Binding of sperm proacrosin/β-acrosin to zona pellucida glycoproteins is sulfate and stereodependent. Synthesis of a novel fertilization inhibitor

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Abstract

Specific binding of spermatozoa to the zona pellucida that surrounds mammalian eggs is a key step in the fertilization process. However, the sperm proteins that recognise zona pellucida receptors remain contentious despite longstanding research efforts to identify them. Here we present evidence that proacrosin, a tissue-specific protein found within the acrosomal vesicle of all mammalian spermatozoa, is a multifunctional protein that mediates binding of acrosome-reacted spermatozoa to zona glycoproteins via a stereospecific polysulfate recognition mechanism. Using sulfated versus non-sulfated forms of chemically defined compounds in binding assays employing native proteins in their normal cellular location or conjugated to FluoSpheres, we have attempted to identify the sulfation ‘code’ required for recognition. Results show that protein conformation is important for specificity and that at least 2 sulfate groups are required to cross-link spatially separated docking sites on proacrosin. The consistently most effective inhibitory compounds were suramin and quercetin-3-β-D-glucoside sulfate. The results support our hypothesis that proacrosin is one of several proteins in the acrosomal matrix that retain acrosome reacted spermatozoa on the zona surface prior to penetration. They also establish, as a proof-of-principle, the feasibility of synthesising sulfated compounds of high specificity as antifertility agents for human or animal use.

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Introduction

A critical event during fertilization in animals is the specific recognition and binding between complementary molecules on the surfaces of spermatozoa and eggs. In marine invertebrates, such as sea urchins (De Angelis and Glabe, 1987; Duhme and Lennarz, 1995) and starfish (Ushiyama et al., 1993), and several species of amphibia (Omata and Katagiri, 1996; Vo et al., 2003; Caputo et al., 2005), sulfated oligosaccharides on vitelline envelope (VE) glycoproteins have been shown to function as low affinity sperm receptors. Given the great diversity in structure afforded by oligosaccharide chains together with their associated modifications and the participation of the polypeptide backbone, cell and species specificity becomes more readily understood. In mammals, receptors on eggs are associated with the zona pellucida (ZP), an extracellular matrix composed of 3–4 large glycoproteins (reviews Rankin and Dean, 2000; Jovine et al., 2005). Extensive biochemical, genetic and biological analyses of the ZP in the mouse suggest that receptor activity is associated with two constituent glycoproteins (ZP2 and ZP3), both of which contain N- and O-linked sulfated oligosaccharides (Bleil and Wassarman, 1980; Shimizu et al., 1983; Jovine et al., 2005). ZP3 participates in the recognition and attachment of acrosome intact spermatozoa (known as primary or pre-acrosome reaction (pre-AR) binding) whereas ZP2 retains acrosome reacted spermatozoa (known as secondary or post-acrosome reaction (post-AR) binding) long enough for them to initiate penetration through the ZP. In the pig on the other hand, hetero-oligomers of ZPB and ZPC glycoproteins have been shown to be required for sperm binding (Yurewicz et al., 1998) as has the intact ZP for completion of the acrosome reaction (Yoshizawa et al., 1994), both findings highlighting the relevance of native protein structure for correct sperm-ZP interactions. Recent genetic knockout and rescue experiments in mice also emphasise the importance of the supra-
molecular structure of ZP glycoproteins (ZPGPs) in the intact zona for taxon-specific sperm binding (Rankin et al., 2003; Dean, 2004).

Identifying egg binding proteins on spermatozoa, however, has proved problematic, partly because of the greater number of sperm components presented to the egg at fertilization and partly because of inconsistencies in the methods used to investigate the process. Probably the best known egg binding protein is sea urchin sperm ‘bindin’ (Vacquier and Moy, 1977) which is normally sequestered within the acrosomal granule and is only exposed following the acrosome reaction when it coats the acrosomal process. In this location it is correctly positioned to bind with low affinity to sulfated oligosaccharides on the genus-specific domain of the receptor in the egg’s VE (Stears and Lennarz, 1997; Maehashi et al., 2003). In mammals, the situation is more contentious and a diverse range of ZP binding molecules has been described (review Bi et al., 2002). Currently, it is not clear whether they function co-operatively or sequentially, at what stage they are active and, most importantly, if their mechanism of binding is compatible with the known properties of the ZP receptor. A proposed orthologue of bindin in vertebrates is the sperm-specific protein proacrosin (Brown and Jones, 1987; Jones, 1991; Urch and Patel, 1991).

Proacrosin, which arose as a gene duplication event from bacterial trypsin ~900 million years ago (Klemm et al., 1991), is found exclusively within the acrosomal vesicle of all mammalian spermatozoa and throughout its long evolutionary history has remained organelle and tissue specific. It fulfils many of the requirements for a post-AR ZP binding protein, namely, it is released at the correct time following acrosomal exocytosis and place (on the ZP surface) and has been shown to bind directly and selectively to polysulfate groups on ZPGPs (Williams and Jones, 1993; Moreno et al., 1998). Our working hypothesis predicts that proacrosin mediates post-AR binding through a sequential process of forming, breaking and reforming low affinity interactions with ZPGPs as a result of the oscillatory movement of the sperm head. This process continues until the acrosomal matrix is eroded and the sperm head has initiated a penetration slit into the ZP. High affinity binding at this stage would obviously be prejudicial as it would impede subsequent ZP penetration.

