High Molecular Mass Egg Fucose Sulfate Polymer Is Required for Opening Both Ca\(^{2+}\) Channels Involved in Triggering the Sea Urchin Sperm Acrosome Reaction* 

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A linear fucose sulfate polymer (FSP), >10\(^6\) daltons, is a major component of sea urchin egg jelly. FSP induces the sperm acrosome reaction (AR), an exocytotic process required for animal fertilization. Two Ca\(^{2+}\) channels active during AR induction, the first opens 1 s after FSP addition, and the second opens 5 s after the first. Mild acid hydrolysis of FSP results in a linear decrease in polymer size. The ability of FSP to induce the AR and activate sperm Ca\(^{2+}\) channels decreases with increasing time of hydrolysis. Hydrolyzed FSP of ~60 kDa blocks intact FSP from inducing the AR. At 44 \(\mu\)g/ml hydrolyzed FSP, Ca\(^{2+}\) entry into sperm is almost equal to that occurring in 3.8 \(\mu\)g/ml intact FSP; however the AR is not induced. The shape of the [Ca\(^{2+}\)] increase curve and use of the Ca\(^{2+}\) channel blockers nifedipine and Ni\(^{2+}\) indicate that hydrolyzed FSP opens the second Ca\(^{2+}\) channel, but not the first, and thus does not induce the AR. The giant size of intact FSP is required to open both Ca\(^{2+}\) channels involved in triggering the AR.

The sperm acrosome reaction (AR)\(^1\) is required for animal fertilization and is a potential target for the development of novel methods of non-hormonal contraception. Sea urchin spermatozoa are ideal for studying signal transduction underlying the animal sperm AR because they can be obtained as pure cells in vast quantities at low cost. The AR is triggered when sperm contact the jelly layer surrounding the egg (EJ). Morphologically, the AR involves the exocytosis of the acrosomal vesicle and the polymerization of actin to form the acrosomal process; both events are required for sperm to bind to and fuse with eggs. Physiologically, the AR requires the influx of Ca\(^{2+}\) and Na\(^+\) and the efflux of H\(^+\) and K\(^+\) ions (1, 2). There are two plasma membrane Ca\(^{2+}\) channels involved in AR induction: the first is receptor-operated and opens 1 s after sperm contact EJ, the second opens 5 s after the first in response to increased intracellular pH (pH\(_i\)). The second channel can also transport Mn\(^{2+}\) (2–4). The Ca\(^{2+}\) channel blocker nisoldipine does not block Mn\(^{2+}\) movement through the second channel but does block the first channel and hence also blocks the AR (3).

Eighty percent of the mass of sea urchin EJ is a fucose sulfate polymer (FSP) of >1 million daltons (5). Purified FSP, having no amino acid content, induces the AR (5, 6). However, oligosaccharides of EJ glycoproteins substantially potentiate the FSP-induced AR, suggesting there is more than one receptor system regulating ion channels that trigger the AR (7). \(^2\) FSP is a linear polymer of \(\alpha\)-1,3-fucose with a species-specific pattern of sulfation of the fucosyl residues (8). The sulfation pattern is responsible for FSP's species-specific induction of the AR (9, 10). FSP is also a potent inhibitor of human blood coagulation through its high affinity binding to heparin cofactor II (11).

Receptor for egg jelly-1 (REJ1) is a 1450-amino acid glycoprotein located in the plasma membrane over the sea urchin sperm acrosomal vesicle and also on the sperm flagellum. Available data support the hypothesis that REJ1 is at least one of the sperm receptors for FSP (6, 12). Purified REJ1 neutralizes the AR activity of EJ (12). An affinity column of REJ1 binds only FSP when crude EJ is applied (6). Monoclonal antibodies to REJ1 induce Ca\(^{2+}\) influx into sperm (13) and induce the AR (14). Approximately 1000 residues of REJ1 consist of a domain named “the REJ module,” which is found in only one other protein family, the polycystin-1s (PKD1; Refs. 12 and 15). PKD1 is mutated in 85% of autosomal dominant polycystic kidney disease, the most frequent human genetic disease (16). The polycystin-1 proteins are a new class of signaling molecules whose function remains to be clarified. Recent work shows that they may be regulators or subunits of nonselective cation channels (17). The “REJ module” homology between REJ1 and PKD1 is restricted to extracellular regions. Both proteins have carbohydrate binding, C-type lectin domains (12, 18). Ligands for PKD1 remain unknown.

