1 **Zona pellucida-induced acrosome reaction in human spermatozoa is potentiated by**
2 **glycodelin-A via down-regulation of extracellular signal-regulated kinases and up-regulation of**
3 **zona pellucida-induce calcium influx**
4

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24 **Abstract**

25 **BACKGROUND**: Glycodelin-A interacts with spermatozoa before fertilization, but its role in 26 modulating sperm functions is not known. Zona pellucida–induced acrosome reaction is crucial to 27 fertilization and its dysfunction is a cause of male infertility. We hypothesized that glycodelin-A, a 28 glycoprotein found in the female reproductive tract, potentiates human spermatozoa for zona 29 pellucida-induced acrosome reaction. **METHODS:** Glycodelin isoforms were 30 immunoaffinity-purified. The sperm intracellular cyclic-adenosine monophosphate concentration, 31 protein kinase-A and extracellular signal-regulated kinase activities, and intracellular calcium were 32 measured by ELISA, kinase activity assay kit and Fluo-4AM technique, respectively. The 33 phosphorylation of inositol 1,4,5-trisphosphate type-1 receptor mediated by extracellular 34 signal-regulated kinase was determined by Western blotting. Zona pellucida-induced acrosome 35 reaction was detected by *Pisum sativum* staining. **RESULTS:** Pre-treatment of spermatozoa with 36 glycodelin-A significantly up-regulated adenylyl cyclase/protein kinase-A activity and 37 down-regulated the activity of extracellular signal-regulated kinase and its phosphorylation of 38 inositol 1,4,5-trisphosphate type-1 receptor, and thereby enhancing zona pellucida-induced calcium 39 influx and zona pellucida-induced acrosome reaction. Glycodelin-F or deglycosylated glycodelin-A 40 did not have these actions. Inhibitor of protein kinase abolished while that of the extracellular 41 signal-regulated kinase pathway mimic the priming activity of glycodelin-A. **CONCLUSIONS:** 42 Glycodelin-A in the female reproductive tract sensitizes spermatozoa for zona pellucida-induced 43 acrosome reaction in a glycosylation-specific manner through activation of the adenylyl 44 cyclase/protein kinase-A pathway, suppression of extracellular signal-regulated kinase activation and 45 up-regulation of zona pellucida-induced calcium influx. The action of glycodelin-A may be important 46 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

76 deglycosylation of the glycoproteins (Chiu et al., 2003a,b; Yeung et al., 2006; Chiu et al., 2007b).
77 The spermatozoa have to pass through the female reproductive tract before reaching the oocyte
78 for fertilization. During this passage, they encounter GdA, which, in addition to the secretory
79 endometrium, is also present in the oviductal fluid, follicular fluid and cumulus matrix (Seppala et al.,
80 2002; Yeung et al., 2006). As glycodelin modulates a variety of sperm functions (Yeung et al., 2006;
81 Seppala et al., 2007), we hypothesized that GdA primes spermatozoa for ZP-induced acrosome
82 reaction. In this study, we demonstrate that GdA potentiates the ZP-induced acrosome reaction in
83 human spermatozoa through down-regulation of extracellular signal-regulated kinase (ERK) activity
84 and up-regulation of zona pellucida-induced calcium influx.
85
86 **Materials and methods**
87 ***Semen samples***
88 The Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong
89 West Cluster approved the research protocol. Spermatozoa with normal semen parameters (WHO,
90 1999) from men attending the infertility clinic at Queen Mary Hospital were processed by Percoll
91 (Pharmacia, Uppsala, Sweden) density gradient centrifugation (Chiu et al., 2003a). The processed
92 spermatozoa were capacitated overnight as reported (Baldi et al., 1991; Whitmarsh et al., 1996;
93 Ficarro et al., 2003; Shetty et al., 2003; Chiu et al., 2008a) in Earle's balanced salt solution
94 containing 0.265 mg/ml calcium chloride, 2.2 mg/ml sodium bicarbonate, 1.09 mg/ml L-lactate,
95 0.033 mg/ml sodium pyruvate, 0.06 mg/ml penicillin G, 0.075 mg/ml streptomycin sulphate (EBSS;
96 Flow Laboratories, Irvine, UK), and 3% bovine serum albumin (BSA) at 37°C in an atmosphere of
97 5% CO_2 in air. The mean percentage of capacitated spermatozoa was 43.2±2.9% (mean ± standard
98 error of mean, s.e.m.) as determined by chlortetracycline staining (Chiu et al., 2005). The capacitated
99 spermatozoa were resuspended in EBSS supplemented with 0.3% BSA (EBSS/BSA).
100
101 ***Purification of glycodelin-A***
102 GdA was purified as described from first trimester amniotic fluid using monoclonal
103 anti-glycodelin (clone F43-7F9) affinity chromatography (Chiu et al., 2003a). In brief, amniotic fluid
104 diluted with Tris-buffered saline (TBS, pH 7.4) containing 0.1% Triton X-100 was loaded onto the

105 column. After successive washing of the column with TBS, 1M NaCl with 1% isopropanol, 10 mM
106 ammonium acetate with 0.1% isoproponal, pH 5 and TBS, GdA was eluted by 20 mM $CaCl_2$
107 containing 0.1% trifluoroacetic acid. Deglycosylated GdA was prepared by denaturation of GdA in
108 0.1% β-mecaptoethanol before incubation with 0.5 mU Peptide-N-Glycosidase F (PNGase F,
109 Sigma-Aldrich Inc., St. Louis, MO) at 37°C for 24 hours. The digest was boiled for 5 minutes to
110 inactivate PNGase F and dialyzed in 2 mM Tris-HCl, pH 7.5. The concentration of purified GdA was
111 determined by a commercial protein assay kit (Bio-Rad, Hercules, USA).
112
113 ***Preparation of solubilized zona pellucida***
114 ZPs were obtained from unfertilized human oocytes from the assisted reproduction programme
115 at Queen Mary Hospital, Hong Kong. The purification of solubilized ZP was reported recently (Chiu
116 et al., 2008b). Briefly, the purification involved separation of the ZP from the oocytes under
117 microscope and heat-solubilization of ZP at 70°C in 5 mM NaH_2PO_4 buffer (pH 2.5) for 90 minutes.
118
119 ***Determination of acrosome reaction***
120 Staining with fluorescein isothiocyanate labeled peanut (*Pisum sativum*) agglutinin (FITC-PSA;
121 Sigma) and Hoechst 33258 (bisBenzimide; Sigma) were used to evaluate the acrosomal status of
122 spermatozoa (Chiu et al., 2005). Processed spermatozoa were incubated with phosphate-buffered
123 saline (PBS, pH 7.4) containing 0.001% (w/v) Hoechst for 10 minutes, centrifuged through 2% (w/v)
124 polyvinylpyrrolidone-40 (Sigma) in PBS, fixed in 300 μl of 95% ethanol and dried on slide before
125 staining with 0.01% (w/v) FITC-PSA in PBS for 10 minutes. The fluorescence patterns of 150
126 spermatozoa in randomly selected fields were determined under a fluorescence microscope (Zeiss,
127 Oberkochen, Germany) with 600x magnification. The filter set used for Hoechst staining consisted of
128 an excitation filter G365, a chromatic beam splitter FT395 and a barrier filter LP420, whereas that for
129 FITC-PSA consisted of an excitation filter BP 450-490, a chromatic beam splitter FT510 and a
130 barrier filter LP520. Acrosome-reacted spermatozoa were defined as those without Hoechst and
131 FITC-PSA staining or with FITC-PSA staining at the equatorial segment only.
132
133 ***Determination of cyclic-adenosine monophosphate (cAMP)***