A central feature of this hypothesis is that proacrosin–ZP interactions depend on the correct alignment between polysulfate groups on ZPGPs and specific arginines, lysines and histidines on the surface of proacrosin in a manner similar to that described for heparin–antithrombin III interactions (Jin et al., 1997). In the latter system 4 sulfonate and 2 carboxyl groups on the heparin pentasaccharide are positioned opposite a cluster of 4 arginines, 3 lysines and 1 glutamate on an exposed groove on the surface of antithrombin III (Whisstock et al., 2000). This weak binding induces a structural change in antithrombin III that leads to its activation. We have speculated that a similar sulfate ‘ docking’ mechanism applies to proacrosin–ZPGP interactions and that it is stereodependent (Crosby et al., 1998; Howes et al., 2001). Both pig ZPB and ZPC glycoproteins contain sulfated N-acetylpoly lactosamine oligosaccharides that represent potential proacrosin receptors (Nakano et al., 1990; Yonezawa et al., 1997). Previous studies, however, have relied on binding assays using denatured or denatured–renatured proteins, raising the possibility of folding artifacts, and sulfated polymers such as fucoidan and dextran sulfate whose three dimensional structures are difficult to predict. These criticisms become acute when the aims are to elucidate the stereochemistry of binding and ultimately to design small molecular weight compounds that will competitively inhibit fertilization.

Our studies in this direction have been facilitated by the discovery that the anti-cancer drug suramin is very effective in inhibiting binding of ZPGPs to proacrosin (Jones et al., 1996; Howes et al., 2001). Suramin is a polysulfonated naphthylurea compound (Mf, 1429) from which many analogues have been synthesised (Jentsch et al., 1987). Its chemistry is well known, analogues are available and consequently it has considerable potential as a tool for dissecting the fertilization process. In this investigation we have compared firstly, the ability of a variety of sulfated compounds (e.g. oligosaccharides, suramin analogues, suradistas) to inhibit proacrosin–ZPGP binding using combinations of denatured and native proteins as well as proteins in their correct cellular position. Secondly, using suramin as a lead compound we have synthesised several new sulfated compounds, one of which has similar efficacy to suramin in blocking fertilization but is potentially less cytotoxic. Taken together these results strengthen our hypothesis that proacrosin (and its activated form β-acrosin) is a stereospecific sulfate binding protein that mediates retention of post-AR spermatozoa on the zona surface. Proacrosin, therefore, is one of a growing number of multifunctional or ‘moonlighting’ proteins that have acquired additional roles during evolution that are distinct from their traditional enzymic activities (Jeffery, 1999; Moore, 2004).

Materials and methods

Materials

All routine chemicals and enzymes were of the highest purity available commercially and were purchased from Merck-Eurolab (Lutterworth, UK) or Sigma-Aldrich (Poole, UK). FluoSpheres sulfate (1 μm diameter, red fluorescence, Cat. F-8851) and AlexaFluor® 648® protein labelling kit (Cat. A-10235) were obtained from Invitrogen (Paisley, UK) and suramin, quercetin, quercetin-3-β-d-glucoside and maltose were supplied by Sigma-Aldrich. Suradistas (NSC651015 and NSC651016) were a generous gift of Dr. Robert Schultz (NCI, NIH, Bethesda, USA). Fragmin (dalteparin sodium) was kindly provided by Dr. Hugues Vilain, France. Ejaculated spermatozoa were collected from boars maintained at the Babraham Institute or were obtained from a commercial breeder (JSR Newsham, Thorpe Willoughby, UK). Pig, cow and sheep ovaries were collected fresh from local slaughterhouses and processed immediately on arrival in the laboratory. Mouse and rat eggs were obtained by standard superovulation protocols. All procedures involving animals followed UK Home Office approved guidelines and appropriate licenses. Boar sperm β-acrosin was purified to homogeneity as described previously (Lo Leggio et al., 1994); part of this preparation had been used earlier for structural studies (Tranter et al., 2000).

The composition of the media and buffers used were as follows. Zona preparation buffer: 130 mM NaCl, 10 mM Na2HPO4, 2 mM EDTA, 11 mM Na citrate, 1 mM p-aminobenzamidine (pAB), pH 7.0. Phosphate buffered saline (PBS): 137 mM NaCl, 1.6 mM Na2HPO4, 4.8 mM NaH2PO4, 2 mM KCl pH 7.2. Egg Medium: PBS supplemented with 1% polyvinylalcohol (PVA), 5 mM glucose, 4 mM pAB, 4 mM Na HEPES, pH 6.9–7.0. TALP medium: 100 mM
NaCl, 3.1 mM KCl, 25 mM NaHCO₃, 3 mM Na₂HPO₄, 21.6 mM Na lactate, 1 mM Na pyruvate, 2 mM CaCl₂, 0.4 mM MgCl₂, 10 mM HEPES, 5 mM glucose, pH 7.3. RIPA buffer: PBS supplemented with 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS.

