that neoglycoproteins containing mannose, GlcNAc, or GalNAc, but not Gal or Glc, induced capacitated mouse sperm to undergo the acrosome reaction [51, 52, 55]. This induction was blocked by the L-type calcium-channel inhibitors, verapamil and diltiazem, but not by the T-type calcium-channel inhibitor, amiloride [51]. The inhibitory activity of verapamil and diltiazem but not of amiloride is significant because current data indicate that ZP3 receptors on mouse sperm, when bound to the ZP, activate a T-type calcium channel [30].

A second model posits that β 4-galactosyltransferase 1 (β 4-Gal T1) is the primary ZP3 receptor on the surface of mouse sperm and, as such, binds nonreducing terminal GlcNAc residues on ZP3 (see [21] for review). Immunocytochemistry localized β 4-Gal T1 to the plasma membrane overlying the acrosome and antibodies to β 4-Gal T1 induced sperm to undergo the acrosome reaction [56, 57]. Forced β 4 galactosylation reduced, but did not eliminate, the ability of ZP3 to act as a competitive inhibitor of sperm-ZP binding [58]. Additionally, de novo expression of β 4-Gal T1 in frog eggs rendered them responsive to ZP3. Finally, in vitro, neither anti- β 4-Gal T1 nor solubilized ZP glycoproteins induce β 4-Gal T1-null sperm to undergo the acrosome reaction. However, other data do not support the view that mouse sperm surface β 4-Gal T1 acts as the primary sperm surface receptor for ZP3. Monovalent fluorescent probes localized β 4-Gal T1 to the posterior sperm head, not to the plasma membrane overlying the acrosome. Those same studies demonstrated that the binding of polyclonal anti- β 4-Gal T1 immunoglobulin G to mouse sperm caused the movement of β 4-Gal T1 from the posterior region of the sperm head to the plasma membrane overlying the acrosome [59]. Additionally, in three of five experiments, sperm from β 4-Gal T1-null mice were shown to bind radiolabeled ZP3 [60]. In experiments in which this binding was observed, the amount of radiolabeled ZP3 bound by β 4-Gal T1-null sperm was 30% to 50% the amount of radiolabeled ZP3 bound to sperm from wild-type mice. Because only a single dose of radiolabeled ZP3 was tested in these experiments, it was impossible to distinguish whether sperm lacking β 4-Gal T1 had fewer ZP3 binding sites or bound ZP3 with lower affinity. Additionally, two laboratories failed to observe inhibition of sperm-ZP binding by oligosaccharides with terminal GlcNAc residues [3, 4]. Finally, in vivo, β 4-Gal T1-null sperm are fertile [60]. Recently, Shur and colleagues [50] presented data supporting the hypothesis that in vitro, sperm from β 4-Gal T1-null mice bind the zona via a ZP3-independent mechanism. The authors raised the possibility that the ligand on the ZP for β 4-Gal T1-null sperm was an adsorbed oviductal glycoprotein. However, it seems unlikely that an adsorbed oviductal glycoprotein would act in vivo as the sole ligand for sperm, which lack β 4-Gal T1. This same protein, which is soluble in oviductal fluid, would compete with the same protein adsorbed on the ZP for binding sites on the sperm surface.

While the data obtained from our studies disagree with data from other laboratories, those other data are still the subject of active debate and experimentation. Therefore, all current models of sperm-ZP interactions, including those posited by our laboratory, deserve further exploration. Emphasis should be placed on testing these models in intact

animals. Our model, presented here and in [5], support the hypothesis that there are two distinct ZP3 binding sites on the sperm surface that can be distinguished by their affinities for Le^x-Lac and Le^a-Lac. Our in vitro studies indicate that these sites are functional receptors, which when bound to ZP3 or other high-affinity ligands, stimulate capacitated mouse sperm to undergo the acrosome reaction via a calcium-dependent mechanism.

ACKNOWLEDGMENTS

We are grateful to Ms. Janet Folmer for technical assistance with fluorescence microscopy; Drs. Jurrien Dean and Philip Castle for advice and instruction on ZP isolation; Dr. Pablo Visconti for technical assistance with sperm protein tyrosine phosphorylation experiments; and Drs. Ronald Schnaar, Martin Charron, and Harvey Florman for informative discussions.

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