134 Intracellular cAMP was extracted in ice-cold 90% ethanol at -20°C for 30 minutes and at 4°C
135 for 30 minutes as described (Chiu et al., 2005). The ethanol extracts were dried in a rotary evaporator.
136 Intracellular cAMP in the extracts was determined using a non-radioactive cAMP ELISA Kit (R&D,
137 Minneapolis, MN) according to the manufacturer's instructions.
138
139 ***Determination of protein kinase A (PKA) activities***
140 Spermatozoa (20 x 10^6) were washed thrice with PBS, sonicated in 100 µl of homogenizing
141 buffer (20 mM PBS, pH 7.4 containing 2 mM EDTA, 1 mM dithiothreitol, 0.1 mM
142 phenylmethanesulfonyl fluoride, 0.1 mM vanadate, 1 mM $MgCl_2$, 100 mM NaCl and 0.05%
143 Triton-X100) for 15 minutes at 4°C as described (Bajpai and Doncel, 2003). The supernatant was
144 obtained after centrifugation at 15,800 g for 30 minutes at 4°C. The PKA activity in the supernatant
145 was determined as described (Chiu et al., 2010) with an ELISA-based PKA (Calbiochem, San Diego,
146 CA) assay kit. Standard curve was run with the test samples in each experiment. One Unit (U) of
147 PKA activity was defined as the amount of enzyme required to catalyze the transfer of 1 pmol of
148 phosphate to the substrates, RFARKGSLRQKNV in 1 minute at 30°C.
149
150 ***Determination of extracellular signal-regulated kinase activity***
151 Sperm proteins (10×10^6 spermatozoa) were extracted, resolved by 12% SDS-PAGE and blotted
152 on a PVDF membrane as described above. Western blotting was performed using a mouse
153 monoclonal anti-phospho ERK1/2 antibody (purified IgG; 1:1000; Cell Signaling, Danvers, MA).
154 Anti-tubulin antibody was used to reveal sample loading. Quantification of protein bands normalized
155 with respect to the tubulin control was carried out with Image J 1.36b software
156 (http://rsbweb.nih.gov/ij/index.html) and expressed as percent of the corresponding control without
157 treatment. Non-radioactive ERK1/2 activity assay kit (Chemicon, Temecula, CA) was used according
158 to the manufacturer's instructions to confirm the results of the Western blotting. Results were
159 expressed as percentage change over the corresponding control without treatment.
160
161 ***Determination of the effects of glycodelin-A on human zona pellucida-induced acrosome reaction***
162 Capacitated spermatozoa at a concentration of 2×10^6 spermatozoa/ml (N=5) were incubated

163 with either EBSS/BSA (control), different concentrations of GdA or GdF (0.3-300 nM) or
164 deglycosylated glycodelin (0.3-300 nM) for 90 minutes at 37°C in an atmosphere of 5% CO_2 in air.
165 After incubation, the spermatozoa were washed twice with EBSS/BSA before further incubation with
166 solubilized ZP (1 μg/ml or ~0.03 ZP/μl) or EBSS/BSA (control) for 60 minutes at 37°C in an
167 atmosphere of 5% CO_2 in air. The acrosomal status of the treated spermatozoa was then evaluated as
168 described above.
169

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

171 Capacitated spermatozoa (2×10^6/ml, N=5) were incubated with 30 nM of GdA, deglycosylated
172 glycodelin or EBSS/BSA (control) at 37°C under 5% CO_2 in air for 90 minutes. The intracellular
173 cAMP level, PKA and ERK activity of the treated spermatozoa were evaluated as described above.
174

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

176 To demonstrate the possible relationship between PKA activation with ERK phosphorylation
177 and priming of ZP-induced acrosome reaction, the ERK activities and acrosome reaction of
178 capacitated human spermatozoa (2×10^6, N=5) treated with 30 nM of GdA for 90 minutes in the
179 presence or absence of 200 μM of Rp-Adenosine 3′,5′-cyclic monophosphorothioate
180 triethylammonium salt (Rp-cAMPS, a PKA inhibitor; Calbiochem) prior to treatment with
181 solubilized ZP (1 μg/ml) were determined. The spermatozoa were washed after treatment and their
182 intracellular ERK activities and acrosomal status were evaluated as described above.
183

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

186 Capacitated spermatozoa (2×10^6/ml, N=5) were incubated with different concentrations of ERK
187 pathway inhibitors, PD98059 (0.1-100 μM), U0126/U0124 (0.01-10 μM), ZM336372 (0.01-10 μM),
188 Raf1 kinase inhibitor I (0.01-10 μM) or DMSO (control) for 90 minutes at 37°C in an atmosphere of
189 5% CO_2 in air. The inhibitors, PD98059 and U0126 (Calbiochem) are inhibitors of ERK1/2
190 activation inhibiting mitogen-activated protein kinase kinase activities through different mechanisms,
191 while U0124 (Calbiochem) is a negative control for U0126. ZM336372 and Raf1 kinase inhibitor I

192 (Calbiochem) are potent and specific inhibitor of the protein kinase c-Raf. The treated spermatozoa
193 were washed twice with EBSS/BSA. They were then incubated with solubilized ZP at a
194 concentration of 1 μg/ml or EBSS/BSA (control) for 60 minutes at 37°C in an atmosphere of 5% CO_2
195 in air. After incubation, the spermatozoa were washed twice before their acrosomal status was
196 evaluated as described above.
197

198 ***Expression and localization of ERK and Raf kinase***

199 Proteins from capacitated spermatozoa were separated by SDS-PAGE and transferred to PVDF
200 membrane for Western blot analysis as described. Affinity purified polyclonal rabbit antibodies
201 against A-Raf (Cell signaling), B-Raf (ECM, Versailles, KY), c-Raf (ECM) and ERK (Cell signaling)
202 were used. Horseradish peroxidase conjugated donkey anti-rabbit antibody at a dilution of 1:5000
203 was used as secondary antibody. For immunostaining, capacitated spermatozoa were collected on
204 glass slides by cytospin (Shandon, Pittsburgh, PA), fixed with 2% paraformaldehyde and
205 permeabilized by 0.2% Triton X-100 for 20 minutes as described (Chiu et al., 2007a,b) before
206 incubation with the anti-Raf (1:50) or anti-ERK (1:50) antibodies for 24 hours in a humidified
207 chamber at 4°C. Bound antibodies were detected by Alexa-594-conjugated secondary antibody
208 (Invitrogen, CA, USA). Antibody preabsorbed with 1:100 blocking peptide (ECM) or slides
209 processed without the primary antibodies were used as controls.
210

