Egg Sialoglycans Increase Intracellular pH and Potentiate
the Acrosome Reaction of Sea Urchin Sperm

Noritaka Hirohashi and Victor D. Vacquier

Center for Marine Biotechnology and Biomedicine
Scripps Institution of Oceanography
University of California San Diego
La Jolla, California 92093-0202

Address correspondence to Noritaka Hirohashi
Tel.: 858-534-2146
Fax: 858-534-7313
E-mail: nhirohashi@ucsd.edu
Running title: Egg Sialoglycans Increase Sperm pHᵢ

Key words: Fertilization, Sea urchin, Acrosome reaction, Sialoglycans, Intracellular pH
SUMMARY

Sea urchin egg jelly (EJ) triggers sperm acrosome reaction (AR), an exocytotic event required for membrane fusion of the gametes. Purified fucose sulfate polymer (FSP) in EJ is one inducer of the AR. Binding of FSP to its receptor regulates opening of two distinct calcium channels and also elevates intracellular pH (pH_i). Egg jelly also contains sialic acid rich glycans (sialoglycans; SG) which were isolated by β-elimination followed by DEAE chromatography. In the presence of limiting amounts of FSP, the SG fraction markedly potentiates the AR, however, by itself SG has no activity. The SG fraction increases the pH_i of sperm without increasing intracellular Ca^{2+}. The SG-induced increase in pH_i is not blocked by nifedipine or high K^+, whereas the FSP-induced pH_i increase is sensitive to both these agents. Treatment of the SG fraction with neuraminidase or mild metaperiodate that specifically cleaves the glycerol side chain of sialic acid, abolishes the AR potentiation and ability of SG to elevate pH_i. These data are the first to show that there are at least two pathways to induce sperm pH_i increase, and that egg surface sialic acid plays a role in triggering the sperm AR.
INTRODUCTION

The acrosome reaction (AR)\(^1\) of animal spermatozoa is necessary for sperm penetration of the egg’s extracellular matrices and fusion of the gamete plasma membranes. The mechanism underlying the AR represents a potential target for novel methods of contraception. Sea urchin spermatozoa are model cells for studying the signal transduction pathways leading to the AR. Molecules in the egg’s jelly coat (EJ) bind to sperm receptors and trigger the AR by activating ion fluxes which result in a net influx of Ca\(^{2+}\) and Na\(^+\) and net efflux of H\(^+\) and K\(^+\) (1-3). The macromolecular fraction of EJ is composed of sialoglycoproteins (4, 5) and a fucose sulfate polymer (FSP; (6)). The FSPs of several sea urchin species are linear polymers of 1→3-linked α-L fucose with species-specific patterns of O-sulfation (7). The pattern of sulfation is what makes FSP a species-specific inducer of the AR (8, 9). FSP induces the sequential activation of two sperm calcium channels. The first channel, which is transiently opened, is blocked by dihydropyridines (10). The second channel, which is insensitive to dihydropyridines, allows a sustained increase in intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) which triggers the AR (11). The second channel may be a store operated channel which is dependent for activation on the increase in intracellular pH (pH\(_i\); (11, 12)). The EJ-induced increase in sperm pH\(_i\) occurs by activation of a voltage dependent Na\(^+\)/H\(^+\) exchanger (12-14).

Sea urchin sperm receptor for egg jelly-1 (suREJ1; (15)) is a single transmembrane segment, 1450 amino acid glycoprotein, localized to the flagellar and acrosomal plasma membranes. suREJ1 binds FSP to induce the AR (16), monoclonal antibodies to suREJ1 induce the AR, and purified suREJ1 neutralizes FSP as an inducer of the AR (15). Sea urchin sperm suREJ3 is a 2,681 amino acid orphan receptor which
localizes exclusively to the cell membrane covering the sperm acrosomal vesicle. suREJ1 and suREJ3 share significant homology with each other and with the human polycystin-1 family (the protein mutated in autosomal dominant polycystic kidney disease; (17)). Although ligands binding suREJ3 remain unknown, it is reasonable to assume that suREJ3 is somehow involved in AR induction.

EJ contains several high molecular weight components. Although purified FSP, devoid of amino acids, induces all the ion channel events triggering the AR (6, 9, 16), earlier data suggested that other EJ macromolecules may also be involved in secondary or redundant signal transduction pathways leading to the AR. For example, protease treatment of EJ, which has no effect on FSP, was found to decrease the AR inducing activity of EJ by as much as 50% (18, 19). Treatment of EJ with sodium metaperiodate decreased AR activity, but left FSP intact (18). Treatment of EJ with PNGase-F releases N-linked oligosaccharides from EJ and decreases its AR activity (20). These results, and other conflicts in the literature (21), caused us to revisit the question of the role of the oligosaccharide fraction of EJ in AR induction.