Chemical sulfation

Compounds to be sulfated (e.g. quercetin-3-O-glucoside, 25 mg, 0.054 mmol) were added to sulfurtioxidetrimethylamine complex (59.9 mg, 0.43 mol, 1 equivalent for each OH) followed by 400 µl dry N,N-dimethyl formamide. The mixture was left at room temperature under argon for 2 h after which time the compounds were fully in solution. Reaction mixtures were heated for 20 h at 55 °C to 60 °C and cooled to room temperature. Diethyl ether (4 ml) was added and the sample left to stand until the product had fully separated either as a solid or an oil at the bottom of the reaction vessel. The diethyl ether layer was decanted and the sample washed with another 4 ml diethyl ether. The product was dried under vacuum overnight, dissolved in a small amount of water and the solution was filtered through a 0.45 µm syringe filter. The filtrate was then diluted in PBS to the appropriate concentration (see Results) and pre-incubated with biotin-NHS (Sigma H-1759) or Alexafluor 488 (Invitrogen A-10235) for 1 h. Incubations were terminated by adding 2 ml of Egg Medium and washing eggs twice more in 1 ml to remove unbound Fluorospheres. Washed eggs, in a final volume of ∼ 8 µl, were added to 2 µl of 3% formaldehyde/PBS in a welled glass slide and sealed with a cover slip. Eggs were viewed by epifluorescence microscopy and the number of beads adhering to the zona surface counted visually. Displacement of bound Fluorospheres-Ac was investigated by transferring the eggs to Egg Medium supplemented with 0.5 mM NaCl or 25 mM HEPES to pH 8.0, or RIPA buffer and incubating at 37 °C for 1 h. Eggs were washed and bound beads counted as above.

In experiments where compounds were to be tested for their ability to inhibit binding of Fluorospheres to eggs, Fluorospheres-Ac or Fluorospheres-BSA were pre-exposed to the compounds for 1 h followed by addition of the eggs (40–50) in ∼ 10 µl Egg Medium. Alternatively, eggs were pre-exposed to the compounds for 1 h, washed <3 followed by addition of Fluorospheres-Ac or Fluorospheres-BSA in fresh Egg Medium. After incubation for 2 h at 37 °C, eggs were washed and beads counted as above. The list of compounds and the concentrations tested are described in Tables 1 and 2.

Sperm-intact ZP assay

Washed spermatozoa were diluted to ∼2 × 10⁵/ml in TALP medium containing 5 mg/ml BSA and capacitated for 4 h at 38.2 °C in 5% CO₂/95% air. Suspensions were centrifuged for 4 min at 2000 × g for 2 min to pellet agglutinated spermatozoa. The upper layer (motile capacitated spermatozoa) was removed and diluted to ∼1.25 × 10⁶ sperm/ml in Egg Medium. Spermatozoa were centrifuged again at 75 × g for 2 min to pellet agglutinated spermatozoa. Suspensions were incubated for 1 h with gentle shaking at room temperature, diluted to 1.25 × 10⁶ sperm/ml in Egg Medium + inhibitors and 100 µl drops added to 30 mm Petri dishes. Eggs (30–35 in 10 µl) were added and incubated at 38.2 °C for a further 30 min, after which time they were washed ×4 in TALP medium to remove unbound spermatozoa and fixed in 4% p-formaldehyde for 20 min. Eggs were stained with 7.1 µM DAPI for 15 min, washed ×3 and the attached spermatozoa counted using confocal microscopy. To assess any direct effects of inhibitors on spermatozoa, samples were observed by phase contrast microscopy and the percentage of motile cells estimated by 2 observers.

FACS analysis, epifluorescence microscopy, confocal microscopy and scanning electron microscopy (SEM)

Intact or permeabilised spermatozoa (1.8 × 10⁶/ml) in Egg Medium (46 µl) were incubated + inhibitors for 1 h at room temperature at which time 3.3 µl Alexafluor 488-ZPGPs (final concentration 100 µg/ml) was added and incubation continued for another 2 h. As a control, spermatozoa were incubated with 20-fold excess of unlabelled ZPGPs (2 mg/ml final concentration). Unbound probe was removed by washing ×2 with TALP medium and the proportion of labelled cells measured using a FACSCalibur cell sorter. Propidium iodide (PI, 10 µg/ml) was added immediately before FACS analysis. Fluorescently labelled spermatozoa and eggs were viewed with Olympus BX40 or Zeiss Axioskop epifluorescence microscopes fitted with CCD cameras or imaged by confocal microscopy on a Zeiss LSM 510 META. For SEM, eggs were fixed in 4% glutaraldehyde in 0.1 M sodium PIPES buffer pH 7.4 for 12 h and washed in 4 changes of 0.2 M PIPES buffer, pH 7.4. Eggs were then treated with 1% OsO₄ washed in 3 changes of PIPES buffer, dehydrated through a graded series of ethanol and critical point dried. Specimens were sputter coated with Au/Pd and viewed with a Philips XL30 FEG SEM (Cambridge University Imaging Facility).