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

213 The relative levels of intracellular calcium within individual spermatozoon were measured
214 using Fluo-4AM as described with modification (Tesarik et al., 1996; Ren et al., 2001). In brief,
215 spermatozoa were loaded with 5 μM Fluo-4AM for 30 minutes at 37°C. Fifty microlitres of the
216 sperm suspension (2×10^6/ml) with or without prior GdA (30 nM; 90 minutes) or U0126 (0.1 μM; 90
217 minutes) treatment were then placed on a tissue culture dish coated with poly-L-lysine (10%
218 poly-L-lysine in double distilled water) as a droplet covered with light mineral oil (Sigma). The
219 spermatozoa were examined on a heated stage at 37°C under a fluorescence inverted microscope
220 (Nikon, Tokyo, Japan) with ×600 magnification. Spermatozoa attached to the culture dish and with a

221 wiggling tail were selected. Fluo-4AM was excited at a wavelength of 488 nm and the emission was
222 monitored at 512 nm. Fluorescence images of the selected spermatozoa were captured every second
223 for a total of 60 seconds through a CCD camera under the control of an Image Pro Plus imaging
224 system (Media Cybernetics, Bethesda, MD). The fluorescence signal associated with each selected
225 spermatozoon in the first 5 seconds of capturing was considered as the resting fluoresecence of the
226 spermatozoon. Two microlitres of solubilized ZP was then added to the sperm droplet to give a final
227 concentration of 1 μg/ml with the use of a micromanipulator (Nikon, Tokyo, Japan) and a
228 microinjector (Narishige, Tokyo, Japan). EBSS/BSA was added to the control droplet. The
229 fluorescent images of the spermatozoon were captured for a further 55 seconds. All the captured
230 images were analyzed as follows: After subtraction of the background signal, each sperm head in the
231 captured image was selected electronically. The intensity of the fluorescence signal within the
232 selected sperm head was extracted as eight-bit grey scale measurements. The signal from the sperm
233 tail or any cell that moved across the selected sperm head in the course of image capturing was
234 excluded from the analysis. The raw intensity values were normalized using the equation, $\Delta F = [(F -$
235 $F_{rest})/F_{rest}]\times 100\%$, where ΔF was the normalized fluorescence intensity, F was the intensity at a given
236 time point and F_{rest} was the mean intensity derived from the 5 images captured before addition of ZP
237 or EBSS/BSA. The experiment was performed 5 times, each involving spermatozoa from a different
238 donor. Twenty spermatozoa were randomly selected from each donor for analysis.
239

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

242 Capacitated spermatozoa (2×10^6/ml, N=3) were incubated with 30 nM of GdA or EBSS/BSA
243 (control) for 90 minutes at 37°C in an atmosphere of 5% CO_2 in air. The protein extract from 10×10^6
244 spermatozoa were resolved by 5% SDS-PAGE and blotted on a PVDF membrane as described above.
245 Western blotting was performed using a rabbit polyclonal anti-IP3R1 antibody (purified IgG; 0.26
246 μg/ml; Abcam, Cambridge, MA). To determine the phosphorylation of IP3R1 mediated by ERK, the
247 membrane was reprobed with a rabbit antibody against the phosphorylated S/TP motif
248 (serine/threonine residue followed by a proline residue) of the receptor (purified IgG; 1.5 μg/ml;
249 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).

279 ***Glycodelin-A increases intracellular cAMP concentration and PKA activity***

280 GdA induced a gradual increase in cAMP concentration, reaching a plateau at 60 m inutes
281 (Figure 2A). The intracellular cAMP levels increased from 43.4±3.6 fmol/10^6 spermatozoa to
282 61.6±5.1 fmol/10^6 spermatozoa ($p<0.05$) after 60 minutes of GdA treatment. Deglycosylated
283 glycodelin treatment did not affect intracellular cAMP concentration of spermatozoa at any of the
284 time points studied.

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

289

290 ***Glycodelin-A reduces extracellular signal-regulated kinase activity***

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

295

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

298 Rp-cAMPS (a PKA inhibitor) at a concentration 200 μM inhibited 35.2±4.5% of the sperm
299 PKA activity (N=10). Compared to the control without treatment (Figure 3A upper, lane 1),
300 Rp-cAMPS treatment significantly ($P<0.05$) increased the level of activated-ERK1/2 (Figure 3A
301 upper, lane 2; Figure 3A lower, column 2) and elevated ERK activity by 59.0±21.4% in the treated
302 spermatozoa (Figure 3B, column 2). Treatment with Rp-cAMPS abolished the inhibitory effect of
303 GdA on activated-ERK1/2 level (Figure 3A upper, lane 3 and 4; Figure 3A lower, column 3 and 4)
304 and ERK activity (Figure 3B, column 3 and 4). Rp-cAMPS also significantly ($P<0.05$) suppressed
305 the effects of GdA on ZP-induced acrosome reaction (Figure 3C); the percentage of ZP-induced
306 acrosome-reacted spermatozoa decreased from 38.7±4.5% (column 4) to 21.2±3.5% (column 5).
307 Rp-cAMPS at the concentrations used did not affect ZP-induced acrosome reaction (column 2 and 3),

308 sperm viability, sperm motility and spontaneous acrosome reaction (Supplementary Table ST1).

309

310 ***Extracellular signal-regulated kinase pathway inhibitors mimic the stimulatory effect of***
311 ***glycodelin-A on zona pellucida-induced acrosome reaction***

312 The percentages of spermatozoa undergoing ZP-induced acrosome reaction with and without
313 pretreatment of ERK pathway inhibitors were compared. The mean percentage of spontaneously
314 acrosome reacted spermatozoa before treatment was 6.9±0.7%. ERK pathway inhibitors, PD98059
315 (Figure 4A), U0126 (Figure 4B), ZM336372 (Figure 4C) and Raf1 kinase inhibitor I (Figure 4C) at
316 concentrations of 1 μM, 0.1 μM, ≥ 1 μM and 1 μM, respectively, significantly ($P<0.05$) increased the
317 ZP-induced acrosome reaction. PD98059 (1 μM) and U0126 (0.1 μM) inhibited 39.2±3.6% and
318 34.5±5.0% respectively of the sperm ERK activity (N=5). At high concentration (10 μM), U0126 and
319 Raf1 kinase inhibitor I inhibited ZP-induced acrosome reaction. U0124 (Figure 4B) had no effect on
320 ZP-induced acrosome reaction except at high concentration (10 μM). The inhibitors at the
321 concentrations used did not affect sperm viability, sperm motility and spontaneous acrosome reaction
322 (Supplementary Table ST1).