FSP is the only ligand known to bind to a protein containing a REJ module. Because FSP is a giant polymer, we studied what effect reduction of its size might have on sperm physiology. Mild acid hydrolysis of purified FSP randomly cleaves the large polymer to smaller size. Treatment of sperm with fragmented FSP triggers the opening of the second but not the first Ca\(^{2+}\) channel and also fails to induce the AR. Further results suggest that intact and fragmented FSP compete for the same binding site(s) and that the binding of fragmented FSP greatly desensitizes the AR response. The giant size of intact FSP is therefore required for the normal opening of both sperm Ca\(^{2+}\) channels involved in AR induction.

EXPERIMENTAL PROCEDURES

Chemicals—Fura-2/AM, BCECF/AM, and nigericin were from Molecular Probes (Eugene, OR). Dimethyl sulfoxide was from Research Org.

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\(^2\) The abbreviations used are: AR, acrosome reaction; EJ, egg jelly; FSP, fucose sulfate polymer; iFSP, intact FSP; hFSP, hydrolyzed FSP; 5b-hFSP, FSP fragments generated by 5 b hydrolysis; REJ1, receptor for egg jelly; PKD1, polycystin-1; ASW, artificial seawater; BCECF, 2',7'-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein; DHP, dihydropyridine; CRD, carbohydrate recognition domain.

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Fucanases (Cleveland, OH). Rhodizonic acid disodium and L-tartric acid were from Aldrich. Ionomycin was from Calbiochem. Other biochemical reagents were from Sigma.

Ganete Handling and Purification of FSP—Sea urchins (Strongylocentrotus purpuratus) were injected with 0.5 M KCl, and eggs spawned into Petri dishes. Settled eggs were washed once and passed through 250-μm Nitex mesh. Eggs were acid-treated to solubilize EJ by adjusting the pH of seawater to 5.0 (2 min) with 0.1 N HCl. Solubilized EJ was recovered by gentle hand centrifugation to sediment dejellied eggs, and the supernatant was then centrifuged at 30,000 × g for 20 min. This crude EJ supernatant was stored at −20°C. The precipitate was sedimented at 100,000 × g for 40 min and was washed three times with 0.1 M NaCl, pH 6.0. The precipitate was obtained by centrifugation at 140,000 × g for 75 min. EJ supernatants were stored at −80°C. Egg suspension, collected as undiluted semen and stored on ice. The purification of FSP was performed as described before (5) with some modifications. Egg jelly was precipitated by the addition of an equal volume of 95% ethanol. The precipitate was sedimented at 1000 × g for 5 min and redissolved in distilled water. β-Elimation was performed by making this solution 0.05 M KOH containing 1 M NaBH4 (24 h at 45°C). An equal volume of acetate buffer (50 mM sodium acetate, pH 5.0) was then added, followed by the addition of 2 M acetic acid to adjust the pH to 5.0. SDS-PAGE followed by silver staining confirmed that the EJ proteins were completely degraded. This solution was applied to a DEAE-cellulose column (14 × 60 mm) equilibrated with the above acetate buffer. After washing with 5-column volumes of acetate buffer, bound material was eluted by a linear gradient of NaCl (0-2.5 M) in acetate buffer. Each fraction was tested by the metachromatic assay for sulfated glycans (19) and the phenol-sulfuric assay for neutral sugars (20). The FSP peak was eluted between 1.5 and 2.5 M NaCl. Fractions containing FSP were pooled, precipitated with 50% ethanol, dissolved in deionized distilled water (ddH2O), and dialyzed against ddH2O.

Fragmentation of FSP by Mild Acid Hydrolysis—The purified FSP (1 mg of fucose/ml) was subjected to mild acid hydrolysis (50 mM sodium citrate, pH 3.9, at 80°C). Hydrolysis was followed with time by removing 1-ml aliquots and adding 0.1 ml of HEPES buffer (1 mM HEPES, pH 8.0 at 4°C). Cleavage of the glycan chains was assessed by SDS-PAGE using a 2-15% linear gradient of polyacrylamide gel, followed by toluidine blue staining (0.1% toluidine blue in 20% methanol, 7% acetic acid; Ref. 6). The hydrolysates were dialyzed against chemical analyses or artificial seawater (for Ca2+-free, 450 mM NaCl, 48 mM MgSO4, 9 mM KCl, 6 mM NaHCO3; for Ca2+-plus, add 10 mM CaCl2) for biological assays. The size of the hydrolyzed FSP (hFSP) was estimated by Sepharose CL-6B gel filtration chromatography (Amersham Biosciences, Inc.). Samples of 1 mg of FSP were applied to the column (12 × 60 cm) equilibrated with 10 mM HEPES-buffered seawater (pH 8.0), and every fraction was tested with the metachromatic assay (8). The void and total volume of the column was determined with dextrans.

