<table>
<thead>
<tr>
<th>Title</th>
<th>Zona pellucida-induced acrosome reaction in human spermatozoa is potentiated by glycodein-A via down-regulation of extracellular signal-regulated kinases and up-regulation of zona pellucida-induced calcium influx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author(s)</td>
<td>Chiu, PCN; Wong, BST; Lee, CL; Lam, KKW; Chung, MK; Lee, KF; Koistinen, R; Koistinen, H; Gupta, SK; Seppälä, M; Yeung, WSB</td>
</tr>
<tr>
<td>Citation</td>
<td>Human Reproduction, 2010, v. 25 n. 11, p. 2721-2733</td>
</tr>
<tr>
<td>Issued Date</td>
<td>2010</td>
</tr>
<tr>
<td>URL</td>
<td><a href="http://hdl.handle.net/10722/139900">http://hdl.handle.net/10722/139900</a></td>
</tr>
<tr>
<td>Rights</td>
<td>This is a pre-copy-editing, author-produced PDF of an article accepted for publication in Human Reproduction following peer review. The definitive publisher-authenticated version Human Reproduction, 2010, v. 25 n. 11, p. 2721-2733 is available online at: <a href="http://humrep.oxfordjournals.org/content/25/11/2721">http://humrep.oxfordjournals.org/content/25/11/2721</a></td>
</tr>
</tbody>
</table>
Zona pellucida-induced acrosome reaction in human spermatozoa is potentiated by glycodein-A via down-regulation of extracellular signal-regulated kinases and up-regulation of zona pellucida-induce calcium influx

Philip CN Chiu\textsuperscript{1,2\#}, Ben ST Wong\textsuperscript{\#}, Cheuk-Lun Lee\textsuperscript{1}, Kevin KW Lam\textsuperscript{1}, Man-Kin Chung\textsuperscript{3}, Kai-Fai Lee\textsuperscript{1,2}, Riitta Koistinen\textsuperscript{4}, Hannu Koistinen\textsuperscript{4}, Satish K Gupta\textsuperscript{5}, Markku Seppälä\textsuperscript{4} and William SB Yeung\textsuperscript{1,2*}

\textsuperscript{1}Department of Obstetrics and Gynaecology, \textsuperscript{2}Centre of Reproduction, Development and Growth, University of Hong Kong, Pokfulam Road, Hong Kong, China.

\textsuperscript{3}Department of Obstetrics and Gynaecology, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong, China.

\textsuperscript{4}Clinical Chemistry, Helsinki University Central Hospital, 00029 HUS Helsinki, Finland,

\textsuperscript{5}Reproductive Cell Biology Laboratory, National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi 110 067, India

\textsuperscript{\#}The two authors contributed equally to this study.

Running title: glycodein and zona pellucida-induced acrosome reaction

Key terms: glycodein, zona pellucida, sperm acrosome reaction, ERK, intracellular calcium

*Address all correspondence and requests for reprints to: Prof. WSB Yeung, Department of Obstetrics and Gynaecology, University of Hong Kong, Queen Mary Hospital, Pokfulam Road, Hong Kong, Tel: 852-28553405; Fax: 852-28175374; Email: wsbyeung@hkucc.hku.hk
Abstract

BACKGROUND: Glycodelin-A interacts with spermatozoa before fertilization, but its role in modulating sperm functions is not known. Zona pellucida–induced acrosome reaction is crucial to fertilization and its dysfunction is a cause of male infertility. We hypothesized that glycodelin-A, a glycoprotein found in the female reproductive tract, potentiates human spermatozoa for zona pellucida-induced acrosome reaction. METHODS: Glycodelin isoforms were immunoaffinity-purified. The sperm intracellular cyclic-adenosine monophosphate concentration, protein kinase-A and extracellular signal-regulated kinase activities, and intracellular calcium were measured by ELISA, kinase activity assay kit and Fluo-4AM technique, respectively. The phosphorylation of inositol 1,4,5-trisphosphate type-1 receptor mediated by extracellular signal-regulated kinase was determined by Western blotting. Zona pellucida-induced acrosome reaction was detected by Pisum sativum staining. RESULTS: Pre-treatment of spermatozoa with glycodelin-A significantly up-regulated adenylyl cyclase/protein kinase-A activity and down-regulated the activity of extracellular signal-regulated kinase and its phosphorylation of inositol 1,4,5-trisphosphate type-1 receptor, and thereby enhancing zona pellucida-induced calcium influx and zona pellucida-induced acrosome reaction. Glycodelin-F or deglycosylated glycodelin-A did not have these actions. Inhibitor of protein kinase abolished while that of the extracellular signal-regulated kinase pathway mimic the priming activity of glycodelin-A. CONCLUSIONS: Glycodelin-A in the female reproductive tract sensitizes spermatozoa for zona pellucida-induced acrosome reaction in a glycosylation-specific manner through activation of the adenylyl cyclase/protein kinase-A pathway, suppression of extracellular signal-regulated kinase activation and up-regulation of zona pellucida-induced calcium influx. The action of glycodelin-A may be important in vivo to ensure full responsiveness of human spermatozoa to the zona pellucida.
**Introduction**

Acrosome reaction is a critical event in fertilization. The zona pellucida (ZP) of the oocyte induces acrosome reaction. It allows the spermatozoa to release acrosomal enzymes, enabling penetration of the spermatozoa through the ZP. The ZP–induced acrosome reaction has been shown to be the major indicator of sperm fertilizing ability (Liu et al., 2007), and spermatozoa from men with defective ZP–induced acrosome reaction have reduced or no ability to penetrate the ZP and fertilize oocytes either *in vivo* or *in vitro* (Liu et al., 2004; 2007).

Only ~48% of the zona pellucida-bound spermatozoa from fertile men are capable of undergoing ZP–induced acrosome reaction *in vitro* (Liu et al., 2003). Human follicular fluid exerts a priming effect on ZP-induced acrosome reaction (Schuffner et al., 2002), i.e., follicular fluid-treated spermatozoa are more sensitive to the ZP-induced acrosome reaction. This is believed to enhance fertilization. Follicular fluid contains progesterone, which has been reported to prime the ZP-induced acrosome reaction in mice (Roldan et al., 1994) and guinea-pigs (Shi et al., 2005). In human, successive treatment with progesterone and ZP induces a higher percentage of acrosome-reacted spermatozoa than treatment with either progesterone or ZP alone (Schuffner et al., 2002). The temporal response to ZP-induced acrosome reaction is different between human spermatozoa pretreated with progesterone and those pretreated with follicular fluid (Schuffner et al., 2002), suggesting that other follicular fluid components are involved in the priming process. Apart from progesterone, no other components in human follicular fluid are known to have a priming effect.