323

324 ***Localization of extracellular signal-regulated kinase and Raf kinase***

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

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

336

337 ***Glycodelin-A potentiates ZP-induced rise in the sperm intracellular calcium concentration***

338 Representative single spermatozoon records are shown in Figure 6. The addition of ZP induced
339 a rapid rise in the sperm intracellular calcium concentration ($[Ca^{2+}]_i$) that peaked at 21 seconds and
340 declined slowly thereafter (Figure 6A). The percentage increase of $[Ca^{2+}]_i$ upon ZP treatment was
341 significantly higher in the GdA- (334.4±43.2%, N=20; Figure 6B) or U0126-pretreated spermatozoa
342 (277.4±9.2%, N=20; Figure 6C) than the control spermatozoa incubated in medium alone
343 (194.3±27.3%, N=20; Figure 6A). In addition, the time taken for the $[Ca^{2+}]_i$ to reach the peak value
344 was shorter for GdA (18 seconds) or U0126-pretreated spermatozoa (16 seconds) (Figure 6B and C).
345 61.9±4.4% and 54.6±3.8% of spermatozoa responded to GdA and U0126 in calcium mobilization
346 respectively. Treatment with GdA (30 nM) or U0126 (0.1 μM) alone or addition of solvent vehicle
347 had no effect on these parameters (data not shown).

348

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

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

355

356 **Discussion**

357 Human oviduct cells express glycodelin mRNA and protein (Laird et al., 1995; Saridogan et al.,
358 1997; Yeung et al., 2006), which would interact with the spermatozoa entering the oviductal lumen.
359 The concentration of glycodelin in the oviduct is higher in the secretory than in the proliferative
360 phase of the menstrual cycle (Julkunen et al., 1986). GdA binds to the acrosome region of human
361 spermatozoa (Chiu et al., 2003a). It does not affect spontaneous acrosome reaction and capacitation
362 (Chiu et al., 2003a, 2005). The present study show that GdA, but not GdF and deglycosylated GdA,
363 enhanced the ZP-induced acrosome reaction of capacitated human spermatozoa, demonstrating that the
364 effect is glycosylation-dependent. We also demonstrate that the priming activity of GdA is mediated by
365 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.,

395 2001; de Lamirande and Gagnon, 2002; Liguori et al., 2005; Almog et al., 2008; Almog and Naor,
396 2008).

397 In this study, we provide the first evidence on the cAMP/PKA-dependent suppression of ERK
398 activity mediated by GdA in human spermatozoa. cAMP/PKA may act through the Raf
399 serine/threonine kinases (Cook and McCormick, 1993; Graves et al., 1993; Sevetson et al., 1993; Wu
400 et al., 1993; Stork and Schmitt, 2002) to phosphorylate MEK, which in turn phosphorylates ERK at
401 the tyrosine and threonine residues of the activation domain (Seger et al., 1994). There are three Raf
402 family members, namely A-Raf, B-Raf and c-Raf. cAMP/PKA activates ERK in many neuronal and
403 endocrine cells (Vossler et al., 1997; Dugan et al., 1999; Grewal et al., 1999) expressing B-Raf
404 (Erhardt et al., 1995; Stork and Schmitt, 2002). In cells that do not express B-Raf, transfection of
405 B-Raf converts cAMP from an inhibitor to an activator of ERKs (Vossler et al., 1997; Dugan et al.,
406 1999). In contrast, cAMP inhibits ERK activation in many nonneuronal cells expressing c-Raf,
407 including NIH 3T3 cells (Schmitt and Stork, 2001), Rat-1 fibroblasts (Cook and McCormick, 1993;
408 Burgering et al., 1993; Wu et al., 1993), myocytes (Graves et al., 1993), and adipocytes (Sevetson et
409 al., 1993). It has been proposed that PKA phosphorylates serine-43 of c-Raf, thereby inhibiting
410 Ras-dependent activation of c-Raf by preventing the binding of c-Raf to Ras (Wu et al., 1993).

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

416 Another novel finding of the present results was that the GdA-induced decrease in ERK activity
417 enhanced the ZP-induced acrosome reaction. Such a relationship was in agreement with the ability of
418 ERK pathway inhibitors, PD98059, U0126, ZM336372 and Raf1 kinase inhibitor I to mimic GdA
419 activity, enhancing the ZP-induced acrosome reaction. But, the enhancement was not related to a
420 direct action of the inhibitors on acrosome reaction (Supplementary Table ST1). The reported actions
421 of ERK pathway inhibitors on human acrosome reaction vary according to the inducer of acrosome
422 reaction. PD98059 or U0126 suppress phorbol myristoyl acetate-induced (Almog et al., 2008),
423 lysophosphatidylcholine- (de Lamirande and Gagnon, 2002), and A23187- (du Plessis et al., 2001),

424 but not progesterone- (Luconi et al., 1998a,b) induced acrosome reaction. PD98059 has also been
425 reported to inhibit ZP-induced acrosome reaction (du Plessis et al., 2001). The reason for the
426 discrepancy between these reports and the present study may be the use of different concentrations of
427 PD98059. While 1 μM of PD98059 demonstrated a priming effect in our study, 50 μM of the same
428 inhibitor inhibited the ZP-induced acrosome reaction in Du Plessis' report (du Plessis et al., 2001). In
429 somatic cells, PD98059 selectively inhibits ERK with IC50 of ~1-2 μM (Pang et al., 1995; Reiners et
430 al., 1998; Li et al., 2008). The inhibitor at concentrations ≥ 10 μM inactivates calcium influx
431 non-specifically (Gould and Stephano, 2000; Pereira et al., 2002). Non-specific activity of U0126 at
432 high concentrations has also been reported (Pereira et al., 2002). Therefore, the high concentrations
433 of PD98059 or U0126 may suppress the ZP-induced acrosome reaction by suppressing calcium
434 influx, which is known to be important for ZP-induced acrosome reaction (Breitbart, 2002;
435 Kirkman-Brown et al., 2002).

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

444 IP3R1 regulates the IP3-gated Ca^{2+} store of spermatozoa (Patel et al., 1999). This is consistent
445 with the expression of IP3R1 in the acrosomal region of human spermatozoa and its reduction after
446 acrosome reaction (Kuroda et al., 1999). In contrast, the expression of IP3R3 remains unchanged
447 after acrosome reaction and IP3R2 is absent in human spermatozoa (Kuroda et al., 1999).
448 Phosphorylation is an important regulatory mechanism of IP3R1 function (Taylor et al., 2009;
449 Vanderheyden et al., 2009). Activated ERK phosphorylates Ser 436 of IP3R1 and reduces its binding
450 to IP3 (Yang et al., 2006; Bai et al., 2006; Taylor et al., 2009). The present results demonstrate that
451 GdA suppresses ERK-mediated IP3R1 phosphorylation. Taken together, an intriguing possibility
452 arises that the suppressive activity of GdA on sperm ERK reduces the phosphorylation of IP3R1,

453 thereby increasing its affinity to IP3 and enhancing the ZP-induced calcium influx and acrosome
454 reaction. *In vitro*, only ~48% of the zona pellucida-bound spermatozoa from fertile men are capable
455 of undergoing ZP–induced acrosome reaction (Liu et al., 2003). Patients with normal semen analysis
456 but with <15% acrosome reaction upon exposure to solubilized ZP had been reported to have poor
457 fertilization results with standard IVF (Esterhuizen et al., 2001). Therefore, the action of GdA may be
458 important *in vivo* to ensure full responsiveness of human spermatozoa to the ZP. This hypothesis is
459 being investigated in our laboratory. Based on the present data as well as others, a schema for the
460 intracellular signaling network of GdA in modulating ZP-induced acrosome reaction is proposed in
461 Figure 8.