Here we present data showing that β-elimination of total EJ, followed by DEAE chromatography and electrodialysis, separates the sialic acid rich-high molecular mass glycans from FSP. This fraction has no AR activity of its own. However, it greatly potentiates the FSP-induced AR, indicating that it works through a receptor pathway that is different from the FSP pathway. The sialoglycan fraction appears to elevate sperm pH, suggesting that its ultimate target might be the Na⁺/H⁺ exchanger responsible for the pH increase (3). Treatment of sialoglycans with neuraminidase, or mild metaperiodate that specifically destroys sialic acid, renders the fraction
inactive. This is the first direct evidence for the involvement of sialic acid as a ligand in the induction of the animal sperm AR.

**EXPERIMENTAL PROCEDURES**

*Materials*—Fura-2 AM, BCECF AM and nigericin were from Molecular Probes. Rhodizonic acid disodium and L-ascorbic acid were from Aldrich. Ionomycin and NeuAc were from Calbiochem. 1,9-dimethylmethylene blue was from Serva. Heparin, *Vibrio cholerae* neuraminidase, L-fucose, nifedipine and DEAE-cellulose were from Sigma. Sepharose CL-6B was from Pharmacia. Affi-Gel Hz was from Bio-Rad Laboratories.

*Gamete handling and fractionation of EJ polysaccharides*—*Strongylocentrotus purpuratus* sperm were collected as undiluted semen by intercoelomic injection of 0.5 M KCl and kept on ice. Eggs were spawned into beakers filled with seawater. Preparation of crude EJ was described (22). Preparation and fractionation of EJ oligo/polysaccharides were performed as described (22). Briefly, crude EJ, dialyzed against deionized water, was subjected to β-elimination (0.05 N KOH, 1 M NaBH₄ 45 °C, 24 h), diluted with 4 × of acetate buffer (50 mM sodium acetate pH 5.0) and the pH adjusted to 5.0 by addition of 2 M acetic acid. The solution containing EJ oligo/polysaccharides was applied to a DEAE-cellulose column equilibrated in the above acetate buffer. The column (1.4 × 6 cm) was then washed with 10 × of acetate buffer. Bound materials were eluted with a 0-3 M NaCl gradient in acetate buffer. Each 3-ml fraction was tested for negatively charged polysaccharides by the metachromatic assay (23). The fractions containing sialoglycans eluted between 0-1 M NaCl. They were pooled and sialoglycans precipitated by addition of 3 × of 95%
ethanol. FSP eluted between 1.5-2.5 M NaCl and was precipitated by adding 1 × of 95% ethanol. Both sialoglycan and FSP precipitates were then sedimented by centrifugation at 12,000 × g for 20 min. The supernatants were decanted and the centrifuge tubes air dried. For FSP, the pellet was dissolved in deionized water (dH₂O) and dialyzed overnight against dH₂O at 4 °C. For some experiments (Fig. 4-7), the sialoglycan fraction was further purified by electrodialysis. For electrodialysis, the sample was placed in a Centricon YM-10 cartridge (10 kDa cut-off; Amicon) and the cartridge placed in the electrodialysis apparatus (Centrilutor, Amicon) and filled with 50 mM sodium acetate pH 5.0. The sample was subjected to 1 watt for 4 h at room temperature. After electrodialysis, sialoglycans remaining in the cartridge were recovered and dialyzed against dH₂O.

**Sepharose CL-6B gel filtration chromatography**—The Sepharose CL-6B column (1.2 × 60 cm) was equilibrated with millipore filtered seawater (MFSW). The sample was applied and 1-ml fractions collected. The fractions were tested in the metachromatic assay for negatively charged polysaccharides, the thiobarbituric acid assay for sialic acid (24), the Dische-Shettle cysteine assay for fucose (25), and the sperm AR potentiation assay.

**Sialidase treatment and metaperiodate oxidation of sialoglycans**—Sialogycans in the concentration range equivalent to 0.1-1 mg heparin/ml (metachromatic assay) were placed in acetate buffer (10 mM sodium acetate, 4 mM CaCl₂, pH 5.5) and *Vibrio cholerae* neuraminidase (0.3 units/mg of sialoglycans) added, and the reaction tube incubated at 37 °C for 24 h. The reaction was stopped by addition of 10 × of quenching buffer (25 mM Tris, 192 mM glycine, 10 mM EDTA, pH 8.3) and the released sialic acid removed by electrodialysis using Centricon YM-10 cartridges.
Sialogycans were treated with NaIO₄ to specifically truncate the glycerol side chain of sialic acid (26). Briefly, the sialoglycans were treated with 2 mM NaIO₄ in phosphate-buffered saline for 30 min on ice in the dark. This reaction mixture was immediately used for crosslinking to Affi-Gel Hz. For biological assays of mild metaperiodate treated sialoglycans, the resulting aldehyde of sialic acid was reduced with 10 mM NaBH₄ in phosphate-buffered saline for 2 h on ice in the dark. The reaction mixture was then dialyzed overnight against seawater at 4 °C.