Analytical Methods—Sugar concentrations of intact FSP (iFSP) and hFSP were determined by the phenol-sulfuric acid assay (20). One hundred μl of sample was placed in a clean glass tube and mixed with 100 μl of 5% phenol. One ml of concentrated sulfuric acid was then added, and the tube was vortexed vigorously and left for 10 min to cool, the absorbance at 490 nm was read, and the sugar concentrations were determined using L-fucose as a standard. The concentration of reducing sugar was determined by the Park-Johnson method (21). One hundred μl each of sample, ferricyanide solution (0.5 μlter K3Fe(CN)6), and carbonate cyanide solution (50 mM Na2CO3, 8.7 mM KCl) were mixed in a glass tube and heated in a boiling water bath for 15 min. After cooling to room temperature, 0.5 ml of ferric iron solution (1.5 g of Fe3O4·H2O, 1 g of SDS in 1 liter of 0.05 M H2SO4) was added, and the absorbance was read at 690 nm. Phosphate content in hFSP was determined by the rhodizonate method (22). Samples containing 0–2 μg of phosphate was placed in 1.5-ml Eppendorf tubes without sealing and hydrolyzed with 0.1 M NaOH at 80°C in a heat block until dry. The absorbance at 490 nm was read, and the phosphate concentrations were determined using L-fucose as a standard. The concentration of reducing sugar was determined by the Park-Johnson method (21). One hundred μl each of sample, ferricyanide solution (0.5 μlter K3Fe(CN)6), and carbonate cyanide solution (50 mM Na2CO3, 8.7 mM KCl) were mixed in a glass tube and heated in a boiling water bath for 15 min. After cooling to room temperature, 0.5 ml of ferric iron solution (1.5 g of Fe3O4·H2O, 1 g of SDS in 1 liter of 0.05 M H2SO4) was added, and the absorbance was read at 690 nm. Phosphate content in hFSP was determined by the rhodizonate method (22). Samples containing 0–2 μg of phosphate was placed in 1.5-ml Eppendorf tubes without sealing and hydrolyzed with 0.1 M NaOH at 80°C in a heat block until dry. The absorbance at 490 nm was read, and the phosphate concentrations were determined using L-fucose as a standard.

Measurements of Intracellular Ca2+ and pH—Sperm were incubated in a 15-ml round bottom polystyrene tube with dimethyl sulfoxide (final concentration 0.6%) containing either fura-2/AM or BCECF/AM at a final concentration of 12 μM, and the tube was incubated at least 8 h before washing. To wash the cells free of the dyes, sperm suspensions were sedimented in a swinging bucket rotor at 430 × g for 7 min. The resulting supernatants were removed, and 4 volumes of fresh dye loading buffer were added, followed by gentle mixing until the pellet of sperm was completely resuspended. This washing procedure was repeated twice. The final cell density was 1010 cells/ml. Sperm suspensions were subsequently resuspended in artificial seawater, pH 8.0 (HEPES-ASW). The tube was mounted in a FluoroMax-2 fluorometer (Jobin Yvon-SPEX) and excited at 340 nm. Fluorescence intensity at excitation wavelength 340 nm, F0, is the fluorescence intensity at excitation wavelength 380 nm, F0, free of Ca2+. The fluorescence intensity at excitation wavelength 380 nm, F0, free of Ca2+ is approximately equal to F0, when all of the fura-2 is Ca2+-bound (3 μM ionomycin).