Glycodelin is an endocrine-regulated glycoprotein with four well defined glycoforms, namely amniotic fluid glycodelin (glycodelin-A, GdA), follicular fluid glycodelin (glycodelin-F, GdF), seminal plasma glycodelin (glycodelin-S, GdS) and cumulus matrix glycodelin (glycodelin-C, GdC) (Chiu et al., 2003a, 2007a; Seppala et al., 2007). These glycoforms have the same protein backbone but different glycosylation profiles. GdA and GdF inhibit spermatozoa-ZP binding (Oehninger et al., 1995, Yeung et al., 2006) by interaction with sperm surface fucosyltransferase-5 (sFUT5), which also binds to the ZP (Chiu et al., 2007b). GdF, but not the other glycoforms, suppresses progesterone-induced acrosome reaction (Chiu et al., 2003a,b), and may thereby prevent premature acrosome reaction. Both GdA and GdF bind to the sperm plasma membrane overlying the acrosome. The binding is greatly reduced after acrosome reaction (Chiu et al., 2004) and is abolished after
deglycosylation of the glycoproteins (Chiu et al., 2003a,b; Yeung et al., 2006; Chiu et al., 2007b).

The spermatozoa have to pass through the female reproductive tract before reaching the oocyte for fertilization. During this passage, they encounter GdA, which, in addition to the secretory endometrium, is also present in the oviductal fluid, follicular fluid and cumulus matrix (Seppala et al., 2002; Yeung et al., 2006). As glycodelin modulates a variety of sperm functions (Yeung et al., 2006; Seppala et al., 2007), we hypothesized that GdA primes spermatozoa for ZP-induced acrosome reaction. In this study, we demonstrate that GdA potentiates the ZP-induced acrosome reaction in human spermatozoa through down-regulation of extracellular signal-regulated kinase (ERK) activity and up-regulation of zona pellucida-induced calcium influx.

Materials and methods

Semen samples

The Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster approved the research protocol. Spermatozoa with normal semen parameters (WHO, 1999) from men attending the infertility clinic at Queen Mary Hospital were processed by Percoll (Pharmacia, Uppsala, Sweden) density gradient centrifugation (Chiu et al., 2003a). The processed spermatozoa were capacitated overnight as reported (Baldi et al., 1991; Whitmarsh et al., 1996; Ficarro et al., 2003; Shetty et al., 2003; Chiu et al., 2008a) in Earle’s balanced salt solution containing 0.265 mg/ml calcium chloride, 2.2 mg/ml sodium bicarbonate, 1.09 mg/ml L-lactate, 0.033 mg/ml sodium pyruvate, 0.06 mg/ml penicillin G, 0.075 mg/ml streptomycin sulphate (EBSS; Flow Laboratories, Irvine, UK), and 3% bovine serum albumin (BSA) at 37°C in an atmosphere of 5% CO₂ in air. The mean percentage of capacitated spermatozoa was 43.2±2.9% (mean ± standard error of mean, s.e.m.) as determined by chlortetracycline staining (Chiu et al., 2005). The capacitated spermatozoa were resuspended in EBSS supplemented with 0.3% BSA (EBSS/BSA).

Purification of glycodelin-A

GdA was purified as described from first trimester amniotic fluid using monoclonal anti-glycodelin (clone F43-7F9) affinity chromatography (Chiu et al., 2003a). In brief, amniotic fluid diluted with Tris-buffered saline (TBS, pH 7.4) containing 0.1% Triton X-100 was loaded onto the
column. After successive washing of the column with TBS, 1M NaCl with 1% isopropanol, 10 mM ammonium acetate with 0.1% isopropanol, pH 5 and TBS, GdA was eluted by 20 mM CaCl₂ containing 0.1% trifluoroacetic acid. Deglycosylated GdA was prepared by denaturation of GdA in 0.1% β-mecaptoethanol before incubation with 0.5 mU Peptide-N-Glycosidase F (PNGase F, Sigma-Aldrich Inc., St. Louis, MO) at 37°C for 24 hours. The digest was boiled for 5 minutes to inactivate PNGase F and dialyzed in 2 mM Tris-HCl, pH 7.5. The concentration of purified GdA was determined by a commercial protein assay kit (Bio-Rad, Hercules, USA).

**Preparation of solubilized zona pellucida**

ZPs were obtained from unfertilized human oocytes from the assisted reproduction programme at Queen Mary Hospital, Hong Kong. The purification of solubilized ZP was reported recently (Chiu et al., 2008b). Briefly, the purification involved separation of the ZP from the oocytes under microscope and heat-solubilization of ZP at 70°C in 5 mM NaH₂PO₄ buffer (pH 2.5) for 90 minutes.

**Determination of acrosome reaction**

Staining with fluorescein isothiocyanate labeled peanut (*Pisum sativum*) agglutinin (FITC-PSA; Sigma) and Hoechst 33258 (bisBenzimide; Sigma) were used to evaluate the acrosomal status of spermatozoa (Chiu et al., 2005). Processed spermatozoa were incubated with phosphate-buffered saline (PBS, pH 7.4) containing 0.001% (w/v) Hoechst for 10 minutes, centrifuged through 2% (w/v) polyvinylpyrrolidone-40 (Sigma) in PBS, fixed in 300 µl of 95% ethanol and dried on slide before staining with 0.01% (w/v) FITC-PSA in PBS for 10 minutes. The fluorescence patterns of 150 spermatozoa in randomly selected fields were determined under a fluorescence microscope (Zeiss, Oberkochen, Germany) with 600x magnification. The filter set used for Hoechst staining consisted of an excitation filter G365, a chromatic beam splitter FT395 and a barrier filter LP420, whereas that for FITC-PSA consisted of an excitation filter BP 450-490, a chromatic beam splitter FT510 and a barrier filter LP520. Acrosome-reacted spermatozoa were defined as those without Hoechst and FITC-PSA staining or with FITC-PSA staining at the equatorial segment only.

**Determination of cyclic-adenosine monophosphate (cAMP)**
Intracellular cAMP was extracted in ice-cold 90% ethanol at -20°C for 30 minutes and at 4°C for 30 minutes as described (Chiu et al., 2005). The ethanol extracts were dried in a rotary evaporator. Intracellular cAMP in the extracts was determined using a non-radioactive cAMP ELISA Kit (R&D, Minneapolis, MN) according to the manufacturer’s instructions.

**Determination of protein kinase A (PKA) activities**

Spermatozoa (20 x 10^6) were washed thrice with PBS, sonicated in 100 µl of homogenizing buffer (20 mM PBS, pH 7.4 containing 2 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethanesulfonyl fluoride, 0.1 mM vanadate, 1 mM MgCl₂, 100 mM NaCl and 0.05% Triton-X100) for 15 minutes at 4°C as described (Bajpai and Doncel, 2003). The supernatant was obtained after centrifugation at 15,800 g for 30 minutes at 4°C. The PKA activity in the supernatant was determined as described (Chiu et al., 2010) with an ELISA-based PKA (Calbiochem, San Diego, CA) assay kit. Standard curve was run with the test samples in each experiment. One Unit (U) of PKA activity was defined as the amount of enzyme required to catalyze the transfer of 1 pmol of phosphate to the substrates, RFARKGSLRQKNV in 1 minute at 30°C.