Crosslinking of oxidized sialoglycans to Affi-Gel Hz — A 50 µl aliquot of Affi-Gel Hz beads was washed twice with 20 × of dH₂O. The beads were sedimented by centrifugation at 12,000 × g for 30 s and the supernatant discarded. Fifty µl of solution containing mild periodate-treated sialogycans was mixed continuously with the beads for 30 min at room temperature (total volume 100 µl). The beads were sedimented by centrifugation, and the supernatant recovered. The beads were resuspended in 50 µl of high salt buffer (2 M NaCl in 10 mM sodium acetate, pH 5.0), centrifuged and the supernatant recovered. The beads were then resuspended in 50 µl of acetate buffer and heated to 100 °C for 10 min, and the supernatant was recovered after centrifugation. The supernatants recovered after different washes were tested in the metachromatic assay and the thiobarbituric acid assay.

Measurements of intracellular Ca²⁺ and pH — Sperm intracellular Ca²⁺ and pH were measured as described (22). Briefly, fresh, undiluted semen was suspended in 4 × of dye loading buffer (450 mM NaCl, 9 mM KCl, 48 mM MgSO₄, 6 mM NaHCO₃, 1 mM CaCl₂, 10 mM HEPES, 50 µg/ml soybean trypsin inhibitor, pH 7.0). The suspension was gently mixed with dimethylsulfoxide (DMSO, final concentration...
0.6%) containing either fura-2 AM or BCECF AM at a final concentration of 12 µM, and the tube incubated on ice at least 8 h before washing. To remove free dye, sperm were sedimented by centrifugation in a swinging bucket rotor at 430 × g for 7 min. The supernatants were removed and 9 × of dye loading buffer added, followed by gently mixing until the pellet of sperm was completely resuspended. This washing procedure was repeated 3 times. The final cell pellet was resuspended in 4 × of dye loading buffer without soybean trypsin inhibitor, and stored on ice in the dark. For the measurements, 50 µl of fura-2 loaded sperm (2 × 10⁸ cells/ml), or 10 µl of BCECF loaded sperm, were placed in a 11 mm diameter round bottomed-glass tube containing 1.5 ml of 10 mM HEPES buffered-millipore filtered seawater pH 8.0 (HEPES-SW). The tube was mounted in a temperature controlled-cell holder (16 °C) in a FluoroMax-2 fluorometer (JOBIN YVON-SPEX), and the fluorescence at excitation/emission wavelength pairs of 380/500 and 340/500 nm (for fura-2), 490/510 and 440/510 nm (for BCECF) were recorded while continuously stirred with a micro magnetic bar. Fura-2 signals were calibrated to the ratio of 340/380 nm and presented as the increased [Ca²⁺]/Kd value or Δ[Ca²⁺]/Kd (27) obtained after 5 min of recording. The calibration of pH_i was performed as described (22).

Monosaccharides analyses—Monosaccharides analyses were done by Glycobiology Core Resource, Glycobiology Research and Training Center (University of California, San Diego). The DEAE-fractionated (dSG) and electrodialyzed (eSG) sialoglycan fractions were hydrolyzed. The hydrolysates were then analyzed by high-pH anion-exchage (HPAE) chromatography on a Dionex CarboPac PA-10 or PA-1 column with pulsed amperometric detector. Standard
mixtures of monosaccharides were run in parallel to calibrate HPAE retention times and to allow qualifications.

*Scoring the AR and the AR potentiation assay*—The AR was evaluated by scoring the number of acrosome intact and reacted sperm under a phase contrast microscope at 1,200 × magnification (16). Briefly, 10 µl of sperm suspension that was freshly diluted 50-fold in ice-cold HEPES-SW was mixed in a 1.5 ml centrifuge tube with 30 µl of HEPES-SW containing the test compound at 16 °C. After 5 min sperm were fixed with 40 µl of 6% glutaraldehyde in HEPES-SW. For the AR potentiation assay, the concentration of FSP resulting in 10-20% AR was determined. FSP at this concentration was then mixed with sialoglycans in the range of 0-25 µg heparin equivalent/ml. These mixtures were then tested in the AR assay.