Results

Mild Acid Hydrolysis of FSP—The β-eliminated, DEAE-purified FSP was subjected to mild acid hydrolysis in 0.1 M sodium citrate, pH 3.9, 80°C for up to 5 h. The hydrolysates were neutralized and analyzed on 2–15% gradient SDS-PAGE gels, which were stained with toluidine blue (Fig. 1A). The apparent size of FSP decreases with time, the toluidine blue staining appearing as a more diffuse band, suggesting that variable length fragments are created by randomly breaking glycosidic bonds. A similar pattern of FSP fragmentation was observed either in acetic or citrate buffers, pH 3.9, or in 0.1 M HCl (data not shown). During acid hydrolysis, the generation of reducing sugar was linear with time, and a decrease in FSP size was seen up to 5 h, the last time point taken (Fig. 1A and B). The size of FSP fragments generated by 5-h hydrolysis was determined by Sepharose CL-6B gel filtration chromatography. The peak appeared at an average molecular weight of about 60 kDa, whereas intact FSP eluted in the void volume fractions (Fig. 1C). Considering a molar ratio of 1.1 to 1.0 sulfate groups per fucose (8), the average FSP fragment after 5 h of hydrolysis has about 220 fucose residues. Loss of sulfate groups during hydrolysis was examined by the rhodizonate assay. After 5 h of
hydrolysis, 66.2% of the sulfate groups remained, whereas 82.0% of the fucose was recovered (Fig. 1D). The actual loss of sulfate groups from the retained fucose polymer was 19.3%. However, acid treatment with 0.1 M HCl caused a 56% loss of sulfate groups after 5 h at 80 °C (data not shown). For this reason, subsequent hydrolysis of FSP to hFSP was done in citrate buffer.

Relationship between the Size of FSP and Increase in Intracellular \([\text{Ca}^{2+}]\) and \(\text{pH}\)—FSPs of various hydrolysis times were dialyzed against seawater and tested for AR induction. All samples were diluted with seawater to the point at which iFSP yielded 80% AR. The AR activity of FSP decreased with increasing times of acid hydrolysis (Fig. 2A). The AR activity reached a minimum of 8% after 3 h of hydrolysis (compared with 3% in the no FSP control). Experiments were performed to determine if the decrease in AR activity was due to a loss of \([\text{Ca}^{2+}]\) influx. hFSP at final concentrations of 14.1 \(\mu\text{g}\) of fucose/ml from various hydrolysis times were mixed with fura-2-loaded sperm (Fig. 2B). With iFSP (0 h), the level of \([\text{Ca}^{2+}]\) reached a plateau in 100 s and continued for more than 300 s (Fig. 2B). This maximum level of \([\text{Ca}^{2+}]\), decreased as a function of FSP hydrolysis time. hFSP from 2–5 h of hydrolysis did not trigger the rapid increase in \([\text{Ca}^{2+}]\), seen in iFSP. hFSP from 2–5 h of hydrolysis caused a small, continuous increase in \([\text{Ca}^{2+}]\). From these data, we conclude that FSP of larger molecular mass is needed to induce the normal opening of sperm \([\text{Ca}^{2+}]\) channels.

High Concentrations of 5h-hFSP Induce Elevated \([\text{Ca}^{2+}]\), and \(\text{pH}\), but Do Not Induce the AR—The effects of treating sperm with high concentrations of 5h-hFSP were also studied. The increase in \([\text{Ca}^{2+}]\), by either iFSP or 5h-hFSP is linear when plotted on a logarithmic scale (Fig. 3A). The plots intersect the horizontal axis at \(-0.1 \mu\text{g}\) of fucose/ml, suggesting that the threshold concentrations to evoke \([\text{Ca}^{2+}]\) influx by iFSP and 5h-hFSP are similar. This also suggests that the affinities of iFSP and 5h-hFSP for the sperm surface are comparable. The difference in \([\text{Ca}^{2+}]\), levels becomes larger as the concentrations of both FSPs increase. However, 5h-hFSP is much poorer at opening sperm \([\text{Ca}^{2+}]\) channels than is iFSP.

A mandatory increase in \(\text{pH}\), of about 0.2 unit occurs along with \([\text{Ca}^{2+}]\) influx to trigger the AR (2). Results similar to the \(\Delta[\text{Ca}^{2+}]/K_d\) curve (Fig. 3A) were obtained when \(\Delta\text{pH}\), was measured, except that the curve for iFSP was not linear (Fig. 3B). The threshold values for \(\Delta\text{pH}\), were higher than those for \(\Delta[\text{Ca}^{2+}]/K_d\) (Fig. 3A compared with 3B). The sperm used for \([\text{Ca}^{2+}]\), measurements were immediately fixed and scored for AR (Fig. 3C). The AR plots for iFSP are similar to those obtained in the \([\text{Ca}^{2+}]\), experiments. In contrast, the percentage of AR triggered by 5h-hFSP was very low even when \([\text{Ca}^{2+}]\), and \(\text{pH}\), were increased with high concentrations of 5h-hFSP. For example, the equivalent increases in \([\text{Ca}^{2+}]\), and \(\text{pH}\), caused by 3 \(\mu\text{g}\) of fucose/ml of iFSP were observed at 50 \(\mu\text{g}\) of fucose/ml of 5h-hFSP. However, when 50% AR was observed at 50 \(\mu\text{g}\) of fucose/ml of 5h-hFSP. These data show that high concentrations of hFSP induce the increases in both \([\text{Ca}^{2+}]\), and \(\text{pH}\), but do not induce the AR.