**Determination of extracellular signal-regulated kinase activity**

Sperm proteins (10×10^6 spermatozoa) were extracted, resolved by 12% SDS-PAGE and blotted on a PVDF membrane as described above. Western blotting was performed using a mouse monoclonal anti-phospho ERK1/2 antibody (purified IgG; 1:1000; Cell Signaling, Danvers, MA). Anti-tubulin antibody was used to reveal sample loading. Quantification of protein bands normalized with respect to the tubulin control was carried out with Image J 1.36b software (http://rsbweb.nih.gov/ij/index.html) and expressed as percent of the corresponding control without treatment. Non-radioactive ERK1/2 activity assay kit (Chemicon, Temecula, CA) was used according to the manufacturer’s instructions to confirm the results of the Western blotting. Results were expressed as percentage change over the corresponding control without treatment.

**Determination of the effects of glycodelin-A on human zona pellucida-induced acrosome reaction**

Capacitated spermatozoa at a concentration of 2×10^6 spermatozoa/ml (N=5) were incubated...
with either EBSS/BSA (control), different concentrations of GdA or GdF (0.3-300 nM) or
deglycosylated glycodelin (0.3-300 nM) for 90 minutes at 37°C in an atmosphere of 5% CO₂ in air.

After incubation, the spermatozoa were washed twice with EBSS/BSA before further incubation with
solubilized ZP (1 µg/ml or ~0.03 ZP/µl) or EBSS/BSA (control) for 60 minutes at 37°C in an
atmosphere of 5% CO₂ in air. The acrosomal status of the treated spermatozoa was then evaluated as
described above.

**Determination of the effects of glycodelin-A on adenylyl cyclase, PKA and ERK activities**

Capacitated spermatozoa (2×10⁶/ml, N=5) were incubated with 30 nM of GdA, deglycosylated
glycodelin or EBSS/BSA (control) at 37°C under 5% CO₂ in air for 90 minutes. The intracellular
cAMP level, PKA and ERK activity of the treated spermatozoa were evaluated as described above.

**Determination of the effects of PKA inhibitor on the priming effect of glycodelin-A**

To demonstrate the possible relationship between PKA activation with ERK phosphorylation
and priming of ZP-induced acrosome reaction, the ERK activities and acrosome reaction of
capacitated human spermatozoa (2×10⁶, N=5) treated with 30 nM of GdA for 90 minutes in the
presence or absence of 200 µM of Rp-Adenosine 3',5'-cyclic monophosphorothioate
triethylammonium salt (Rp-cAMPS, a PKA inhibitor; Calbiochem) prior to treatment with
solubilized ZP (1 µg/ml) were determined. The spermatozoa were washed after treatment and their
intracellular ERK activities and acrosomal status were evaluated as described above.

**Determination of the effects of ERK pathway inhibitors on human zona pellucida-induced
acrosome reaction**

Capacitated spermatozoa (2×10⁶/ml, N=5) were incubated with different concentrations of ERK
pathway inhibitors, PD98059 (0.1-100 µM), U0126/U0124 (0.01-10 µM), ZM336372 (0.01-10 µM),
Raf1 kinase inhibitor I (0.01-10 µM) or DMSO (control) for 90 minutes at 37°C in an atmosphere of
5% CO₂ in air. The inhibitors, PD98059 and U0126 (Calbiochem) are inhibitors of ERK1/2
activation inhibiting mitogen-activated protein kinase kinase activities through different mechanisms,
while U0124 (Calbiochem) is a negative control for U0126. ZM336372 and Raf1 kinase inhibitor I
(Calbiochem) are potent and specific inhibitor of the protein kinase c-Raf. The treated spermatozoa were washed twice with EBSS/BSA. They were then incubated with solubilized ZP at a concentration of 1 μg/ml or EBSS/BSA (control) for 60 minutes at 37°C in an atmosphere of 5% CO2 in air. After incubation, the spermatozoa were washed twice before their acrosomal status was evaluated as described above.

Expression and localization of ERK and Raf kinase

Proteins from capacitated spermatozoa were separated by SDS-PAGE and transferred to PVDF membrane for Western blot analysis as described. Affinity purified polyclonal rabbit antibodies against A-Raf (Cell signaling), B-Raf (ECM, Versailles, KY), c-Raf (ECM) and ERK (Cell signaling) were used. Horseradish peroxidase conjugated donkey anti-rabbit antibody at a dilution of 1:5000 was used as secondary antibody. For immunostaining, capacitated spermatozoa were collected on glass slides by cytopsin (Shandon, Pittsburgh, PA), fixed with 2% paraformaldehyde and permeabilized by 0.2% Triton X-100 for 20 minutes as described (Chiu et al., 2007a,b) before incubation with the anti-Raf (1:50) or anti-ERK (1:50) antibodies for 24 hours in a humidified chamber at 4°C. Bound antibodies were detected by Alexa-594-conjugated secondary antibody (Invitrogen, CA, USA). Antibody preabsorbed with 1:100 blocking peptide (ECM) or slides processed without the primary antibodies were used as controls.

Determination of the effects of glycodelin-A and U0126 on human zona pellucida-induced calcium influx

The relative levels of intracellular calcium within individual spermatozoon were measured using Fluo-4AM as described with modification (Tesarik et al., 1996; Ren et al., 2001). In brief, spermatozoa were loaded with 5 μM Fluo-4AM for 30 minutes at 37°C. Fifty microlitres of the sperm suspension (2×10⁶/ml) with or without prior GdA (30 nM; 90 minutes) or U0126 (0.1 μM; 90 minutes) treatment were then placed on a tissue culture dish coated with poly-L-lysine (10% poly-L-lysine in double distilled water) as a droplet covered with light mineral oil (Sigma). The spermatozoa were examined on a heated stage at 37°C under a fluorescence inverted microscope (Nikon, Tokyo, Japan) with ×600 magnification. Spermatozoa attached to the culture dish and with a
wiggling tail were selected. Fluo-4AM was excited at a wavelength of 488 nm and the emission was
monitored at 512 nm. Fluorescence images of the selected spermatozoa were captured every second
for a total of 60 seconds through a CCD camera under the control of an Image Pro Plus imaging
system (Media Cybernetics, Bethesda, MD). The fluorescence signal associated with each selected
spermatozoon in the first 5 seconds of capturing was considered as the resting fluorescence of the
spermatozoon. Two microlitres of solubilized ZP was then added to the sperm droplet to give a final
contentration of 1 μg/ml with the use of a micromanipulator (Nikon, Tokyo, Japan) and a
microinjector (Narishige, Tokyo, Japan). EBSS/BSA was added to the control droplet. The
fluorescent images of the spermatozoon were captured for a further 55 seconds. All the captured
images were analyzed as follows: After subtraction of the background signal, each sperm head in the
captured image was selected electronically. The intensity of the fluorescence signal within the
selected sperm head was extracted as eight-bit grey scale measurements. The signal from the sperm
tail or any cell that moved across the selected sperm head in the course of image capturing was
excluded from the analysis. The raw intensity values were normalized using the equation, \( \Delta F = \left[ \frac{F - F_{\text{rest}}}{F_{\text{rest}}} \right] \times 100\% \), where \( \Delta F \) was the normalized fluorescence intensity, \( F \) was the intensity at a given
time point and \( F_{\text{rest}} \) was the mean intensity derived from the 5 images captured before addition of ZP
or EBSS/BSA. The experiment was performed 5 times, each involving spermatozoa from a different
donor. Twenty spermatozoa were randomly selected from each donor for analysis.