**RESULTS**

*Isolation of the EJ sialoglycan fraction from FSP*—EJ was subjected to β-elimination which released the O-linked oligosaccharides and FSP from total EJ. The EJ oligosaccharides were then separated from FSP by DEAE chromatography. Each eluted fraction was tested in the metachromatic assay for negatively charged glycans such as sulfated glycosaminoglycans (28) and sulfated fucans (9). The first peak appeared in the fractions at −0.7 M NaCl (Fig. 1A). Monosaccharide compositional analysis of this sharp peak showed that it contained two major constituents: N-glycolylneuraminic acid (NeuGc; 78.5% of total sugar) and fucose (12.3% of total sugar; Table 1). The high molar ratio of NeuGc to fucose suggests the presence of polysialic acid in this fraction (4). The metachromatic assay is a novel method for
the detection of sulfated glycosaminoglycans, so the first peak was examined for uronic acid. Neither glucuronic acid (GlcA) nor galacturonic acid (GalA) were detected (Table 1). This first peak is hereafter referred to as the DEAE-purified sialoglycans (dSG). The second broad peak eluting at 1.6–2.5 M NaCl is FSP as determined in previous experiments (6, 9, 16). The elution profile of the FSP peak has a shoulder, which probably represents the two isotypes of FSP present in S. purpuratus EJ which differ in sulfate content (9). Fractions 16-20, representing the dSG peak, and fractions 40-75, representing the FSP peak, were pooled and both pools were separated by electrophoresis on a 4-15% SDS-polyacrylamide gel which was stained with 0.1% toluidine blue (Fig. 1B). Samples were equalized on the basis of heparin equivalents as determined by the metachromatic assay. FSP appeared as a smear in the high molecular mass range, whereas no staining appeared in the dSG lane. Since toluidine blue stains the fragmented FSP (22), the lack of stain in the dSG lane does not represent the degradation of FSP.

The dSG fraction potentiates the FSP-induced AR—The specific activity of purified FSP to induce the AR was examined in comparison with total EJ. Sugar concentrations of EJ and FSP were determined by the phenol-sulfuric acid method and are presented as hexose equivalents. Although sperm obtained from different batches differ in sensitivity of response to EJ (16), EJ is always a better inducer of the AR than purified FSP (Fig. 2A). In this experiment, 10 μg hexose/ml of FSP was needed to achieve 50% AR, whereas only 0.5 μg/ml total EJ induced 50% AR. Although purified FSP is an AR inducer, these data suggest that EJ contains other components that enhance AR induction. The dSG fraction was tested for AR inducing activity, alone or in combination with FSP (Fig. 2B). EJ at 3 μg hexose/ml
induced 98% AR, whereas 1% AR occurred with only seawater as the negative control. FSP at 3 µg/ml induced 20% AR, whereas 3 µg/ml dSG induced 2%. However, when FSP and dSG (3 µg/ml each) were combined, the AR reached 95%. Decreasing both FSP and dSG to 1.5 µg/ml each resulted in 90% AR. A 5-fold increase in FSP to 15 µg/ml induced 92% AR, however, the dSG at 15 µg/ml yielded only 1% AR. These data clearly show that the dSG fraction potentiates the FSP-induced AR.

The AR potentiation activity resides in 100 kDa glycans—The dSG fraction was chromatographed on Sepharose CL-6B. Fractions were tested in the metachromatic assay and the peak found to have an average mass of approximately 100 kDa (Fig. 3A). Since the dSG fraction contains fucose at 12.3% mole of total monosaccharides (Table 1), fucose content in each fraction was also determined by the Dische-Shettles cysteine method. The peak for fucose elution was identical to the peak determined by the metachromatic assay (Fig. 3B). Sepharose CL-6B fractions were further tested in the AR potentiation assay in the presence of limiting amount of FSP. The maximum activity for AR potentiation corresponded to the peak determined by the metachromatic assay (Fig. 3C).

Sialic acid is a component of the metachomasia positive glycans—The dSG fraction was subjected to further purification by electrodialysis. After 4 h electrodialysis, 23% of total sialic acid was removed (data not shown). A molar ratio between fucose and NeuGc was approximately 1:6 (Table 1). The electrodialyzed sialoglycan fraction (eSG) was tested in the AR potentiation assay. The AR specific activity of the eSG increased approximately 4-fold relative to the dSG fraction when both fractions were normalized to heparin equivalents (Fig. 4A). To be specific, the
concentrations of dSG or eSG that are required for 50% AR were 13.5 or 3.0 µg/ml, respectively. eSG was chromatographed on Sepharose CL-6B. Two peaks, one representing the metachromatic assay, and the other sialic acid, closely overlapped, but were not perfectly superimposable (Fig 4B). The eSG fractions were treated with *Vibrio cholerae* neuraminidase. After a 24 h incubation, the reaction mixture was rechromatographed on Sepharose CL-6B. The peaks of metachromasia and sialic acid were now superimposable (Fig. 4C). The small peak at fraction 57 is monosialic acid released by the neuraminidase. However, the majority of sialic acid remained associated with the original glycans. The reason that the two peaks are not perfectly superimposable (Fig. 4B) may be due to the heterogeneous sialylation of the SG molecules (4). The time course of sialic acid release (Fig. 4D) shows maximum release (30% of total) by 1 h. These results show that approximately 70% of total sialic acid in the eSG is neuraminidase-resistant. The parallel decreases in both metachromasia and sialic acid suggest that the glycans detected by the metachromatic assay contain sialic acid (Fig. 4D).