hFSP Blocks AR Induction by iFSP—One explanation for the loss of biological activity of hFSP is the loss of affinity for its receptor. Another explanation is that FSP retains the capability to bind to its receptor, but this does not open sperm \([\text{Ca}^{2+}]\) channels.
channels. In the later case, hFSP would be expected to act as an antagonist and competitively block the AR triggered by iFSP. Sperm were preincubated for 5 min with 5h-hFSP and then mixed with iFSP (final concentration 0.67 μg of fucose/ml) under conditions yielding 70% AR in the control. The iFSP-induced AR was blocked by 5h-hFSP in a dose-dependent manner (Fig. 4A). Next, 5h-hFSP was mixed with iFSP prior to the addition of sperm, and the cells were fixed after 5 min and scored for AR induction. The percentage AR declined in a concentration-dependent manner (Fig. 4A). The IC50 of 5h-hFSP was estimated to be 4 μg of fucose/ml in both experiments. This amount is 6-fold greater than that of iFSP needed to induce 70% AR. The inhibition curve shows two phases, the first being a steep decline from 0–4 μg of fucose/ml and a second, gradual decline from 4–65 μg of fucose/ml.

Sperm were pretreated for 5 min with 5h-hFSP at a concentration of 4 μg of fucose/ml, then various concentrations of iFSP were added, and the cells were fixed in 5 min. High concentrations of iFSP induced the AR (Fig. 4B). These concentrations of iFSP are at least 10 times higher than those required to induce similar percentages of AR without prior treatment with 5h-hFSP (Fig. 4A). These data indicate that iFSP and hFSP recognize the same binding site(s) on sperm. The increase in [Ca2+]i, in response to 5h-hFSP and iFSP was measured. Sperm treated with 5h-hFSP increased [Ca2+]i, in a concentration-dependent manner (Fig. 4C, filled columns) and reached a steady state after 5 min (data not shown). Subsequently, 5 μg of fucose/ml of iFSP was added, and [Ca2+]i was recorded for 5 min. The increase in the [Ca2+]i/Kd value decreased with increasing concentrations of 5h-hFSP. Reductions in Δ[Ca2+]i/Kd in AR are not equivalent. For example, sperm treated with 10 μg of fucose/ml of 5h-hFSP showed 26.3% reduction in Δ[Ca2+]i/Kd, whereas they were reduced by 85.1% in AR induction. Although the AR induction is tightly associated with the increase in [Ca2+]i (Fig. 3, A and B), pretreatment of sperm with hFSP somehow desensitizes the sperm’s response to the iFSP-induced AR. The desensitization does not involve blocking Ca2+ uptake.

The AR Depends on a Rapid, Transient Increase in [Ca2+]i— When iFSP was added at 3.8 μg of fucose/ml, sperm [Ca2+]i increased rapidly and then decreased to a steady level (Fig. 5). As found by others, this rapid transient peak in [Ca2+]i represents the opening of the first Ca2+ channel (4). The peak was maximum at 20–30 s after iFSP addition. Occasionally, at higher concentrations of iFSP, the first peak is obscured by the subsequent, sustained rise in [Ca2+]i, through the second channel (Fig. 2B). However, the rapid, transient Ca2+ peak is not observed in 5h-hFSP at any concentration (4.6–44 μg of fucose/ml) tested (Fig. 5). The steady state [Ca2+]i, level produced by 0.5 μg of fucose/ml of iFSP was similar to that of 22 μg of fucose/ml of 5h-hFSP. In agreement with the results mentioned
iFSP evokes the rapid, transient Ca\(^{2+}\) peak. However, a relatively large amount of 5h-hFSP (44 \(\mu\)g of fucose/ml) causes a rapid increase (within a few seconds) in [Ca\(^{2+}\)], but does not induce the AR. These results suggest that 5h-hFSP does not open the first Ca\(^{2+}\) channel but at high enough concentrations does open the second channel.