**Determination of the effects of glycodelin-A on the phosphorylation of inositol 1,4,5-trisphosphate
type-1 receptor (IP3R1)**

Capacitated spermatozoa (2×10^6/ml, N=3) were incubated with 30 nM of GdA or EBSS/BSA
(control) for 90 minutes at 37°C in an atmosphere of 5% CO₂ in air. The protein extract from 10×10^6
spermatozoa were resolved by 5% SDS-PAGE and blotted on a PVDF membrane as described above.
Western blotting was performed using a rabbit polyclonal anti-IP3R1 antibody (purified IgG; 0.26
μg/ml; Abcam, Cambridge, MA). To determine the phosphorylation of IP3R1 mediated by ERK, the
membrane was reprobed with a rabbit antibody against the phosphorylated S/TP motif
(serine/threonine residue followed by a proline residue) of the receptor (purified IgG; 1.5 μg/ml;
Abcam).
Statistical Analysis

All the data were expressed as mean and s.e.m.. The data were analyzed by statistical softwares (SigmaPlot 8.02, Jandel Scientific, San Rafael, CA). For all experiments, the non-parametric repeated measures ANOVA on Rank test for multiple comparisons were used. If the data were normally distributed, Tukey Test or Parametric Student t-test was used where appropriate as the post-test. A probability value <0.05 was considered to be statistically significant.

Results

Glycodelin-A enhances ZP-induced acrosome reaction in a glycosylation-dependent manner

Dose-dependent increase in acrosome reaction of spermatozoa by solubilized ZP was observed (Supplementary Figure S1). Statistically significant increase in acrosome reaction was detected at concentrations of ≥ 1 µg/ml for solubilized ZP. At a concentration of 1 µg/ml, solubilized ZP significantly increased the percentage of acrosome-reacted spermatozoa to 24.3±4.0%. This concentration of ZP was used in subsequent experiments in order to reduce the amount of ZP used and to avoid masking of the priming effect by excessive ZP stimulation at high concentrations.

Figure 1A shows the percentages of acrosome-reacted spermatozoa above the background (spontaneous acrosome reaction in medium alone) in sperm samples treated with ZP or GdA alone, or sequentially with GdA and ZP. The extent of priming (priming index; Figure 1B) was determined by the difference in the percentages of acrosome-reacted spermatozoa between sequential treatment and sum of the two individual treatments. GdA at concentrations of ≥ 30 nM exhibited priming activity (Figure 1A); the percentages of acrosome-reacted spermatozoa induced by sequential GdA→ZP treatment were significantly (P<0.05) higher than the sum of the percentages of acrosome-reacted spermatozoa induced by GdA alone at the corresponding concentrations and by ZP alone. The priming index increased dose-dependently with the concentration of GdA used. At 30 nM, it increased 16.0±6.6% of the ZP-induced acrosome reaction (Figure 1B). Pre-incubation with differently glycosylated GdF or deglycosylated GdA did not affect the ZP-induced acrosome reaction at all the concentrations tested (data not shown).
Glycodelin-A increases intracellular cAMP concentration and PKA activity

GdA induced a gradual increase in cAMP concentration, reaching a plateau at 60 minutes (Figure 2A). The intracellular cAMP levels increased from 43.4±3.6 fmol/10^6 spermatozoa to 61.6±5.1 fmol/10^6 spermatozoa (p<0.05) after 60 minutes of GdA treatment. Deglycosylated glycodelin treatment did not affect intracellular cAMP concentration of spermatozoa at any of the time points studied.

Consistent with the stimulatory effect on cAMP levels, GdA also significantly increased the PKA activity of the treated spermatozoa when compared to those incubated in culture medium only (Figure 2B). The PKA activity increased from 27.5±3.4 to 40.7±4.1 U/10^6 spermatozoa. Deglycosylated glycodelin treatment had no effect on PKA activity.

Glycodelin-A reduces extracellular signal-regulated kinase activity

GdA at a concentration of 30 nM suppressed the expression of activated-ERK (Figure 2C) and reduced ERK activity by 63.3±2.3% (P<0.001, Figure 2D). Lower concentrations of GdA did not affect ERK activity. Again, treatment with deglycosylated glycodelin had no significant effect on either the activated-ERK expression or ERK activity.

Protein kinase inhibitor abolishes the inhibitory effects of glycodelin-A on activated ERK1/2 level, ERK activity and ZP-induced acrosome reaction

Rp-cAMPS (a PKA inhibitor) at a concentration 200 μM inhibited 35.2±4.5% of the sperm PKA activity (N=10). Compared to the control without treatment (Figure 3A upper, lane 1), Rp-cAMPS treatment significantly (P<0.05) increased the level of activated-ERK1/2 (Figure 3A upper, lane 2; Figure 3A lower, column 2) and elevated ERK activity by 59.0±21.4% in the treated spermatozoa (Figure 3B, column 2). Treatment with Rp-cAMPS abolished the inhibitory effect of GdA on activated-ERK1/2 level (Figure 3A upper, lane 3 and 4; Figure 3A lower, column 3 and 4) and ERK activity (Figure 3B, column 3 and 4). Rp-cAMPS also significantly (P<0.05) suppressed the effects of GdA on ZP-induced acrosome reaction (Figure 3C); the percentage of ZP-induced acrosome-reacted spermatozoa decreased from 38.7±4.5% (column 4) to 21.2±3.5% (column 5).

Rp-cAMPS at the concentrations used did not affect ZP-induced acrosome reaction (column 2 and 3),
sperm viability, sperm motility and spontaneous acrosome reaction (Supplementary Table ST1).