462 ZP stimulation activates phospholipase C (Roldan and Shi, 2007), which generates IP3, thereby
463 inducing release of intracellular Ca^{2+} through IP_3-gated channels. Although GdA modulates the
464 IP3R1 activity, our preliminary data show that it has no effect on the phospholipase C activity in
465 spermatozoa. The differential action of GdA and ZP on phospholipase C-IP3-gated calcium channels
466 may explain the lack of effect of GdA on acrosome reaction while exhibiting a priming effect on
467 ZP-induced acrosome reaction.

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

482 to ZP-induced acrosome reaction.

483

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488 **References**

489 Aitken RJ, Harkiss D, Knox W, Paterson M and Irvine DS. A novel signal transduction cascade in
490 capacitating human spermatozoa characterised by a redox-regulated, cAMP-mediated
491 induction of tyrosine phosphorylation. *J Cell Sci* 1998; **111**:645-656.

492 Almog T, Lazar S, Reiss N, Etkovitz N, Milch E, Rahamim N, Dobkin-Bekman M, Rotem R, Kalina
493 M, Ramon J et al. Identification of extracellular signal-regulated kinase 1/2 and p38 MAPK as
494 regulators of human sperm motility and acrosome reaction and as predictors of poor
495 spermatozoan quality. *J Biol Chem* 2008; **283:**14479-14489.

496 Almog T and Naor Z. Mitogen activated protein kinases (MAPKs) as regulators of spermatogenesis
497 and spermatozoa functions. *Mol Cell Endocrinol* 2008; **282:**39-44.

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

501 Bajpai M and Doncel GF. Involvement of tyrosine kinase and cAMP-dependent kinase cross-talk in
502 the regulation of human sperm motility. *Reproduction* 2003; **126:**183-195.

503 Baldi E, Casano R, Falsetti C, Krausz C, Maggi M and Forti G. Intracellular calcium accumulation
504 and responsiveness to progesterone in capacitating human spermatozoa. *J Androl* 1991; **12:**
505 323-330.

506 Branham MT, Bustos MA, De Blas GA, Rehmann H, Zarelli VE, Treviño CL, Darszon A, Mayorga
507 LS and Tomes CN. Epac activates the small G proteins Rap1 and Rab3A to achieve exocytosis.
508 *J Biol Chem* 2009; **284:**24825-24839.

509 Breitbart H. Intracellular calcium regulation in sperm capacitation and acrosomal reaction. *Mol Cell*
510 *Endocrinol* 2002; **187:**139-144.

511 Burgering BM, Pronk GJ, van Weeren PC, Chardin P and Bos JL. cAMP antagonizes p21ras-directed
512 activation of extracellular signal-regulated kinase 2 and phosphorylation of mSos nucleotide
513 exchange factor. *EMBO J* 1993; **12:**4211-4220.

514 Chiu PC, Koistinen R, Koistinen H, Seppala M, Lee KF and Yeung WS. Zona-binding inhibitory
515 factor-1 from human follicular fluid is an isoform of glycodelin. *Biol Reprod* 2003a;
516 **69:**365-372.

517 Chiu PC, Koistinen R, Koistinen H, Seppala M, Lee KF and Yeung WS. Binding of zona binding
518 inhibitory factor-1 (ZIF-1) from human follicular fluid on spermatozoa. *J Biol Chem* 2003b;
519 **278:**13570-13577.
520 Chiu PC, Tsang HY, Koistinen R, Koistinen H, Seppala M, Lee KF and Yeung WS. The contribution
521 of D-mannose, L-fucose, N-acetylglucosamine, and selectin residues on the binding of
522 glycodelin isoforms to human spermatozoa. *Biol Reprod* 2004; **70:**1710-1719.
523 Chiu PC, Chung MK, Tsang HY, Koistinen R, Koistinen H, Seppala M, Lee KF and Yeung WS.
524 Glycodelin-S in human seminal plasma reduces cholesterol efflux and inhibits capacitation of
525 spermatozoa. *J Biol Chem* 2005; **280:**25580-25589.
526 Chiu PC, Chung MK, Koistinen R, Koistinen H, Seppala M, Ho PC, Ng EH, Lee KF, Yeung WS
527 Cumulus oophorus-associated glycodelin-C displaces sperm-bound glycodelin-A and -F and
528 stimulates spermatozoa-zona pellucida binding. *J Biol Chem* 2007a; **282:**5378-5388.
529 Chiu PC, Chung MK, Koistinen R, Koistinen H, Seppala M, Ho PC, Ng EH, Lee KF and Yeung WS.
530 Glycodelin-A interacts with fucosyltransferase on human sperm plasma membrane to inhibit
531 spermatozoa-zona pellucida binding. *J Cell Sci* 2007b; **120:**33-44.
532 Chiu PC, Wong BS, Chung MK, Lam KK, Pang RT, Lee KF, Sumitro SB, Gupta SK and Yeung WS.
533 Effects of native human zona pellucida glycoproteins 3 and 4 on acrosome reaction and zona
534 pellucida binding of human spermatozoa. *Biol Reprod* 2008a; **79:**869-877.
535 Chiu PC, Wong BS, Lee CL, Pang RT, Lee KF, Sumitro SB, Gupta SK and Yeung WS. Native
536 human zona pellucida glycoproteins: purification and binding properties. *Hum Reprod* 2008b;
537 **23:**1385-1393.
538 Chiu PC, Liao S, Lam KK, Tang F, Ho JC, Ho PC, O WS, Yao YQ and Yeung WS. Adrenomedullin
539 Regulates Sperm Motility and Oviductal Ciliary Beat via Cyclic Adenosine
540 5'-Monophosphate/Protein Kinase A and Nitric Oxide. *Endocrinology* 2010; **151:**3336-3347.
541 Cook SJ and McCormick F. Inhibition by cAMP of Ras-dependent activation of Raf. *Science* 1993;
542 **262:**1069-1072.
543 de Jonge CJ. The cAMP-dependent kinase pathway and human sperm acrosomal exocytosis. *Front*
544 *Biosci* 1996; **1:**d234-40