**Nifedipine, a Ca\(^{2+}\) Channel Blocker Specific for the First Ca\(^{2+}\) Channel, Does Not Block hFSP-induced Ca\(^{2+}\) Influx**—Ca\(^{2+}\) channel antagonists such as the dihydropyridines (DHPs) and verapamil, inhibit the AR (26). Nisoldipine selectively blocks the first Ca\(^{2+}\) channel but not the second channel (3). Ca\(^{2+}\) influx is absent when sperm are treated with 10 \(\mu\)M nisoldipine prior to the addition of FSP (3), suggesting that the second Ca\(^{2+}\) channel is up-regulated by the opening of the first channel. Because an increase in [Ca\(^{2+}\)], triggered by hFSP occurs without a rapid, transient peak, we postulated that 1) hFSP regulates Ca\(^{2+}\) channels other than the first channel or 2) the FSP-triggered transient Ca\(^{2+}\) peak is due to the opening and closing of the first channel. To test the first hypothesis we asked whether DHPs would block the 5h-hFSP-triggered Ca\(^{2+}\) influx. We chose nifedipine instead of nisoldipine because nifedipine has been most frequently used among DHPs, and it also blocks the mammalian sperm AR (27, 28). \(IC_{50}\) for inhibition of the AR by this compound has been determined to be 26 \(\mu\)M (26). In the absence of nifedipine, 3.6 \(\mu\)g/ml iFSP increased the \([\text{Ca}^{2+}]_{\text{i}}/K_d\) by 0.13 in 60 s. However, in the presence of 50 \(\mu\)M nifedipine, this value was only 0.05, and in the seawater control, 0.04 (Fig. 6A). With 10.6 \(\mu\)g/ml 5h-hFSP, the increased \([\text{Ca}^{2+}]_{\text{i}}/K_d\) value was 0.08 both with and without nifedipine, whereas seawater control was 0.03 (Fig. 6B). We conclude that, although the rates of [Ca\(^{2+}\)], increase differ in the two sets of experiments, nifedipine does not block Ca\(^{2+}\) influx triggered by hFSP. The data suggest that hFSP regulates Ca\(^{2+}\) channels other than the first Ca\(^{2+}\) channel.

Ni\(^{2+}\), an Inhibitor of the Second Ca\(^{2+}\) Channel Blocks the hFSP-triggered Ca\(^{2+}\) Influx—Ni\(^{2+}\) is known to block Ca\(^{2+}\) influx through the second, but not the first, Ca\(^{2+}\) channel (4). To test whether 5h-hFSP opens the second channel, Ni\(^{2+}\) was used at 300 \(\mu\)M, a concentration at which neither a sustained [Ca\(^{2+}\)], increase nor the AR are induced (4). A rapid, transient [Ca\(^{2+}\)], increase followed by a sustained [Ca\(^{2+}\)], increase is observed when 10 \(\mu\)g of fucose/ml iFSP is added to sperm (Fig. 7A). However, in the presence of Ni\(^{2+}\) only a rapid, transient [Ca\(^{2+}\)], increase is observed. In contrast, with 50 \(\mu\)g of fucose/ml of 5h-hFSP, only the sustained [Ca\(^{2+}\)], increase occurs, but it is completely blocked by Ni\(^{2+}\) (Fig. 7B). From the above data it is concluded that 5h-hFSP opens the second, but not the first, Ca\(^{2+}\) channel. Although the [Ca\(^{2+}\)], level is elevated by hFSP, it is insufficient to induce AR.

**DISCUSSION**

Linear polymers of \(\alpha\)-1,3-sulfoglycosaminoglycans are only known in echinoderms (10), the single invertebrate phylum leading to the evolution of vertebrates (29). In addition to egg jelly coats, these polymers are also found in the extracellular matrix of the adult body wall in echinoderms (30). The egg FSPs mediate signal transduction in sperm and are also potent inhibitors of human blood coagulation (11, 31). Their biosynthesis has not been studied, and glycosidases that degrade them have not been described. The mouse sperm AR is also triggered by carbohydrate components of the egg’s extracellular matrix; however, these are oligosaccharide chains of unknown structure of the glycoprotein ZP3 (32). Also of interest is the fact that the sulfation pattern of FSP is responsible for its species-specific induction of the AR (8–10). Sulfation pattern is a relatively unknown structural mechanism to confer specificity on a cell-cell interaction leading to signal transduction and physiological interactions.
activation. It is also unusual that a pure carbohydrate, completely lacking amino acids (6), should induce signal transduction leading to exocytosis in animal cells.