The eSG fraction was treated with 2 mM NaIO₄ (mild metaperiodate) to remove the glycerol side chain and form an aldehyde in the sialic acid (26). Following the treatment, the aldehyde group of eSG was used to covalently couple it to Affi-Gel Hz (beads containing hydrazine groups). Using the TBA assay, 86.2% of total sialic acid bound to the beads. However, in the absence of NaIO₄ oxidation, 34.4% bound to the beads (Fig. 4E). Using the metachromatic assay, 81.2% of total negatively charged glycans bound to the beads, whereas in the no NaIO₄ control, only 11.3% bound to the beads. These data show that the metachromasia positive glycans responsible for AR potentiation contain sialic acid.
Sialoglycans increase sperm pH\textsubscript{i} but do not increase [Ca\textsuperscript{2+}]\textsubscript{i}—To understand the underlying mechanism of AR potentiation by sialoglycans, we first examined the effect on sperm [Ca\textsuperscript{2+}]\textsubscript{i}. The eSG alone, when added to fura-2 loaded-sperm, had no effect on [Ca\textsuperscript{2+}]\textsubscript{i} (Fig. 5A). FSP at 0.25 µg/ml induced 30% of the sperm to AR; these sperm showed a moderate increase in [Ca\textsuperscript{2+}]\textsubscript{i} compared to that of total EJ. FSP mixed with eSG increased the percentage of AR in a concentration dependent manner (0-10 µg/ml). However, this mixture did not significantly alter the [Ca\textsuperscript{2+}]\textsubscript{i} in comparison to FSP alone. Increases in [Ca\textsuperscript{2+}]\textsubscript{i} were quantified and are represented as \(\Delta[Ca^{2+}]_{i}/K_d\) as described under “Experimental Procedures”. Purified FSP caused a concentration dependent increase in [Ca\textsuperscript{2+}]\textsubscript{i} (Fig. 5B). However, eSG did not increase [Ca\textsuperscript{2+}]\textsubscript{i} either in the presence or absence of FSP. These results show that sialoglycans do not up-regulate sperm Ca\textsuperscript{2+} channels. Next, we examined the effect on sperm pH\textsubscript{i}, because an increase in pH\textsubscript{i} is a crucial event in AR induction (3, 29). The eSG alone increased sperm pH\textsubscript{i} in a concentration dependent manner (Fig. 6A). The pH\textsubscript{i} change produced by FSP was approximately 3-fold greater than that produced by eSG. The FSP-induced pH\textsubscript{i} increase is known to be blocked by Ca\textsuperscript{2+} channel blockers such as nifedipine (22), or extracellular high K\textsuperscript{+} (10). In fact, 50 µM nifedipine, which completely blocks the opening of the first sperm Ca\textsuperscript{2+} channel (11), inhibited the FSP-induced pH\textsubscript{i} increase (Fig. 6B). The FSP-induced pH\textsubscript{i} increase was also inhibited by 50 mM K\textsuperscript{+}. Three hundred mM Ni\textsuperscript{2+}, which blocks the second sperm Ca\textsuperscript{2+} channel (11), also inhibited the FSP-induced pH\textsubscript{i} increase (Fig. 6B). In contrast, nifedipine and 50 mM K\textsuperscript{+} were only moderately effective in
blocking the eSG-induced pH increase (Fig. 6B). However, Ni\(^{2+}\) completely blocked the eSG-induced pH increase. These data show that EJ sialoglycans induce sperm pH increases by a different pathway than the one used by FSP.

*Sialic acid residues of sialoglycans are crucial for AR potentiation*—The eSG fractions, treated either with neuraminidase or mild metaperiodate, were tested in the AR potentiation assay. The non-treated eSG fraction (plus FSP) potentiated the AR in a concentration dependent manner and a maximum percentage AR was obtained at 80 µg heparin/ml (Fig. 7A). The eSG’s potentiation activity was completely lost when it was treated with either neuraminidase or mild metaperiodate (Fig. 7A). The percentage AR was constant at 15 ± 5% in either neuraminidase or mild metaperiodate treated eSGs up to 160 µg/ml, showing that neither sample negatively effects AR induction. These results demonstrate that a glycerol side chain of NeuGc residues is essential for potentiation activity. Finally, we tested the effects of the treated eSGs on sperm pH. The non-treated eSG at 50 µg heparin/ml caused a 0.05 unit increase in pH (Fig. 7B). In contrast, the eSG fractions treated with either neuraminidase or mild metaperiodate were completely ineffective at increasing pH. In conclusion, sialoglycans, consisting mainly NeuGc, increase sperm pH by an unknown mechanism which is independent of the FSP-regulated [Ca\(^{2+}\)]\(_i\) increasing pathway. These sialoglycans markedly potentiate the FSP-induced AR.