545 de Jonge CJ, Barratt CL, Radwanska E and Cooke ID. The acrosome reaction-inducing effect of
546 human follicular and oviductal fluid. *J Androl* 1993; **14:**359-365.
547 de Lamirande E and Gagnon C. The extracellular signal-regulated kinase (ERK) pathway is involved
548 in human sperm function and modulated by the superoxide anion. *Mol Hum Reprod* 2002; **8:**
549 124-135.
550 de Lamirande E, Leclerc P and Gagnon C. Capacitation as a regulatory event that primes
551 spermatozoa for the acrosome reaction and fertilization. *Mol Hum Reprod* 1997; **3:**175-194.
552 du Plessis SS, Page C and Franken DR. The zona pellucida-induced acrosome reaction of human
553 spermatozoa involves extracellular signal-regulated kinase activation. *Andrologia* 2001;
554 **33:**337-342.
555 Dugan LL, Kim JS, Zhang Y, Bart RD, Sun Y, Holtzman DM and Gutmann DH. Differential effects
556 of cAMP in neurons and astrocytes. Role of B-raf. *J Biol Chem* 1999; **274:**25842-25848.
557 Erhardt P, Troppmair J, Rapp UR and Cooper GM. Differential regulation of Raf-1 and B-Raf and
558 Ras-dependent activation of mitogen-activated protein kinase by cyclic AMP in PC12 cells.
559 *Mol Cell Biol* 1995; **15:**5524-5530.
560 Esterhuizen AD, Franken DR, Lourens JG and van Rooyen LH. Clinical importance of zona
561 pellucida-induced acrosome reaction and its predictive value for IVF. *Hum Reprod* 2001;
562 **16:**138-144.
563 Ficarro S, Chertihin O, Westbrook VA, White F, Jayes F, Kalab P, Marto JA, Shabanowitz J, Herr JC,
564 Hunt DF et al. Phosphoproteome analysis of capacitated human sperm. Evidence of tyrosine
565 phosphorylation of a kinase-anchoring protein 3 and valosin-containing protein/p97 during
566 capacitation. J Biol Chem 2003; **278:**11579-11589.
567 Gould MC and Stephano JL. Inactivation of Ca(2+) action potential channels by the MEK inhibitor
568 PD98059. *Exp Cell Res* 2000; **260:**175-179.
569 Graves LM, Bornfeldt KE, Raines EW, Potts BC, Macdonald SG, Ross R and Krebs EG. Protein
570 kinase A antagonizes platelet-derived growth factor-induced signaling by mitogen-activated
571 protein kinase in human arterial smooth muscle cells. *Proc Natl Acad Sci USA* 1993;
572 **90:**10300-10304.

573 Grewal SS, York RD and Stork PJ. Extracellular-signal-regulated kinase signalling in neurons. *Curr*
574 *Opin Neurobiol* 1999; **9:**544-553.
575 Julkunen M, Wahlström T and Seppälä M. Human fallopian tube contains placental protein 14. *Am J*
576 *Obstet Gynecol* 1986; **154:**1076-1079.
577 Kinukawa M, Oda S, Shirakura Y, Okabe M, Ohmuro J, Baba SA, Nagata M and Aoki F. Roles of
578 cAMP in regulating microtubule sliding and flagellar bending in demembranated hamster
579 spermatozoa. *FEBS Lett* 2006; **580:**1515-1520.
580 Kirkman-Brown JC, Punt EL, Barratt CL and Publicover SJ. Zona pellucida and
581 progesterone-induced Ca2+ signaling and acrosome reaction in human spermatozoa. *J Androl*
582 2002; **23:**306-315.
583 Kolch W. Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein
584 interactions. *Biochem. J* 2000; **351:**289-305.
585 Kuroda Y, Kaneko S, Yoshimura Y, Nozawa S and Mikoshiba K. Are there inositol 1,4,5-triphosphate
586 (IP3) receptors in human sperm? *Life Sci* 1999; **65:**135-143.
587 Laird SM, Hill CJ, Warren MA, Tuckerman EM and Li TC. The production of placental protein 14
588 by human uterine tubal epithelial cells in culture. *Hum Reprod* 1995; **10:**1346-1351.
589 Lefievre L, Jha KN, de LE, Visconti PE and Gagnon C. Activation of protein kinase A during human
590 sperm capacitation and acrosome reaction. *J Androl* 2002; **23:**709-716.
591 Li L, Tatake RJ, Natarajan K, Taba Y, Garin G, Tai C, Leung E, Surapisitchat J, Yoshizumi M, Yan
592 C et al. Fluid shear stress inhibits TNF-mediated JNK activation via MEK5-BMK1 in
593 endothelial cells. *Biochem Biophys Res Commun* 2008; **370:**159-163.
594 Liguori L, de LE, Minelli A and Gagnon C. Various protein kinases regulate human sperm acrosome
595 reaction and the associated phosphorylation of Tyr residues and of the Thr-Glu-Tyr motif. *Mol*
596 *Hum Reprod* 2005; **11:**211-221.
597 Liu DY, Stewart T and Baker HW. Normal range and variation of the zona pellucida-induced
598 acrosome reaction in fertile men. *Fertil Steril* 2003; **80:**384-389.
599 Liu DY, Garrett C, Baker HW. Clinical application of sperm-oocyte interaction tests in in vitro
600 fertilization--embryo transfer and intracytoplasmic sperm injection programs. *Fertil Steril* 2004;
601 **82:**1251-1263.

602 Liu DY, Liu ML, Garrett C and Baker HW. Comparison of the frequency of defective sperm-zona
603 pellucida (ZP) binding and the ZP-induced acrosome reaction between subfertile men with
604 normal and abnormal semen. *Hum Reprod* 2007; **22:**1878-1884.
605 Luconi M, Krausz C, Barni T, Vannelli GB, Forti G and Baldi E. Progesterone stimulates p42
606 extracellular signal-regulated kinase (p42erk) in human spermatozoa. *Mol Hum Reprod* 1998a;
607 **4:**251-258.
608 Luconi M, Barni T, Vannelli GB, Krausz C, Marra F, Benedetti PA, Evangelista V, Francavilla S,
609 Properzi G, Forti G et al. Extracellular signal-regulated kinases modulate capacitation of
610 human spermatozoa. *Biol Reprod* 1998b; **58:**1476-1489.
611 Nixon B, Bielanowicz A, McLaughlin EA, Tanphaichitr N, Ensslin MA and Aitken RJ. Composition
612 and significance of detergent resistant membranes in mouse spermatozoa. *J Cell Physiol* 2009;
613 **218:**122-134.
614 Nolan MA, Babcock DF, Wennemuth G, Brown W, Burton KA and McKnight GS. Sperm-specific
615 protein kinase A catalytic subunit Calpha2 orchestrates cAMP signaling for male fertility. *Proc*
616 *Natl Acad Sci USA* 2004; **101:**13483-13488.
617 Oehninger S, Coddington CC, Hodgen GD and Seppala M. Factors affecting fertilization:
618 endometrial placental protein 14 reduces the capacity of human spermatozoa to bind to the
619 human zona pellucida. *Fertil Steril* 1995; **63:**377-383.
620 O'Flaherty C, de LE and Gagnon C. Phosphorylation of the Arginine-X-X-(Serine/Threonine) motif
621 in human sperm proteins during capacitation: modulation and protein kinase A dependency.
622 *Mol Hum Reprod* 2004; **10:**355-363.
623 O'Flaherty C, Breininger E, Beorlegui N and Beconi MT. Acrosome reaction in bovine spermatozoa:
624 role of reactive oxygen species and lactate dehydrogenase C4. *Biochim Biophys Acta* 2005;
625 **1726:**96-101.
626 O'Flaherty C, de LE and Gagnon C. Reactive oxygen species and protein kinases modulate the level
627 of phospho-MEK-like proteins during human sperm capacitation. *Biol Reprod* 2005;
628 **73:**94-105.