In the present study, the fragmentation of purified FSP was achieved by mild acid hydrolysis, creating a size-heterogeneous population of fragments (Fig. 1A). Glycosidic bond cleavage was confirmed by an increase in reducing sugar with time (Fig. 1B).

By 30 min of hydrolysis, gel analysis shows that FSP is still relatively large; however, it has already lost much of its ability to induce Ca\(^{2+}\)/H\(_{11001}\) influx (Fig. 2B).

FIG. 5. iFSP and hFSP induce different patterns of [Ca\(^{2+}\)]\(_i\) increases in sperm. Either iFSP (bold lines) or 5h-hFSP was mixed with fura-2-loaded sperm at 80 s. Concentrations of iFSP and 5h-hFSP used are indicated on the right. After 300 s, sperm were fixed and scored for AR. The percentage AR is indicated in the parentheses on the right.

FIG. 6. Effects of nifedipine on [Ca\(^{2+}\)]\(_i\) influx triggered by iFSP and 5h-hFSP. iFSP at a final concentration of 3.6 \(\mu\)g/ml (A) or 5h-hFSP at 10.6 \(\mu\)g/ml (B) was added to fura-2-loaded sperm in the presence (+Nif) or absence (–Nif) of 50 \(\mu\)M nifedipine. The arrows indicate time of iFSP/hFSP additions. Seawater (SW) was added as a negative control.

FIG. 7. Effects of Ni\(^{2+}\) on iFSP- and hFSP-induced increase in [Ca\(^{2+}\)]\(_i\). A, top trace shows the increase in [Ca\(^{2+}\)]\(_i\) induced by iFSP added at the arrow. The second trace shows the [Ca\(^{2+}\)]\(_i\) increase by iFSP in 300 \(\mu\)M NiCl\(_2\). The trace shows increasing and decreasing of [Ca\(^{2+}\)]\(_i\) regulated by the first Ca\(^{2+}\) channel. B, same conditions as above except that 5h-hFSP was added instead of iFSP. Bottom trace is the seawater control in 300 \(\mu\)M NiCl\(_2\).
induction has decreased from 80 to 20% (Fig. 2A). By 5 h of hydrolysis gel filtration shows that FSP chromatographs as a broad peak with a relative mass of 60 kDa (Fig. 1C). We cannot distinguish whether the small amount of sulfate loss, or the decrease in polymer size, are singly, or in combination, responsible for the loss of biological activity of hFSP. However, physically braking FSP by sonication at pH 8.0 yields the same data presented in Figs. 1A and 4A, suggesting that loss of polymer size is responsible for the characteristics of hFSP described herein (data not shown). In starfish, the giant pentasaccaride repeat polymer of EJ contains two sulfate groups per repeat. Solvolysis of the polymer shows that the loss of one sulfate per repeat greatly decreases its ability to induce the AR of starfish sperm (33).

hFSP samples showed a marked decrease in the ability to induce Ca\(^{2+}\) influx after 30 min of hydrolysis (Fig. 2B), yet the polymer size was still relatively large (Fig. 1A). The concentration dependences of iFSP and 5h-hFSP to stimulate increases in Ca\(^{2+}\) and pH, and to induce the AR show that iFSP is always a more potent inducer than 5h-hFSP (Fig. 3). 5h-hFSP blocked iFSP from inducing the AR, and this inhibitory effect was restored by an excess amount of iFSP, suggesting that both types of FSP bind the same site(s) (Fig. 4, A and B). Although 22 \(\mu\)g/ml hFSP induced a final [Ca\(^{2+}\)] of 7000 \(\mu\)M, elevation equivalent to 0.5 \(\mu\)g/ml FSP, AR did not occur in the hFSP-treated cells (Fig. 5). As previously documented by others, nisoldipine blocks the first Ca\(^{2+}\) channel but not the second (3). Our experiments show that the hFSP-induced increase in Ca\(^{2+}\) is not inhibited by 50 \(\mu\)M nifedipine, suggesting that hFSP up-regulates the second channel but not the first (Fig. 6B). This hypothesis is supported by the data showing that Ni\(^{2+}\), which blocks the second sperm Ca\(^{2+}\) channel but not the first (4), completely blocks the 5h-hFSP-induced increase in Ca\(^{2+}\) (Fig. 7).