β-acrosin activity assays

The activity of purified β-acrosin was measured against N₂-Benzoyl-l-arginine 4-nitroanilide hydrochloride ((b)-BAPNA) (Sigma) substrate (Lo Legg et al., 1994) in 96-well flat-bottomed microtitre plates using a BIO-TEK
Effects of sulphated and non-sulphated compounds on the binding of biotinylated ZPGPs to proacrosin on western blots

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Mean±SD (%)</th>
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<tbody>
<tr>
<td>Control</td>
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<th>Group I</th>
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<tr>
<td>Fucoidan</td>
<td>1 mg/ml</td>
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<tr>
<td>Dextran 506 k</td>
<td></td>
<td>134.2±15.9</td>
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<tr>
<td>Galactan</td>
<td></td>
<td>102.5±21.2</td>
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<td>Xylan</td>
<td></td>
<td>5.9±8.8</td>
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<td>Heparin</td>
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<tr>
<td>Fragmin</td>
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<td>35.5±13.0</td>
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<td>Suramin 2 mM</td>
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<td>7.7±5.0</td>
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<tr>
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<td>1 mM</td>
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<tr>
<td>Quercetin 1 mM</td>
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<td>Quercetin</td>
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<td>27.3±3.9</td>
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<td>Quercetin-3β-D-glucoside</td>
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<tr>
<td>Quercetin-3β-D-glucoside SO4</td>
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<td>1.8±0.9</td>
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</table>

Results are expressed as a percentage of the control value (no inhibitor added) which has been adjusted to 100%. Values shown are mean±standard deviation (SD) (n=2 or 5 depending on compound see Materials and methods).

Synergy HT microplate reader. Changes in absorbance at 405 nm were recorded over 40 min at 23 °C. Data were analysed using KC4 software.

Statistics

Statistical analysis was performed using SPSS version 14.0 (www.SPSS.com). Data from binding assays were standardised due to their non-normal distribution and confidence intervals of the mean at the 95%, 99% and 99.9% levels were compared. Significance levels: *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

Results

In previous work we have shown that boar proacrosin (residues 1–399), its activated form β-acrosin (residues 1–322), and a bacterially expressed truncated form (residues 47–272) are quantitatively similar in their capacity to bind homologous ZPGPs (Jansen et al., 1995). Therefore, in this investigation proacrosin and β-acrosin have been used interchangeably with proacrosin as the target in the protein–protein binding assays and β-acrosin conjugated to Fluospheres as the probe in the fluorescent bead assay. The presence of 2 mM pAB in buffers throughout ensured that enzymic activity was not a factor in the binding mechanism. Additionally, we have used the total mixture of solubilised egg ZPGPs rather than individual purified proteins because (a) in the pig all 4 glycoproteins have been shown to bind to boar proacrosin/β-acrosin (Brown and Jones, 1987) and (b) there is evidence for co-operativity between ZPGPs (Yoshizawa et al., 1994; Yurewicz et al., 1998), a property that would be overlooked with purified proteins.

Western blot assays

The degree of proacrosin refolding following SDS-PAGE and Western blotting onto nylon membranes is not known. Although heat-solubilised ZPGPs are likely to undergo some spontaneous refolding at room temperature, they will be dissociated from one another and not organised within the filamentous structure characteristic of the intact ZP. Therefore,
for the purpose of this assay both sets of proteins will be regarded as being in a non-native state.

The efficacy of various sulfated and non-sulfated compounds to inhibit ZPGP binding to proacrosin is shown in Table 1. The compounds were classified into 3 groups. Group I: polymers of various sugars of different molecular weights, sulfated and non-sulfated; Group II: sulfonated naphthylurea compounds such as suradistas, suramin and suramin analogues. Group III: flavonoids and their derivatives, sulfated and non-sulfated. Chemical formulae of selected compounds from Groups II and III are shown in Fig. 1. Of the sugar polymers in Group I, fucoidan, dextran sulfate, xylan and heparin inhibited binding of ZPGPs by >88% whereas galactan and cellobiose were ineffective. Non-sulfated dextran was also without inhibitory activity. The importance of sulfation is shown in the series of low molecular weight polymers of maltose. Polymers ranging from 2 to 7 linear sugar molecules were not inhibitory but their sulfated counterparts showed increasing inhibition, beginning with ∼20% for maltotetraose sulfate, ∼27% for maltopentaose sulfate, ∼32% for maltohexaose sulfate and ∼80% for maltoheptaose sulfate. It should be noted that sulfation of the sugar polymers was not position specific.

In Group II, >80% inhibition was achieved by 2 mM suramin, its analogues NF031, NF062, NF064 and NF065, and 1 mM suradistas NSC651015 and NSC651016. Suramin analogue NF035 on the other hand was not inhibitory while NF063 was less effective than suramin. In Group III, quercetin and quercetin sulfate inhibited ZPGP binding by ∼51% and ∼72% respectively. Quercetin-3β-D-glucoside was non-inhibitory but its sulfated form was highly effective, blocking binding of ZPGPs by ∼98%.