629 O'Flaherty C, de LE and Gagnon C. Positive role of reactive oxygen species in mammalian sperm
630 capacitation: triggering and modulation of phosphorylation events. *Free Radic Biol Med* 2006;
631 **41:**528-540.
632 Pang L, Sawada T, Decker SJ and Saltiel AR. Inhibition of MAP kinase kinase blocks the
633 differentiation of PC-12 cells induced by nerve growth factor. *J Biol Chem* 1995;
634 **270:**13585-13588.
635 Patel S, Joseph SK and Thomas AP. Molecular properties of inositol 1,4,5-trisphosphate receptors.
636 *Cell Calcium* 1999; **25:**247-264.
637 Pereira DB, Carvalho AP and Duarte CB. Non-specific effects of the MEK inhibitors PD098,059 and
638 U0126 on glutamate release from hippocampal synaptosomes. *Neuropharmacology* 2002;
639 **42:**9-19.
640 Reiners JJ, Lee JY, Clift RE, Dudley DT and Myrand SP PD98059 is an equipotent antagonist of the
641 aryl hydrocarbon receptor and inhibitor of mitogen-activated protein kinase kinase. *Mol*
642 *Pharmacol* 1998; **53:**438-445.
643 Ren D, Navarro B, Perez G, Jackson AC, Hsu S, Shi Q, Tilly JL and Clapham DE. A sperm ion
644 channel required for sperm motility and male fertility. *Nature* 2001; **413:**603-609.
645 Roldan ER, Murase T and Shi QX. Exocytosis in spermatozoa in response to progesterone and zona
646 pellucida. *Science* 1994; **266:**1578-1581.
647 Roldan ER and Shi QX Sperm phospholipases and acrosomal exocytosis. *Front Biosci* 2007;
648 **12:**89-104.
649 Saridogan E, Djahanbakhch O, Kervancioglu ME, Kahyaoglu F, Shrimanker K and Grudzinskas JG.
650 Placental protein 14 production by human Fallopian tube epithelial cells in vitro. *Hum Reprod*
651 1997; **12:**1500-1507.
652 Schmitt JM and Stork PJ. Cyclic AMP-mediated inhibition of cell growth requires the small G
653 protein Rap1. *Mol Cell Biol* 2001; **21:**3671-3683.
654 Schuffner AA, Bastiaan HS, Duran HE, Lin ZY, Morshedi M, Franken DR and Oehninger S. Zona
655 pellucida-induced acrosome reaction in human sperm: dependency on activation of pertussis
656 toxin-sensitive G(i) protein and extracellular calcium, and priming effect of progesterone and
657 follicular fluid. *Mol Hum Reprod* 2002; **8:**722-727.

658 Seger R, Seger D, Reszka AA, Munar ES, Eldar-Finkelman H, Dobrowolska G, Jensen AM,
659 Campbell JS, Fischer EH and Krebs EG. Overexpression of mitogen-activated protein kinase
660 kinase (MAPKK) and its mutants in NIH 3T3 cells. Evidence that MAPKK involvement in
661 cellular proliferation is regulated by phosphorylation of serine residues in its kinase
662 subdomains VII and VIII. *J Biol Chem* 1994; **269:**25699-25709.

663 Seino S and Shibasaki T. PKA-dependent and PKA-independent pathways for cAMP-regulated
664 exocytosis. *Physiol Rev* 2005; **85:**1303-1342.

665 Seppala M, Taylor RN, Koistinen H, Koistinen R and Milgrom E. Glycodelin: a major lipocalin
666 protein of the reproductive axis with diverse actions in cell recognition and differentiation.
667 *Endocr Rev* 2002; **23:**401-430.

668 Seppala M, Koistinen H, Koistinen R, Chiu PC and Yeung WS. Glycosylation related actions of
669 glycodelin: gamete, cumulus cell, immune cell and clinical associations. *Hum Reprod Update*
670 2007; **13:**275-287.

671 Sevetson BR, Kong X and Lawrence JC. Increasing cAMP attenuates activation of mitogen-activated
672 protein kinase. *Proc Natl Acad Sci USA* 1993; **90:**10305-10309.

673 Shetty J, Wolkowicz MJ, Digilio LC, Klotz KL, Jayes FL, Diekman AB, Westbrook VA, Farris EM,
674 Hao Z, Coonrod SA et al. SAMP14, a novel, acrosomal membrane-associated,
675 glycosylphosphatidylinositol-anchored member of the Ly-6/urokinase-type plasminogen
676 activator receptor superfamily with a role in sperm-egg interaction. *J Biol Chem* 2003;
677 **278:**30506-30515.

678 Shi QX, Chen WY, Yuan YY, Mao LZ, Yu SQ, Chen AJ, Ni Y and Roldan ER. Progesterone primes
679 zona pellucida-induced activation of phospholipase A2 during acrosomal exocytosis in guinea
680 pig spermatozoa. *J Cell Physiol* 2005; **205:**344-354.

681 Stork PJ and Schmitt JM. Crosstalk between cAMP and MAP kinase signaling in the regulation of
682 cell proliferation. *Trends Cell Biol* 2002; **12:**258-266.