In regard to the mechanism of AR induction in different animal species, the egg ligands and their sperm receptors appear to be highly variable and evolutionarily unrelated (34). However, the intracellular mechanism of the animal sperm AR appears to be conserved in that elevation of Ca\(^{2+}\) and pH are required in all species (2). For example, planar lipid bilayer experiments show that sea urchin and mouse sperm possess a readily detectable, Ca\(^{2+}\)-selective, sperm-specific, high conductance, multistate, voltage-dependent channel with similar voltage dependence (35, 36).

In the NH\(_2\)-terminal extracellular portion of both proteins called the “REJ module,” sea urchin REJ1 and human PKD1 have structural motifs identifying them as “C-type lectin-like,” carbohydrate-binding proteins (12, 18). Ligands for PKD1 are unknown, but the available evidence suggests that FSP binds REJ1 with high affinity (6). REJ1 has two carbohydrate recognition domains (CRDs) that could bind FSP (12). REJ1 has only one transmembrane segment at its extreme COOH terminus with only 15 residues being putatively cytoplasmic; therefore, REJ1 cannot be a pore-forming ion channel subunit. Because some (but not all) monoclonal antibodies to REJ1 induce Ca\(^{2+}\) elevations and the AR, REJ1 must be a regulator of sperm Ca\(^{2+}\) channels (12, 13). The location of REJ1 over the acrosomal vesicle supports its role in AR regulation.

Given a molecular mass of ~60 kDa in 5h-hFSP and an average molar ratio of 1.1 sulfate groups per fucoxide residue (8), the average 5h-hFSP fragment has about 220 fucoxide residues. This would seem large enough to bridge many CRDs of many REJ1 proteins. In addition to binding the plasma membrane receptor REJ1, egg FSP also has affinity for bindin, the protein released from the acrosomal vesicle that species-specifically attaches the sperm to the sea urchin egg (37). Studies on how the size of FSP affects its affinity to bindin have shown that the large size is again important. Little binding occurred below an FSP average size of 15 kDa. In addition, sulfate groups were essential for the binding of FSP to bindin (38). Why is such a giant size of FSP required for the normal physiological response of opening both Ca\(^{2+}\) channels? As with other CRD-containing proteins (39), the two CRDs of REJ1 should recognize terminal sugar residues of oligosaccharides; therefore, perhaps only the first and last residues bind REJ1. 5h-hFSP must bind REJ1 receptors because its AR-inhibiting activity is overridden by an excess amount of iFSP, and it can also induce Ca\(^{2+}\) increases in sperm.

Our data, and the data of others (4), show that mere elevation of [Ca\(^{2+}\)], to a certain concentration is not what induces the AR; it is the pathway leading to induction that is important. It could be that hFSP uncouples components of the pathway, a phenomenon that can be demonstrated in these cells by other means. For example, seawater is ~10 mM Ca\(^{2+}\). Treatment of sperm with EJ in 2 mM Ca\(^{2+}\) makes the cells refractory to AR induction and to increases in [Ca\(^{2+}\)], when the Ca\(^{2+}\) is returned to 10 mM (40, 41). This AR inactivation is hypothesized to result from uncoupling the linkage between the sperm EJ receptors and Ca\(^{2+}\) channels. Pretreatment of sperm with 5h-hFSP causes a marked decrease in the AR response, yet [Ca\(^{2+}\)] increases to reasonably high levels. Desensitization of the AR response by hFSP is thus different from AR inactivation. This might be due to opening of the second Ca\(^{2+}\) channel before the first channel opens, the order of opening being crucial to the AR. Normally, the first channel causes rapid increases in [Ca\(^{2+}\)], whereas the second is responsible for the sustained high level of [Ca\(^{2+}\)]. The first channel may be activated by the binding of FSP to REJ1 (6), whereas the second channel, which is normally activated by the up-regulation of the first channel, has characteristics of a store-operated channel (4). That binding of hFSP to the cell surface bypasses the first channel and up-regulates the second channel suggests that the sensor regulating the second channel could involve unidentified cell surface receptors.

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