Extracellular signal-regulated kinase pathway inhibitors mimic the stimulatory effect of glycodegin-A on zona pellucida-induced acrosome reaction

The percentages of spermatozoa undergoing ZP-induced acrosome reaction with and without pretreatment of ERK pathway inhibitors were compared. The mean percentage of spontaneously acrosome reacted spermatozoa before treatment was 6.9±0.7%. ERK pathway inhibitors, PD98059 (Figure 4A), U0126 (Figure 4B), ZM336372 (Figure 4C) and Raf1 kinase inhibitor I (Figure 4C) at concentrations of 1 μM, 0.1 μM, ≥ 1 μM and 1 μM, respectively, significantly (P<0.05) increased the ZP-induced acrosome reaction. PD98059 (1 μM) and U0126 (0.1 μM) inhibited 39.2±3.6% and 34.5±5.0% respectively of the sperm ERK activity (N=5). At high concentration (10 μM), U0126 and Raf1 kinase inhibitor I inhibited ZP-induced acrosome reaction. U0124 (Figure 4B) had no effect on ZP-induced acrosome reaction except at high concentration (10 μM). The inhibitors at the concentrations used did not affect sperm viability, sperm motility and spontaneous acrosome reaction (Supplementary Table ST1).

Localization of extracellular signal-regulated kinase and Raf kinase

The antibody against non-phosphorylated ERK primarily localized ERK immunoreactivities to the equatorial and tail region of human capacitated spermatozoa (Supplementary Figure S2). Immunoreactivities were found in the acrosomal, equatorial and tail regions with the use of anti-phosphorylated ERK antibody (Supplementary Figures S2). The omission of the primary antibodies greatly reduced the signal (data not shown).

A-raf, B-raf and c-Raf have reported molecular size of ~67, ~84 and ~73 kDa respectively. Anti-c-Raf antibody recognized a protein band of ~73 kDa in the human sperm extract (Figure 5B). Pre-absorption of the antibody with blocking peptide greatly reduced the signal. The antibodies against A-Raf and B-Raf did not detect the presence of A-Raf, B-Raf in the extract (Figure 5B). These results were consistent with the immunostaining data for Raf kinases; only positive c-Raf immunoreactivities were demonstrated over the head and tail of human spermatozoa (Figure 5A).
Glycodelin-A potentiates ZP-induced rise in the sperm intracellular calcium concentration

Representative single spermatozoon records are shown in Figure 6. The addition of ZP induced a rapid rise in the sperm intracellular calcium concentration ([Ca^{2+}]_i) that peaked at 21 seconds and declined slowly thereafter (Figure 6A). The percentage increase of [Ca^{2+}]_i upon ZP treatment was significantly higher in the GdA- (334.4±43.2%, N=20; Figure 6B) or U0126-pretreated spermatozoa (277.4±9.2%, N=20; Figure 6C) than the control spermatozoa incubated in medium alone (194.3±27.3%, N=20; Figure 6A). In addition, the time taken for the [Ca^{2+}]_i to reach the peak value was shorter for GdA (18 seconds) or U0126-pretreated spermatozoa (16 seconds) (Figure 6B and C).

61.9±4.4% and 54.6±3.8% of spermatozoa responded to GdA and U0126 in calcium mobilization respectively. Treatment with GdA (30 nM) or U0126 (0.1 μM) alone or addition of solvent vehicle had no effect on these parameters (data not shown).

Glycodelin A suppresses phosphorylation at the S/TP motif of IP3R1

IP3R1 is phosphorylated and subsequently downregulated by ERK in mouse cerebellum and B-cells (Yang et al., 2006; Bai et al., 2006; Taylor et al., 2009). Spermatozoa after GdA treatment had IP3R1 levels similar to the control (Figure 7). Western blotting using antibody recognizing the phosphorylated S/TP motif, the consensus motif phosphorylated by ERK, showed that the phosphorylation at the motif of IP3R1 was significantly suppressed after GdA treatment (Figure 7).

Discussion

Human oviduct cells express glycodelin mRNA and protein (Laird et al., 1995; Saridogan et al., 1997; Yeung et al., 2006), which would interact with the spermatozoa entering the oviductal lumen. The concentration of glycodelin in the oviduct is higher in the secretory than in the proliferative phase of the menstrual cycle (Julkunen et al., 1986). GdA binds to the acrosome region of human spermatozoa (Chiu et al., 2003a). It does not affect spontaneous acrosome reaction and capacitation (Chiu et al., 2003a, 2005). The present study show that GdA, but not GdF and deglycosylated GdA, enhanced the ZP-induced acrosome reaction of capacitated human spermatozoa, demonstrating that the effect is glycosylation-dependent. We also demonstrate that the priming activity of GdA is mediated by down-regulation of ERK1/2 activities through the cAMP/PKA signaling pathway. This is based on
the findings showing that 1) GdA treatment significantly enhances the cAMP/PKA activity and suppresses the ERK activity; 2) PKA inhibitor diminishes the priming activity of GdA and the GdA-induced suppression of sperm ERK; and 3) ERK inhibitors mimic the priming effect of GdA on ZP-induced acrosome reaction.

A principle target of cAMP in spermatozoa is PKA, though cAMP can also exert its effects on sperm functions by PKA-independent pathway such as through the exchange protein directly activated by cAMP (Epac) (Kinukawa et al., 2006; Branham et al., 2009). The cAMP/PKA pathway regulates exocytosis in a variety of secretory cells (Seino and Shibasaki, 2005). The importance of PKA in sperm capacitation (Visconti et al., 1995; de Lamirande et al., 1997; Aitken et al., 1998; O'Flaherty et al., 2004) and acrosome reaction (de Jonge, 1996; Breitbart, 2002) is also well established. During acrosome reaction, cAMP levels and PKA activity are increased in spermatozoa (Lefievre et al., 2002). PKA inhibitor (KT5720 and H89) completely prevents follicular fluid- (de Jonge et al., 1993) and lysophosphatidylcholine- (O'Flaherty et al., 2005) induced acrosome reaction of human spermatozoa. This is of interest because male mice lacking the testis-specific PKA catalytic subunit C are infertile and have capacitation-related defects (Nolan et al., 2004). Previous studies demonstrated that PKA inhibitor reduced acrosome reaction induced by high concentration of solubilized ZP (Bielfeld et al., 1994; De Jonge et al., 1996). We have similar observation when the concentration of ZP used is ≥10 µg/ml. In this study, the lack of suppressive action of PKA inhibitor on ZP-induced acrosome reaction might be due to the reduced amount of ZP (1 µg/ml) used for induction of acrosome reaction, which was to avoid masking of the priming effect by excessive ZP stimulation at high concentrations. These observations suggest different signaling pathways are induced by ZP depending on the dosage used.