**DISCUSSION**

Approximately 80% of the mass of *S. purpuratus* EJ is FSP and 20% comprises the sialoglycoproteins (6). The purified FSP alone will induce the increase in
intracellular Ca\textsuperscript{2+} and pH needed to trigger the AR (9, 16, 22), but it is much less active than whole EJ (Fig. 2A). Treatment of EJ with mild periodate which has no effect on FSP, decreases the AR activity (18). These results suggest that other EJ components, especially periodate-sensitive glycans, must be involved in AR induction though an ancillary pathway, or as co-factors that facilitate the FSP-induced AR.

β-elimination of whole EJ degrades the protein components and releases O-linked oligosaccharides. Sialoglycans (SG) can be completely separated from FSP by DEAE chromatography (Fig. 1). Monosaccharide analysis of the SG fraction shows that its molar ratio of fucose to sialic acid is approximately 1 to 6 with small amounts of other monosaccharides (Table 1). Other workers have also found that the oligosaccharides in EJ are rich in sialic acid, and that only NeuGc and not NeuAc is present (30). \textit{S. purpuratus} EJ contains a unique polysialic acid; [ Neu5Gc\textsubscript{α2→5-ΟglycolylNeu5Gc]}\textsubscript{n} which is O-glycosidically linked to a 250 kDa glycoprotein (4). Also, a disaccharide, Fuc\textsubscript{p1→4}NeuGc, was found in the acid hydrolysate of \textit{Pseudocentrotus depressus} EJ (31). Although some of these investigators had tried to induce the AR with fractionated SG, until now no one had reported attempts to use the SG mixed with limiting amounts of FSP to look for possible synergistic effects on AR induction.

This paper demonstrates for the first time that the SG fraction is a potent potentiator of the FSP-induced AR. When tested alone, no AR inducing activity was found in the SG fraction (Fig. 2B), showing that FSP is a necessary component. The AR potentiation activity was found in glycans of an apparent molecular mass of 100 kDa that are strongly negatively-charged and contain fucosyl residues (Fig. 3).
Further purification of dSG by electrodialysis removed 23% of the total sialic acid and resulted in a 4-fold increase in AR potentiation activity (Fig. 4A). The evidence that sialic acid is a constituent of the metachromasia positive glycans was demonstrated by Sepharose CL-6B chromatography of eSG after digestion with *Vibrio cholerae* neuraminidase (Fig. 4B, 4C). This enzyme is known to cleave a unique EJ polysialic acid (32). Mild metaperiodate treatment of the eSG fraction immobilized the metachromasia positive glycans to hydrazine beads (Fig. 4E). The sialyl residues of the eSG fraction are crucial for the AR potentiation, because the above treatments, which specifically destroy sialyl residues, caused a loss of biological activity (Fig7A).

The eSG fraction had no effect on the $[Ca^{2+}]_i$ of fura-2 loaded sperm (Fig. 5). However, eSG increased pH$_i$ even in the absence of FSP (Fig. 6A). These data suggest that the SG-induced AR potentiation signaling pathway is different from the FSP-induced pathway. This hypothesis was further supported by the differential sensitivities of the FSP and eSG responses to the Ca$^{2+}$ channel blocker, nifedipine and high extracellular K$^+$ (Fig. 6B). The eSG-induced pH$_i$ increase was abolished by neuraminidase or mild metaperiodate treatment, suggesting that sialyl residues of sialoglycans are responsible for the AR potentiation.

Our previous studies showed that suREJ1 is a receptor for FSP (15, 16). Antibodies showed that suREJ1 localizes to the entire flagellum as well as the acrosomal region (33). suREJ3, a homologue of suREJ1, localizes only to the acrosomal region (17). Because suREJ3 also has extracellular carbohydrate recognition domains (17, 34), we speculate that it might be a receptor for SG. The eSG fraction increased pH$_i$ by 0.08 units at maximum concentration, whereas a pH$_i$
increase of 0.23 units occurred in FSP (Fig. 6A). If SG binds to suREJ3 to evoke the pH$_i$ increase, this small increase (compared to FSP) may be due to a local pH$_i$ increase that might occur only in the acrosomal region.

Two different sperm plasma membrane Ca$^{2+}$ channels mediate the FSP-induced AR (11). Our data suggest that there may be two distinct channel/exchanger systems to regulate the pH$_i$ increase occurring during AR induction. Several parallel signaling pathways may underlie AR induction by whole EJ (3). To our knowledge, this is the first demonstration for a role of sialic acid residues in the induction of the animal sperm AR. Structure studies of SG and molecular cloning of its receptor will increase our understanding of this unique signal transduction pathway.