Quantitative titration assays gave the following IC₅₀ data: suramin, 10 μM; NF064, 5.7 μM; NSC651015, 10 μM; NSC651016, 24 μM; quercetin-3β-D-glucoside sulfate, 55 μM, maltoheptaose sulfate, 1700 μM.

Binding of FluoSpheres-Ac to ZP-intact eggs

In this assay target ZPGPs are retained in their native state as ZP-intact eggs with β-acrosin conjugated to FluoSpheres as the probe. FACS analyses of the suspension of FluoSpheres-Ac and FluoSpheres-BSA showed they were monodisperse after sonication and that a 1:100 dilution of the 2% solution contained 1.05 x 10⁷ beads/ml and 1.1 x 10⁷ beads/ml respectively.

Fig. 1. Chemical structures of selected inhibitory compounds. Suramin is a polysulfonated naphthylurea compound composed of a linker region (b) and two terminal naphthalene rings carrying sulfonate groups positioned as shown (a). Suramin analogues NF065 and NF065 have the same linker section as suramin but differ in the number and position of sulfonate groups (a′ and a″). Analogue NF035 and NF064 are as shown. Suradistas NSC651015 and NSC651016 are also sulfonated with a comparable structure to suramin but differ in the linker region. Quercetin-3β-D-glucoside is shown here in its non-sulfated form.
Preliminary experiments were designed to optimize and characterize the conditions for binding of FluoSpheres-Ac to ZP-intact eggs. Results showed a linear increase in the mean number of beads bound to reach 32 beads/egg at 120 min whereas FluoSpheres-BSA remained at <1 bead/egg (Figs. 2A and B). Uptake of FluoSpheres-Ac was optimal between pH 6.0 and 7.5 and was significantly reduced by the presence of 0.227 M and 0.416 M NaCl in the medium (Fig. 2C). Pre-exposure of FluoSpheres-Ac to soluble ZPGPs (0.24 mg/ml) for 1 h before addition of eggs reduced their binding ability by ~95% (Fig. 2D). Pre-bound FluoSpheres-Ac could be displaced by 35% with 0.5 M NaCl, 59% by pH 8.0 and 92% by RIPA buffer (Fig. 2E). The efficacy of RIPA buffer can be attributed to its mixture of detergents partially solubilising the ZP. Collectively, these properties indicate that the binding of FluoSpheres-Ac to the intact ZP is primarily a strong ionic interaction.

Scanning EM of eggs revealed the fibrous nature of the surface of the ZP with pores of varying sizes (results not shown). Bound FluoSpheres-Ac were readily identifiable from their size and smooth regular outline in contrast to the remnants of cumulus cellular debris.

Binding of FluoSpheres-Ac to ZP-intact mouse, rat and sheep eggs were 8%, 14% and 20% respectively of binding to pig eggs (Fig. 3). Cow eggs by contrast, bound FluoSpheres-Ac at a level comparable to pig eggs. Binding of FluoSpheres-BSA

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**Fig. 2.** Binding of FluoSpheres-Ac and FluoSpheres-BSA to ZP-intact pig oocytes. (A) Time course of binding to the ZP. (B) Confocal microscopy (B.1 and B.3, Differential Interference Contrast (DIC) microscopy; B.2 and B.4 corresponding epifluorescence micrograph of FluoSpheres-Ac (B.1 and B.2) and FluoSpheres-BSA (B.3 and B.4) bound to the ZP. (C) Effect of pH and NaCl concentration on the binding of FluoSpheres-Ac. (D) Inhibition of FluoSpheres-Ac binding to the ZP by soluble ZPGPs. (E) Displacement of bound FluoSpheres-Ac by 0.5 M NaCl, pH 8.0 and RIPA buffer. Scale bar, 18 μm. Significance: **p<0.01. ***p<0.001.

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**Fig. 3.** Relative binding of FluoSpheres-Ac to homologous and heterologous ZP-intact oocytes. Pig eggs are taken as 100%. Binding of FluoSpheres-BSA was <5% for all species. Significance: ***p<0.001.
to eggs of all species was negligible (<1.0 bead/egg). These species differences most likely represent variations in the levels of sulfation and/or the stereochemistry of reacting groups on ZPGPs with respect to boar β-acrosin.

In the Fluospheres-Ac assay (Table 2) compounds in Group I had noticeably different inhibitory activity to that found with the Western blot assay. Heparin, fragmin (truncated heparin containing the sulfated pentasaccharide), xylan and maltpentaose significantly reduced binding of Fluospheres-Ac to eggs whereas dextran sulfate, dextran, galactan, cellobiose, sulfated maltpentaose and fucoidan, either had no significant effect or enhanced binding. Fucoidan was particularly effective in the latter respect causing an 807% increase in the number of bound Fluospheres-Ac. This appears to be due to a cross-linking effect of fucoidan as SEM of these eggs revealed the presence of large amounts of amorphous material (presumably fucoidan) covering both the zona and Fluospheres-Ac (results not shown). The aforementioned polymers, however, had no detectable effect on the binding of Fluospheres-BSA to eggs which remained at <1 bead/egg throughout.