The ERK pathway consists of several components including Raf (Mitogen-activated protein kinase kinase kinase), MEK (Mitogen-activated protein kinase kinase), and ERKs (ERK1 and ERK2) (Kolch, 2000). Components of the ERK cascade, such as Raf (de Lamirande and Gagnon, 2002) and ERK (Luconi et al., 1998a,b) had been detected in human spermatozoa, and were shown to be positively involved in capacitation (Luconi et al., 1998a,b; de Lamirande and Gagnon, 2002; O'Flaherty et al., 2005, 2006; Almog and Naor, 2008). In contrast to the cAMP/PKA pathway, there are only few studies on the participation of the ERK pathway in acrosome reaction (du Plessis et al.,
In this study, we provide the first evidence on the cAMP/PKA-dependent suppression of ERK activity mediated by GdA in human spermatozoa. cAMP/PKA may act through the Raf serine/threonine kinases (Cook and McCormick, 1993; Graves et al., 1993; Sevetson et al., 1993; Wu et al., 1993; Stork and Schmitt, 2002) to phosphorylate MEK, which in turn phosphorylates ERK at the tyrosine and threonine residues of the activation domain (Seger et al., 1994). There are three Raf family members, namely A-Raf, B-Raf and c-Raf. cAMP/PKA activates ERK in many neuronal and endocrine cells (Vossler et al., 1997; Dugan et al., 1999; Grewal et al., 1999) expressing B-Raf (Erhardt et al., 1995; Stork and Schmitt, 2002). In cells that do not express B-Raf, transfection of B-Raf converts cAMP from an inhibitor to an activator of ERKs (Vossler et al., 1997; Dugan et al., 1999). In contrast, cAMP inhibits ERK activation in many nonneuronal cells expressing c-Raf, including NIH 3T3 cells (Schmitt and Stork, 2001), Rat-1 fibroblasts (Cook and McCormick, 1993; Burgering et al., 1993; Wu et al., 1993), myocytes (Graves et al., 1993), and adipocytes (Sevetson et al., 1993). It has been proposed that PKA phosphorylates serine-43 of c-Raf, thereby inhibiting Ras-dependent activation of c-Raf by preventing the binding of c-Raf to Ras (Wu et al., 1993).

Although Raf has been detected in human spermatozoa (de Lamirande and Gagnon, 2002), the identity of the Raf isoform remains unknown. The present study detected c-Raf, but not A-Raf and B-Raf in human spermatozoa. Furthermore, Raf inhibitor promoted ZP-induced acrosome reaction, suggesting that the priming effect of GdA was mediated through PKA-dependent suppression of c-Raf, followed by down regulation of ERK activity.

Another novel finding of the present results was that the GdA-induced decrease in ERK activity enhanced the ZP-induced acrosome reaction. Such a relationship was in agreement with the ability of ERK pathway inhibitors, PD98059, U0126, ZM336372 and Raf1 kinase inhibitor I to mimic GdA activity, enhancing the ZP-induced acrosome reaction. But, the enhancement was not related to a direct action of the inhibitors on acrosome reaction (Supplementary Table ST1). The reported actions of ERK pathway inhibitors on human acrosome reaction vary according to the inducer of acrosome reaction. PD98059 or U0126 suppress phorbol myristoyl acetate-induced (Almog et al., 2008), lysophosphatidylcholine- (de Lamirande and Gagnon, 2002), and A23187- (du Plessis et al., 2001),
but not progesterone- (Luconi et al., 1998a,b) induced acrosome reaction. PD98059 has also been reported to inhibit ZP-induced acrosome reaction (du Plessis et al., 2001). The reason for the discrepancy between these reports and the present study may be the use of different concentrations of PD98059. While 1 μM of PD98059 demonstrated a priming effect in our study, 50 μM of the same inhibitor inhibited the ZP-induced acrosome reaction in Du Plessis’ report (du Plessis et al., 2001). In somatic cells, PD98059 selectively inhibits ERK with IC50 of ~1-2 μM (Pang et al., 1995; Reiners et al., 1998; Li et al., 2008). The inhibitor at concentrations ≥ 10 μM inactivates calcium influx non-specifically (Gould and Stephano, 2000; Pereira et al., 2002). Non-specific activity of U0126 at high concentrations has also been reported (Pereira et al., 2002). Therefore, the high concentrations of PD98059 or U0126 may suppress the ZP-induced acrosome reaction by suppressing calcium influx, which is known to be important for ZP-induced acrosome reaction (Breitbart, 2002; Kirkman-Brown et al., 2002).

It is generally accepted that capacitative Ca\(^{2+}\) entry resulting from depletion of an intracellular inositol 1,4,5-trisphosphate (IP3)-gated Ca\(^{2+}\) store is a fundamental mediator of acrosome reaction (Breitbart, 2002; Kirkman-Brown et al., 2002). Therefore, the effect of GdA on Ca\(^{2+}\) influx was studied in spermatozoa. Interestingly, although GdA treatment alone did not affect sperm [Ca\(^{2+}\)], it significantly enhanced the ZP-induced Ca\(^{2+}\) influx in terms of increasing the [Ca\(^{2+}\)], and shortening the time to peak [Ca\(^{2+}\)] upon exposure to ZP. A similar effect was observed with the use of inhibitor of the ERK pathway, U0126, indicating that ERK mediates the action of GdA on ZP-induced Ca\(^{2+}\) influx.

IP3R1 regulates the IP3-gated Ca\(^{2+}\) store of spermatozoa (Patel et al., 1999). This is consistent with the expression of IP3R1 in the acrosomal region of human spermatozoa and its reduction after acrosome reaction (Kuroda et al., 1999). In contrast, the expression of IP3R3 remains unchanged after acrosome reaction and IP3R2 is absent in human spermatozoa (Kuroda et al., 1999). Phosphorylation is an important regulatory mechanism of IP3R1 function (Taylor et al., 2009; Vanderheyden et al., 2009). Activated ERK phosphorylates Ser 436 of IP3R1 and reduces its binding to IP3 (Yang et al., 2006; Bai et al., 2006; Taylor et al., 2009). The present results demonstrate that GdA suppresses ERK-mediated IP3R1 phosphorylation. Taken together, an intriguing possibility arises that the suppressive activity of GdA on sperm ERK reduces the phosphorylation of IP3R1,
thereby increasing its affinity to IP3 and enhancing the ZP-induced calcium influx and acrosome reaction. In vitro, only ~48% of the zona pellucida-bound spermatozoa from fertile men are capable of undergoing ZP–induced acrosome reaction (Liu et al., 2003). Patients with normal semen analysis but with <15% acrosome reaction upon exposure to solubilized ZP had been reported to have poor fertilization results with standard IVF (Esterhuizen et al., 2001). Therefore, the action of GdA may be important in vivo to ensure full responsiveness of human spermatozoa to the ZP. This hypothesis is being investigated in our laboratory. Based on the present data as well as others, a schema for the intracellular signaling network of GdA in modulating ZP-induced acrosome reaction is proposed in Figure 8.