ACKNOWLEDGMENTS

We thank Drs. B. Hayes, A. Varki, J. Esko and H. van Halbeek for helpful discussions. We thank Drs. Meredith Gould, Sawako Oshima and Jose Stephano for the collection of gametes. We thank Dr. B. Galindo and Sheryl Huffman for technical suggestions and discussions. This work was supported by NIH grants HD12986 and 1P50DK57325.

REFERENCES


FOOT NOTE

The abbreviations used are: AR, acrosome reaction; EJ, egg jelly; FSP, fucose sulfate polymer; [Ca$^{2+}$], intracellular Ca$^{2+}$; intracellular pH, pH$_i$; sea urchin receptor for egg jelly-1, suREJ1; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; ASW, artificial seawater; BCECF, 2',7'-bis-(2-carboxyl)-5-(and-6-)carboxyfluorecein; dSG, DEAE-purified sialoglycans; eSG, electrodialyzed sialoglycans.

FIGURE LEGENDS

FIG. 1. DEAE-cellulose chromatography of $\beta$-eliminated EJ glycans. A, EJ was subjected to $\beta$-elimination, neutralized with acetate buffer and applied to a DEAE-cellulose column. After washing the column, the bound glycans were eluted with a linear gradient of 0-3 M NaCl in acetate buffer. Elution was monitored for negatively charged polysaccharides by the metachromatic assay (●) and the fractions of the first peak (closed bar) and the second broad peak (open bar) were pooled. B, the samples (2.5 µg heparin equivalent) obtained from the first peak (SG) and the second peak (FSP) were subjected to 4-15% SDS-PAGE and the gel stained with 0.1% toluidine blue. Molecular mass standards are shown on the left.

FIG. 2. Sialoglycans potentiate the FSP induced-AR. A, total EJ (●) and purified FSP (○) were tested in the AR assay. The samples were normalized to equal concentrations of neutral sugars as determined by the phenol sulfuric acid assay. Sperm at a 200-fold dilution of semen were incubated with either EJ or FSP for 5
min in HEPES-SW and then fixed with 6% glutaraldehyde in seawater. The percent AR was scored by observing 200 sperm per data point. B, EJ, FSP and DEAE purified-sialoglycans (dSG) were tested in the AR assay, separately or in combination. The concentration of neutral sugar (µg hexose/ml) in each compound is indicated in the parenthesis.

FIG. 3. Sepharose CL-6B gel filtration chromatography. A, DEAE purified-sialoglycans dSG; at 1 mg heparin/ml) were subjected to Sepharose CL-6B chromatography. Even numbered fractions were tested in the metachromatic assay (●). The arrowheads indicate the void volume (V₀) and the total volume (Vₜ) of the column. B, the even numbered fractions were tested in the Dische-Shettle cysteine assay for fucose. C, in addition to all even numbered fractions, odd numbered fractions between 39-51 were tested in the AR potentiation assay. An equal volume of each fraction was mixed with 1 µg/ml FSP. At this concentration FSP alone induced 43% of the sperm to AR. This percentage is represented as 0% potentiation activity. The highest activity (80% AR) was seen in fraction 46 of the metachromatic assay, which was normalized as 100% potentiation activity. AR potentiation activity of each fraction was normalized relative to 80% AR. In all chromatograms fraction 46 is marked by the arrows.

FIG. 4. Treatments of electrodialysis purified sialoglycans (eSG) with neuraminidase or mild metaperiodate. A, the dSG was further purified by electrodialysis as described under “Experimental Procedures”. The dSG (●) and the eSG (❍) were tested for AR potentiation activity. There was approximately a 4-fold increase in AR potentiation activity in the eSG sample. B, the eSG was chromatographed on a Sepharose CL-6B column. C, the eSG was treated with Vibrio
*cholerae* neuraminidase for 24 h and the reaction mixture subjected to Sepharose CL-6B chromatography. The pooled fractions were then tested in the metachromatic assay (●) and the thiobarbituric acid assay (○). The *arrowhead* indicates a monosialic acid peak. D, time course eSG-digestion with neuraminidase was monitored up to 24 h. The released sialic acids were removed by electrodialysis. The electrodialized eSG samples were tested in the metachromatic assay (●) and the thiobarbituric acid assay (○). E, the eSG was treated with 2 mM NaIO₄ (mild periodate-treated) or PBS (non-treated), and mixed for 30 min with (+), or without (−) Affi-Gel Hz beads. The supernatants were recovered and tested in the metachromatic assay (closed columns) and the thiobarbituric acid assay (open columns).