In Group II, suramin and suradistas NSC651015 and NSC651016 reduced binding of Fluospheres-Ac to <11% of controls (Table 2). Suramin analogues NF063, NF064 and NF065 were also effective reducing binding to <29% controls but NF035 had very poor inhibitory activity.

In Group III, the compounds quercetin, quercetin sulfate and quercetin-3β-D-glucoside had no significant effects whereas quercetin-3β-D-glucoside sulfate reduced binding of Fluospheres-Ac to <16% of controls (Table 2).

In the above assays inhibitors were present in the medium throughout incubation of eggs with Fluospheres-Ac and hence it was not possible to know if they were acting against Fluospheres-Ac or the intact ZP, or both. To determine the site of action of the inhibitor, eggs were pre-incubated with the aforementioned compounds for 1 h at room temperature, washed ×3 and then incubated with Fluospheres-Ac in inhibitor-free Egg Medium for another 2 h at 37 °C. Results showed that binding of Fluospheres-Ac was significantly reduced by pre-exposure of eggs to fucoidan, heparin and fragmin whereas other Group I compounds had no significant inhibitory effects (Table 2).

Unexpectedly, dextran sulphate increased Fluospheres-Ac binding by 763%. Pre-exposure of eggs to suramin, NF035, NF063, NF064, NF065, quercetin, quercetin-3β-D-glucoside or quercetin-3β-D-glucoside sulfate had no significant effect on binding of Fluospheres-Ac to <15% of controls. By contrast, quercetin-3β-D-glucoside had no significant inhibitory effect.

To investigate possible effects of the aforementioned compounds on motility, capacitated boar spermatozoa were incubated in the presence of compounds (1 mM) for 1 h at room temperature and the percentage of motile cells estimated visually. Control samples showed high motility (80%) with little or no agglutination. However, suramin, NSC651015, NSC651016 all caused significant head-head agglutination with a reduction in the proportion of motile individual spermatozoa to ∼10%. It is noteworthy that in all samples agglutinated spermatozoa were vigorously motile and the sperm aggregates (3–9 cells) moved rapidly through the medium. Quercetin-3β-D-glucoside and quercetin-3β-D-glucoside sulfate caused similar levels of agglutination but the motility of individual spermatozoa remained high at ~50%. Thus, the possibility that the above compounds were acting indirectly cannot be excluded entirely, although it does not explain how quercetin-3β-D-glucoside and quercetin-3β-D-glucoside sulfate had similar effects.

**Fig. 4.** Effects of selected competitors on the number of spermatozoa bound to the zona pellucida of pig oocytes. Capacitated spermatozoa were incubated for 1 h with selected compounds (1 mM final concentration) followed by addition of ZP-intact pig oocytes and further incubation for 30 min. After washing, eggs were fixed, stained with DAPI and the number of spermatozoa bound per oocyte counted. Sulfated compounds=striped bars, non-sulfated compound=open bar. Photomicrographs show DIC and confocal fluorescence microscopy of sperm binding to an oocyte from a control group (1 and 2) compared to an oocyte incubated in the presence of suramin (3 and 4). Scale bar, 15 μm. Significance: ***p<0.001.

**Binding of spermatozoa to ZP-intact eggs**

In previous work it was shown that suramin was very effective in displacing bound spermatozoa from the ZP surface in ‘pulse-chase’ experiments (Howes et al., 2001). It was presumed that spermatozoa must have initiated their acrosome reactions to allow access of the drug to proacrosin/β-acrosin since suramin does not normally penetrate intact plasma mem-

branes (Jones et al., 1996). In view of the differences between denatured versus native proteins as highlighted by the previous two binding assays, selected compounds in Groups II and III were investigated for their ability to inhibit binding of whole spermatozoa to ZP-intact eggs, a situation where all proteins should be in their native conformation. As shown in Fig. 4, 1 mM suramin, NF064, NSC651015, NSC651016, and quercetin-3β-D-glucoside sulfate reduced the number of spermatozoa bound to the ZP to <15% of controls. By contrast, quercetin-3β-D-glucoside had no significant inhibitory effect.
on sperm motility and agglutination yet gave very different results in the sperm-ZP binding assay (Fig. 4).

**Binding of AlexaFluor<sup>488</sup>-ZPGPs to intact and permeabilised spermatozoa**

The above results strongly suggest that the sulfated Group II and III compounds are effective inhibitors because they interfere with post-AR binding to the ZP. It is possible, however, they might also interfere with the pre-AR zona binding molecules on the plasma membrane overlying the anterior sperm head. It was important, therefore, to visualise ZPGP binding sites on spermatozoa and determine their sensitivity to Group II and III compounds. Results showed that AlexaFluor<sup>488</sup>-ZPGPs bind to the anterior edge of the head of ~90% of intact spermatozoa (Fig. 5A). Pre-incubation for 1 h with 1 mM suramin, NSC651016, quercetin-3β-D-glucoside or quercetin-