ZP stimulation activates phospholipase C (Roldan and Shi, 2007), which generates IP3, thereby inducing release of intracellular Ca\(^{2+}\) through IP3-gated channels. Although GdA modulates the IP3R1 activity, our preliminary data show that it has no effect on the phospholipase C activity in spermatozoa. The differential action of GdA and ZP on phospholipase C-IP3-gated calcium channels may explain the lack of effect of GdA on acrosome reaction while exhibiting a priming effect on ZP-induced acrosome reaction.

GdA suppresses spermatozoa-ZP binding by blocking the binding of sFUT-5 to the ZP (Oehninger et al., 1995; Chiu et al., 2007b). A recent study demonstrated that sFUT5 was concentrated in the membrane raft at the anterior region of the sperm head in a capacitation-dependent manner (Nixon et al., 2009). However, sFUT5 is unlikely to be involved in the priming effect of GdA as sFUT5 acceptors do not have priming activity (Chiu PCN and Yeung WSB unpublished observation), though the acceptors can compete for the GdA binding sites on human spermatozoa (Chiu et al., 2007b). It is likely that another receptor of GdA yet to be identified is responsible for the priming activity of GdA on ZP-induced acrosome reaction. Based on the present results and the production of significant amount of glycodeolin by the oviductal cells (Laird et al., 1995; Saridogan et al., 1997), we propose that GdA in the oviductal fluid binds to spermatozoa, potentiating them for ZP-induced acrosome reaction via the cAMP/PKA/ERK/IP3R1 pathway. During cumulus cell penetration, GdC in the matrix displaces sperm-bound GdA and promotes the zona binding capacity of the penetrated spermatozoa (Chiu et al., 2007a). It remains to be determined whether removal of the sperm-bound GdA would affect the sensitivity of the penetrated spermatozoa
to ZP-induced acrosome reaction.

Acknowledgments

This work was supported by grants from the Research Grant Council, Hong Kong (HKU 7514/05M and HKU 7647/06M), Centre of Reproduction, Development and Growth, the Academy of Finland and the Helsinki University Central Hospital Research Fund.
References


Bai GR, Yang LH, Huang XY and Sun FZ. Inositol 1,4,5-trisphosphate receptor type 1 phosphorylation and regulation by extracellular signal-regulated kinase. Biochem Biophys Res Commun 2006; 348:1319-1327.


de Jonge CJ. The cAMP-dependent kinase pathway and human sperm acrosomal exocytosis. *Front Biosci* 1996; **1**:d234-40


de Lamirande E, Leclerc P and Gagnon C. Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. *Mol Hum Reprod* 1997; **3**:175-194.


Esterhuizen AD, Franken DR, Lourens JG and van Rooyen LH. Clinical importance of zona pellucida-induced acrosome reaction and its predictive value for IVF. *Hum Reprod* 2001; **16**:138-144.


O’Flaherty C, de LE and Gagnon C. Positive role of reactive oxygen species in mammalian sperm
629 capacitation: triggering and modulation of phosphorylation events. Free Radic Biol Med 2006;
630 41:528-540.

Pang L, Sawada T, Decker SJ and Saltiel AR. Inhibition of MAP kinase kinase blocks the
633 270:13585-13588.

Patel S, Joseph SK and Thomas AP. Molecular properties of inositol 1,4,5-trisphosphate receptors.

Pereira DB, Carvalho AP and Duarte CB. Non-specific effects of the MEK inhibitors PD098,059 and
637 U0126 on glutamate release from hippocampal synaptosomes. Neuropharmacology 2002;
638 42:9-19.

Reiners JJ, Lee JY, Clift RE, Dudley DT and Myrand SP PD98059 is an equipotent antagonist of the
639 aryl hydrocarbon receptor and inhibitor of mitogen-activated protein kinase kinase. Mol

Ren D, Navarro B, Perez G, Jackson AC, Hsu S, Shi Q, Tilly JL and Clapham DE. A sperm ion

Roldan ER, Murave T and Shi QX. Exocytosis in spermatozoa in response to progesterone and zona

Roldan ER and Shi QX Sperm phospholipases and acrosomal exocytosis. Front Biosci 2007;
646 12:89-104.

Saridogan E, Djahanbakhch O, Kervancioglu ME, Kahyaoglu F, Shrimanker K and Grudzinskas JG.
649 Placental protein 14 production by human Fallopian tube epithelial cells in vitro. Hum Reprod

Schmitt JM and Stork PJ. Cyclic AMP-mediated inhibition of cell growth requires the small G

Schuffner AA, Bastiaan HS, Duran HE, Lin ZY, Morshed M, Franken DR and Oehninger S. Zona
654 pellucida-induced acrosome reaction in human sperm: dependency on activation of pertussis
655 toxin-sensitive G(i) protein and extracellular calcium, and priming effect of progesterone and


Taylor CW, Rahman T, Tovey SC, Dedos SG, Taylor EJ and Velamakanni S. IP3 receptors: some lessons from DT40 cells. *Immunol Rev* 2009; **231**:23-44.


Yang LH, Bai GR, Huang XY and Sun FZ. ERK binds, phosphorylates InsP3 type 1 receptor and regulates intracellular calcium dynamics in DT40 cells. *Biochem Biophys Res Commun* 2006; 349:1339-1344.

Figure 1. Effect of different concentrations of GdA pretreatment on ZP-induced acrosome reaction. (A) Solubilized ZP (1 μg/ml) was used to induce the acrosome reaction (N=5). *P<0.05 when compared with the corresponding ZP alone control. †P<0.05 when compared with the sum of acrosome-reacted spermatozoa after ZP alone and after GdA alone treatments. (B) Priming index of GdA pretreatment on ZP-induced acrosome reaction. Priming index is defined as the difference in the percentages of acrosome-reacted spermatozoa between sequential treatment and sum of the two individual treatments.

Figure 2. Effects of 30 nM GdA and deglycosylated glycodeolin on: (A) the intracellular cAMP level of human spermatozoa (N=5). *P<0.05 when compared with the corresponding control without glycodeolin treatment; (B) PKA activity (N=5). *P<0.05 for comparison with control without treatment; (C) activated-ERK1/2 level (pERK1/2) (N=3). Upper: Representative images of the western blot result for activated-ERK1/2. Sperm protein extract (10×10^6/lane) were resolved by SDS-PAGE, transferred to PVDF membrane and probed with anti-phospho ERK1/2 antibody (1:1000). Anti-tubulin antibody was used to reveal sample loading. Horseradish peroxidase conjugated anti-rabbit antibody at a dilution of 1:5000 was used as secondary antibody. Lower: Semi-quantitative comparison of the pERK1/2 level. The data were mean ± s.e.m. of densitometric measurements from three independent experiments and expressed as percent of control without treatment and (D) ERK1/2 activity (N=3). The results shown are obtained from three experiments using three samples. *P<0.05 for comparison with corresponding control without treatment.