**Fig. 5.** Sialoglycans do not increases sperm \([\text{Ca}^{2+}]_{i}\). A, 0.25 µg hexose/ml of FSP and/or eSG (0-10 µg heparin/ml) were added to fura-2 loaded-sperm as indicated by the *arrow*. The concentrations of eSG used are indicated (µg heparin/ml) on the right. After the 5-min measurement of \([\text{Ca}^{2+}]_{i}\), sperm were fixed and the %AR determined and shown in the parentheses. B, sperm \([\text{Ca}^{2+}]_{i}\) in response to various concentrations of FSP and eSG was measured. Data show increased \([\text{Ca}^{2+}]_{i}\) /Kd values after the addition of either FSP (●) or eSG (○). Combinations of a constant amount of FSP (0.24 µg heparin/ml) and varying amounts of eSG were also examined (❒). FSP and eSG concentrations were determined by the metachromatic assay and are indicated as heparin equivalents.

**Fig. 6.** Sialoglycans increase sperm pH. A, changes in sperm pH were measured at various concentrations of FSP and eSG. The increased pH (ΔpH) values obtained 5
min after addition of FSP (●) or eSG (○) are presented. B, 2.5 µg heparin/ml FSP or 25 µg heparin/ml eSG were tested in the absence or presence of 50 µM nifedipine (Nif), 50 mM KCl (K⁺) and 300 µM NiCl₂ (Ni²⁺). The columns show mean ± SE from three independent experiments.

Fig. 7. Treatments of eSG with neuraminidase or mild metaperiodate abolish the AR potentiation activity and ability to increase pHᵢ. A, the eSG fractions treated with either *Vibrio cholerae* neuraminidase or mild metaperiodate were tested in the AR potentiation assay. The concentration of each sample was normalized to heparin equivalents. FSP at 1.0 µg hexose/ml alone induced 15% AR. Each eSG sample was mixed with 1.0 µg hexose/ml FSP. Samples are: non-treated (●), neuraminidase treated (□) and mild metaperiodate treated (○) eSG. B, the ability of these samples to alter sperm pHᵢ was examined. The increased values of pHᵢ (ΔpHᵢ) are represented as % ΔpHᵢ relative to the ΔpHᵢ obtained by non-treated eSG. The non-treated eSG (intact) at 50 µg heparin/ml increased pHᵢ by 0.05 units. The neuraminidase treated (Sialidase), and mild metaperiodate treated (NaIO₄) eSG did not increase pHᵢ.
### Table I

**Monosaccharide composition analyses of EJ sialoglycan fractions**

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>dSG molar ratio</th>
<th>eSG molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fucose</strong></td>
<td>1.00 (c)</td>
<td>1.00 (c)</td>
</tr>
<tr>
<td><strong>Mannose/Xylose</strong></td>
<td>0.30</td>
<td>0.22</td>
</tr>
<tr>
<td><strong>Galactose</strong></td>
<td>0.06</td>
<td>0.05</td>
</tr>
<tr>
<td><strong>GlcNAc</strong></td>
<td>0.22</td>
<td>0.08</td>
</tr>
<tr>
<td><strong>GalNAc</strong></td>
<td>0.16</td>
<td>0.06</td>
</tr>
<tr>
<td><strong>Neu5Ac</strong></td>
<td>ND (e)</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Neu5Gc</strong></td>
<td>6.39</td>
<td>5.85</td>
</tr>
<tr>
<td><strong>GlcA</strong></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>GalA</strong></td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(a\) The only monosaccharide present in FSP is fucose (9, 21).
\(b\) The error margins in the values are ±10%.
\(c\) Arbitrarily set to 1.00.
\(d\) The peak in the HPAE profile may consist of a mixture of mannose and xylose. Mannose and xylose elute at the same retention time under these conditions.
\(e\) ND, not detected.
\(f\) GlcA, glucuronic acid; GalA, galacturonic acid.
Figure 2

A) Graph showing the relationship between sugar concentration (μg hexose/ml) and the percentage of acrosome reaction (% Acrosome reaction).

B) Bar chart comparing the percentage of acrosome reaction for different treatments:
- SW
- EJ (3)
- FSP (3)
- dSG (3)
- FSP (3) + dSG (3)
- FSP (1.5) + dSG (1.5)
- FSP (15)
- dSG (15)
Fig. 7

A

B

% AR

\[ \text{Sialoglycans (µg heparin/ml)} \]

\[ \Delta \text{pH}_i (%) \]

Intact  Sialidase  NaIO_4
Egg sialoglycans increase intracellular pH and potentiate the acrosome reaction of sea urchin sperm
Noritaka Hirohashi and Victor D. Vacquier

J. Biol. Chem. published online January 2, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M110661200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2002/01/02/jbc.M110661200.citation.full.html#ref-list-1