![Fig. 5. Binding of AlexaFluor<sup>488</sup>-ZPGPs to intact and permeabilized spermatozoa. Combined FACS analysis and confocal fluorescence microscopy of spermatozoa incubated with AlexaFluor<sup>488</sup>-ZPGPs (green) and propidium iodide (PI, red). Intact (A) and permeabilized (C) spermatozoa were incubated in the presence of AlexaFluor<sup>488</sup>-ZP±20 times excess of unlabelled ZPGPs. Geometrical means of AlexaFluor<sup>488</sup>-ZPGPs fluorescence intensities for the Lower Right (LR) or Upper Right (UR) parts of the quadrants are shown inside the dot plots (a.u.=arbitrary units). Corresponding confocal (superimposed DIC and fluorescence) pictures are shown below the FACS dot plots. (B) and (D) Mean fluorescence intensity from FACS analysis of intact and permeabilized spermatozoa incubated as above in the presence of selected compounds on the binding of AlexaFluor<sup>488</sup>-ZPGPs. Values are means±SEMs from FACS counts of 10,000 cells. Significance: different to intact control, †††<i>p</i>&lt;0.001, *<i>p</i>&lt;0.05. Different to permeabilized control, ***<i>p</i>&lt;0.001. Scale bar, 8 μm.
3β-D-glucoside sulfate did not significantly inhibit uptake of AlexaFluor488-ZPGPs as assessed by FACS analysis (Fig 5B). More than 90% of spermatozoa permeabilised by cold shock, however, labelled strongly over the anterior acrosomal region (Fig. 5C; note that the equatorial segment domain does not label). Irregular particulate labelling was sometimes present on the postacrosomal region. FACS analysis showed that permeabilised spermatozoa bound ~5 times more AlexaFluor 488-ZPGPs than intact spermatozoa and that fluorescence was reduced to <25% of control levels (which was similar to intact spermatozoa) by pre-incubation with 1 mM suramin, NSC651016 and quercetin-3β-D-glucoside sulfate but not with quercetin-3β-D-glucoside (Fig. 5D). Pre-incubation of permeabilised spermatozoa with a 20-fold excess of ‘cold’ ZPGPs (2 mg/ml) completely inhibited binding of AlexaFluor488-ZPGPs to the anterior acrosome (Fig. 5C). These results are consistent with the view that sulfated compounds in Groups II and III only inhibit post-AR binding of spermatozoa to the ZP and not pre-AR binding that involves membrane-intact spermatozoa.

Effects of Group II and III compounds on enzymic activity of β-acrosin

Suramin is known to inhibit the enzymic activity of some serine proteases (e.g. neutrophil elastase) but not others (e.g. chymotrypsin) (Cadène et al., 1997) suggesting that its binding sites on the surface of different proteins are not equivalent, even within the same protein family. To ascertain if Group II and III compounds have any effect on the activity of β-acrosin, varying concentrations (from 1 nM to 1 mM) were incubated with the enzyme and BAPNA substrate. Inhibition was observed with suramin, NF064, NSC651015 and NSC651016 (Fig. 6; calculated IC50 were 100 μM, 12 μM, 700 μM and 700 μM respectively) but not with quercetin-3β-D-glucoside nor quercetin-3β-D-glucoside sulfate. This suggests that at least some of the binding sites on β-acrosin for quercetin-3β-D-glucoside sulfate are different from those for suramin.

Discussion

This investigation has shown that (a) ZPGP-binding proteins on boar spermatozoa are present in 2 different cellular locations, the plasma membrane overlying the anterior acrosome and within the acrosome itself (i.e. the acrosomal matrix and acrosomal membranes); (b) native protein structure and presentation is important for stereodependent polysulfate recognition between sperm proacrosin and ZPGPs; (c) using suramin as a lead compound it is feasible to design and synthesise novel inhibitors of fertilization on the basis of their ability to bind to proacrosin/β-acrosin. One such compound, quercetin-3β-D-glucoside sulfate, has potential as an antifertility agent if released near the site of fertilization.

In the mouse paradigm, pre-AR binding of membrane-intact spermatozoa to zona glycoprotein ZP3 induces exocytosis of the acrosomal vesicle thereby making components within the acrosomal matrix and its surrounding membranes available for post-AR binding to glycoprotein ZP2. The relative duration and significance of these stages varies between species, so that for mouse spermatozoa pre-AR binding is important whereas for guinea pig spermatozoa, which lose their acrosomes very quickly, post-AR binding is dominant (Howes and Jones, 2002). Within this spectrum the pig system appears closer to the mouse. Ultrastructural observations on in vitro fertilization have shown that acrosome intact boar spermatozoa initially attach to the ZP via the plasma membrane overlying the anterior tip or rostral ridge (Peterson et al., 1981). After initiation of the acrosome reaction, however, binding extends over the whole acrosomal region. The present observations, and those of others, are consistent with this scenario (Jones, 1991; Yurewicz et al., 1993; Yonezawa et al., 1995; Harkema et al., 1998; Burkin and Miller, 2000; van Gestel et al., 2005; Bou Khalil et al., 2005). Alexa488-ZPGPs bound primarily to the rostral plasma membrane of intact spermatozoa but following permeabilisation under conditions that inhibited dispersal of the acrosomal matrix, they bound to the entire acrosomal area except for the equatorial segment.