Figure 3. Effects of 30 nM GdA on activated-ERK1/2 level (pERK1/2), ERK1/2 activity and ZP-induced acrosome reaction in the presence and absence of PKA inhibitor, Rp-cAMPS. (A) Upper: Representative images of the western blot result for activated ERK1/2. Sperm protein extract (10×10^6/lane) were resolved by SDS-PAGE, transferred to PVDF membrane and probed with anti-phospho ERK1/2 antibody (1:1000). Anti-tubulin antibody was used to reveal sample loading. Horseradish peroxidase conjugated anti-rabbit antibody at a dilution of 1:5000 was used as secondary antibody. Lower: Semi-quantitative comparison of the pERK1/2 level. The data were mean ± s.e.m. of densitometric measurements from three independent experiments and expressed as percent of control without treatment (N=3). (B) Comparison of the ERK1/2 activity. The results shown are obtained from three experiments using three samples. *P<0.05 for comparison with the no treatment control. †P<0.05 for comparison with the corresponding control without PKA inhibitor. (C) ZP-induced acrosome reaction (N=5). 1 μg/ml solubilized ZP was used to induce the acrosome reaction. *a-b, e-f, g-h P<0.05.

Figure 4. Effect of different concentrations of (A) PD98059 (0.1-100 μM) (B) U0126/U0124 (0.01-10 μM) and (C) rap kinase inhibitor (0.01-10 μM) on ZP-induced acrosome reaction (N=3). 1 μg/ml solubilized ZP was used to induce the acrosome reaction. *P<0.05 when compared with the control without inhibitor treatment.

Figure 5. (A) Immuno-localization of Raf kinases in human spermatozoa. Capacitated spermatozoa were collected on glass slides by cytospin (Shandon), fixed with 2% paraformaldehyde and permeabilized by 0.2% Triton X-100 for 20 minutes before incubation with the 1:50 anti-A-Raf, anti-B-Raf and anti-c-Raf antibodies for 24 hours in a humidified chamber at 4°C. Bound antibodies were detected by Alexa-594-conjugated secondary antibody. Slides processed without the primary antibodies were used as controls. Scale bar: 10 μm (B) Sperm protein extract (2×10^6/lane) were resolved by SDS-PAGE, transferred to PVDF membrane and probed with anti-A-Raf, anti-B-Raf and anti-c-Raf antibodies (1:1000). Horseradish peroxidase conjugated anti-rabbit antibody at a dilution of 1:5000 was used as secondary antibody. Antibody preabsorbed with 1:100 blocking peptide was used as controls. PA: Anti-c-Raf pre-absorbed with 1:100 blocking peptide (control). Arrow indicates the 73 kDa c-Raf.

Figure 6. Time courses of solubilized ZP (1 μg/ml)-induced Ca^2+ changes in the head of capacitated human spermatozoa (A) without pretreatment, (B) with GdA pretreatment (30 nM) or (C) with
U0126 pretreatment (0.1 μM). The bar indicates the period of solubilized ZP exposure.
Representative responses of 5 cells (colored traces). Mean response of all the cells (N=20 from 5
sperm samples) in the experiment is also shown (black traces). Five greyscale images are included
showing the signal intensity of the same spermatozoon at the time points indicated. Similar results
were obtained in 4 other experiments. Scale bar: 4 μm.

**Figure 7.** Effects of 30 nM GdA on the phosphorylation of IP3R1 in human spermatozoa (N=3).
Sperm protein extract (10×10⁶/lane) were resolved by SDS-PAGE, transferred to PVDF membrane
and probed with anti-IP3R1 antibodies (0.26 μg/ml). Phosphorylation of IP3R1 was determined by
reprobing the membrane with antibody that recognized phosphorylated serine or threonine residue
adjacent to a proline (pS/TP), the consensus motif phosphorylated by ERK.

**Figure 8.** A proposed intracellular signaling network of the priming activity of glycodelin-A on
ZP-induced acrosome reaction. The cAMP/PKA-dependent suppression of GdA on sperm ERK
reduces the phosphorylation of IP3R1, thereby increasing its affinity to IP3 and enhancing the
ZP-induced calcium influx and acrosome reaction.
Figure 1.

A

B
**Figure 2.**

Panel A: Graph showing the effect of GdA and Deglycosylated glycodelin on cAMP levels (fmol/10^6 sperm) over time (min).

Panel B: Bar graph comparing PKA activity (U/10^6 sperm) between Control, GdA, and Deglycosylated glycodelin.

Panel C: Western blot analysis showing the expression levels of pERK1, pERK2, and Tubulin in GdA and Deglycosylated glycodelin conditions.

Panel D: Bar graph illustrating the ERK1/2 activity (%) control at different GdA concentrations (0.3, 3, 30 nM).
Figure 3.

A

Solubilized ZP (1 μg/ml) - + + + +
GdA (30 nM) - - - + +
Rp-cAMPS (200 μM) - + - + +

B

ERK1/2 activity (% control)

C

% Acrosome reacted sperm

Solubilized ZP (1 μg/ml) - + + + + +
GdA (30 nM) - - - + +
Rp-cAMPS (200 μM) - - + - +
Figure 4.

A

B

C

% Acrosome reacted sperm

PD98059 concentration (μM)

% Acrosome reacted sperm

U0126/U0124 concentration (μM)

% Acrosome reacted sperm

Inhibitor concentration (μM)
Figure 5.

A

Phase contrast  Alexa-594

Control

A-Raf

B-Raf

c-Raf

B

A-Raf  B-Raf  PA  c-Raf  kDa

-150
-100
-75
-50
-37
-25
-20
Figure 6.

A

1 µg/ml solubilized ZP

Control

ΔF (%)

Seconds

B

1 µg/ml solubilized ZP

Glycolin-A

ΔF (%)

Seconds

C

1 µg/ml solubilized ZP

U0126

ΔF (%)

Seconds
Figure 7.

[Image: An image showing a gel with bands labeled Anti-IP3R1 and Anti-pS/TP. The gel has lane markers for GdA and Control, with molecular weight markers in kDa (100, 150, 250).]
Figure 8.

- Zona pellucida
  - ↑ IP3
  - ↓ IP3 binding affinity of IP3R1
  - ↓ [Ca^{2+}]_i
  - ↑ Acrosome reaction

- Glycodelin-A
  - ↓ cAMP
  - ↓ c-Raf
  - ↓ ERK
  - ↓ Phosphorylation of S/TP motif of IP3R1