Fig. 6. Effects of selected compounds on amidase activity of β-acrosin. (A) and (B) β-acrosin activity was measured spectrophotometrically in the presence of varying concentrations of selected compounds as shown. Values are the mean difference in OD 405 nm/min over the first 15 min of incubation.

Labelling of fixed spermatozoa would not distinguish between these 2 locations as the amount of AlexaFluo488-ZPGPs bound to permeabilized acrosomes is quantitatively ~6 times greater than that bound to the rostral plasma membrane, i.e., there would be a masking effect. Thus, whatever the nature of the primary binding protein(s) on the anterior plasma membrane (reviewed by Shur et al., 2006), they are less abundant than those within the acrosomal vesicle. It is also noteworthy that suramin, suradista NSC651016 and quercetin-3β-D-glucoside sulfate have little or no effect on binding of AlexaFluo488-ZPGPs to receptors on the rostral plasma membrane whereas they reduce binding to acrosomal matrices to the same level as that found on intact cells. These quantitative comparisons are possible because of the robust nature of FACS analysis which quantifies the fluorescence intensity from 10,000 individual cells. These data imply that the ZPGP binding receptors overlying the rostral plasma membrane of boar spermatozoa do not utilise a polysulfate recognition mechanism.

A central tenet of our hypothesis is the importance of stereochemical compatibility between polysulfate groups on receptors and basic residues on proacrosin/β-acrosin. This prediction was based largely on assays that utilised denatured or denatured–renatured proteins. To overcome these problems, we devised 2 additional assays of greater stringency using native protein structure and that at least 2 hydroxyls were modified, 1 on the sugar moiety and 1 on the flavonoid rings. This would provide a ‘span’ between the active sulfates. There is increasing evidence that specific sulfation motifs function as molecular recognition elements—the so-called sulfation code (Bulow and Hobert, 2004; Holt and Dickson, 2005)—and that such a mechanism has a long evolutionary history. In several classes of sea urchins for example, it is thought to be the driving force for speciation. Sulfated fucans in the egg jelly are known to be species-specific inducers of the acrosome reaction and that this property depends on the appropriate position of sulfate groups and the type of glycosidic linkage between L-fucose sugars in the fucan polymer (reviewed by Mourão, 2007). Using a chemical synthesis approach, Gama et al. (2006) have provided compelling evidence for a position-dependent sulfation code in mammalian chondroitin sulfates CS-E, CS-C and CS-A. We hypothesise that a similar situation applies to sulfated oligosaccharides on ZPGPs for recognition of proacrosin and that this accounts for some of the cell and species specificity of binding between gametes.

Other potential ZPGP binding proteins described within the acrosomes of spermatozoa are zonadhesin (Hardy and Garbers, 1995), Sp38 (Mori et al., 1995) and sp56 (Foster et al., 1997). However, little is known about their relative abundance, mechanism of binding or their susceptibility to suramin or quercetin-3β-D-glucoside sulfate. Zonadhesin is a large mosaic protein and has the merit of species specificity but the disadvantage of showing very strong binding to ZPGPs, so strong in fact that it requires RIPA buffer for dissociation (Hardy and Garbers, 1995). Arguably, this would be disadvantageous as the fertilizing spermatozoon would not be able to release itself from the ZP surface and form a penetration slit. A potential role for zonadhesin is retention of the acrosomal ‘ghost’ on the ZP to act as a pivot point for the oscillating sperm head as it creates a penetration slit (Yanagimachi and Phillips, 1983). Acrosomal ghosts are commonly found on the ZP of fertilized eggs. Less is
known about Sp38. It has been suggested that an internal 11-residue peptide containing 5 basic amino acids is analogous to a dibasic 8-residue peptide (365–372) in the C-terminal region of proacrosin that binds ZPGPs (Mori et al., 1995). However, the 8-residue peptide in proacrosin is well outside the minimum binding fragment (47–272) identified in earlier work by fragmentation analysis (Jansen et al., 1995) and seems unlikely to bind ZPGPs. The cellular distribution of sp56 is contentious. It has been described on the plasma membrane overlying the acrosome (Suzuki-Toyota et al., 1995) and within the acrosomal matrix (Kim et al., 2001). Current evidence favours an intracellular location but its mechanism of binding to ZPGPs remains to be elucidated.

In conclusion, the present results support the hypothesis that proacrosin/β-acrosin recognizes a specific sulfation code on ZPGPs and that this interaction mediates post-AR binding of spermatozoa to the ZP long enough for penetration to begin. Proacrosin/β-acrosin, therefore, is one of a growing list of multifunctional or moonlighting proteins (Jeffery, 1999; Moore, 2004). The results also demonstrate the feasibility of synthesizing sulfated compounds based on oligosaccharide or flavanoid structures as antifertility agents for human or animal use, provided they are present at the site of fertilization. It will be important in future work to elucidate the stereochemistry of the sulfation code for ZPGP-proacrosin binding.

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