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

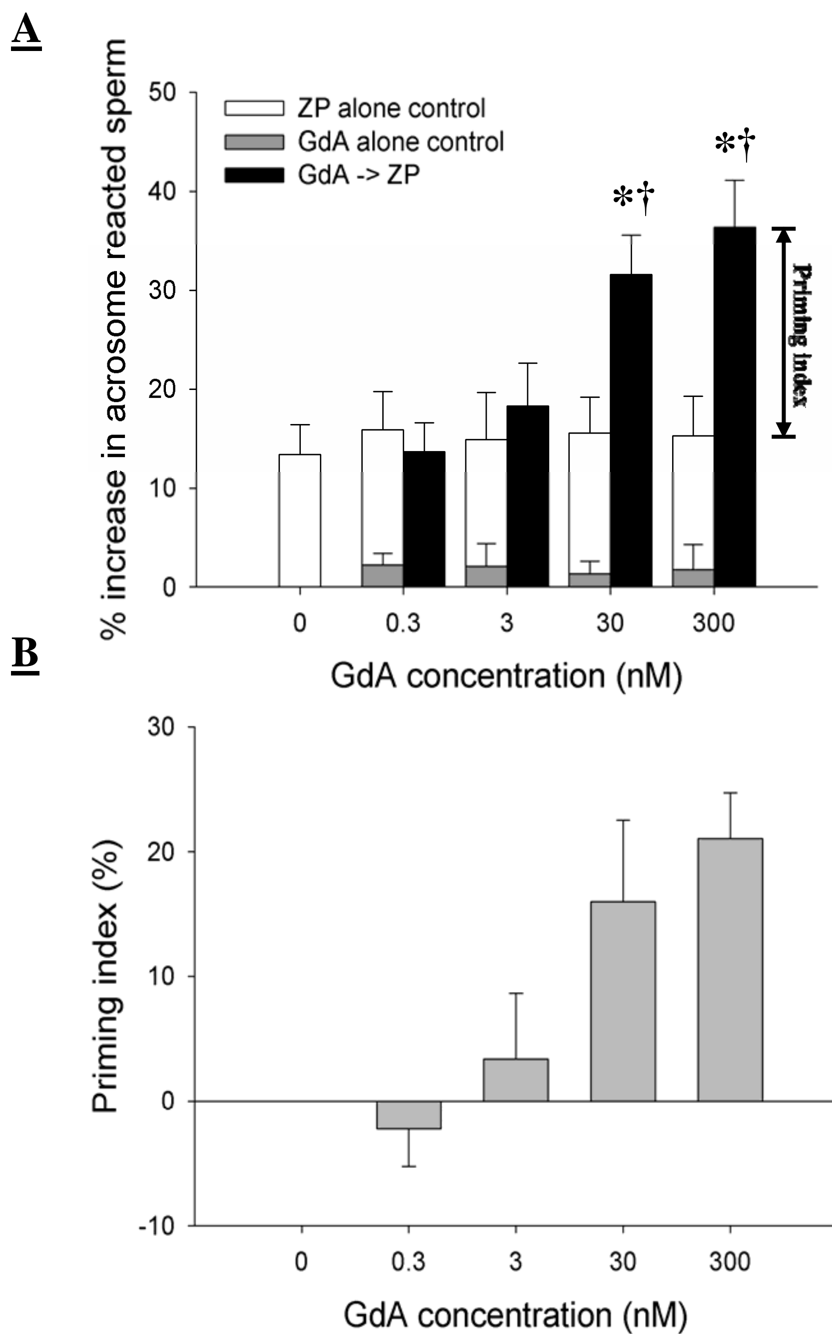
685 Tesarik J, Carreras A and Mendoza C. Single cell analysis of tyrosine kinase dependent and
686 independent Ca2+ fluxes in progesterone induced acrosome reaction. *Mol Hum Reprod* 1996;
687 **2:**225-232.
688 Vanderheyden V, Devogelaere B, Missiaen L, De Smedt H, Bultynck G. and Parys JB. Regulation of
689 inositol 1,4,5-trisphosphate-induced Ca2+ release by reversible phosphorylation and
690 dephosphorylation. *Biochim Biophys Acta* 2009; **1793:**959-970.
691 Visconti PE, Moore GD, Bailey JL, Leclerc P, Connors SA, Pan D, Olds-Clarke P and Kopf GS.
692 Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are
693 regulated by a cAMP-dependent pathway. *Development* 1995; **121:**1139-1150.
694 Vossler MR, Yao H, York RD, Pan MG, Rim CS and Stork PJ. cAMP activates MAP kinase and
695 Elk-1 through a B-Raf- and Rap1-dependent pathway. *Cell* 1997; **89:**73-82.
696 Whitmarsh AJ, Woolnough MJ, Moore HD, Hornby DP and Barratt CL. Biological activity of
697 recombinant human ZP3 produced in vitro: potential for a sperm function test. *Mol Hum*
698 *Reprod* 1996; **2:**911-919.
699 World Health Organization. Laboratory manual for the examination of human semen and
700 semen-cervical mucus interaction. 1999. Cambridge University Press, Cambridge, UK.
701 Wu J, Dent P, Jelinek T, Wolfman A, Weber MJ and Sturgill TW. Inhibition of the EGF-activated
702 MAP kinase signaling pathway by adenosine 3',5'-monophosphate. *Science* 1993;
703 **262:**1065-1069.
704 Yang LH, Bai GR, Huang XY and Sun FZ. ERK binds, phosphorylates InsP3 type 1 receptor and
705 regulates intracellular calcium dynamics in DT40 cells. *Biochem Biophys Res Commun* 2006;
706 **349:**1339-1344.
707 Yeung WS, Lee KF, Koistinen R, Koistinen H, Seppala M, Ho PC and Chiu PC. Roles of glycodelin
708 in modulating sperm function. *Mol Cell Endocrinol* 2006; **250:**149-156.

709 ## Figure Legends

710 **Figure 1.** Effect of different concentrations of GdA pretreatment on ZP-induced acrosome reaction.
711 **(A)** Solubilized ZP (1 μg/ml) was used to induce the acrosome reaction (N=5). *$P<0.05$ when
712 compared with the corresponding ZP alone control. †$P<0.05$ when compared with the sum of
713 acrosome-reacted spermatozoa after ZP alone and after GdA alone treatments. **(B)** Priming index of
714 GdA pretreatment on ZP-induced acrosome reaction. Priming index is defined as the difference in the
715 percentages of acrosome-reacted spermatozoa between sequential treatment and sum of the two
716 individual treatments.
717
718 **Figure 2.** Effects of 30 nM GdA and deglycosylated glycodelin on: **(A)** the intracellular cAMP level
719 of human spermatozoa (N=5). $^{*}P<0.05$ when compared with the corresponding control without
720 glycodelin treatment; **(B)** PKA activity (N=5). *$P<0.05$ for comparison with control without
721 treatment; **(C)** activated-ERK1/2 level (pERK1/2) (N=3). **Upper:** Representative images of the
722 western blot result for activated-ERK1/2. Sperm protein extract (10×10^6/lane) were resolved by
723 SDS-PAGE, transferred to PVDF membrane and probed with anti-phospho ERK1/2 antibody
724 (1:1000). Anti-tubulin antibody was used to reveal sample loading. Horseradish peroxidase
725 conjugated anti-rabbit antibody at a dilution of 1:5000 was used as secondary antibody. **Lower:**
726 Semi-quantitative comparison of the pERK1/2 level. The data were mean ± s.e.m. of densitometric
727 measurements from three independent experiments and expressed as percent of corresponding
728 control without treatment and **(D)** ERK1/2 activity (N=3). The results shown are obtained from three
729 experiments using three samples. *$P<0.05$ for comparison with corresponding control without
730 treatment.
731
732 **Figure 3.** Effects of 30 nM GdA on activated-ERK1/2 level (pERK1/2), ERK1/2 activity and
733 ZP-induced acrosome reaction in the presence and absence of PKA inhibitor, Rp-cAMPS. **(A) Upper:**
734 Representative images of the western blot result for activated ERK1/2. Sperm protein extract
735 (10×10^6/lane) were resolved by SDS-PAGE, transferred to PVDF membrane and probed with
736 anti-phospho ERK1/2 antibody (1:1000). Anti-tubulin antibody was used to reveal sample loading.
737 Horseradish peroxidase conjugated anti-rabbit antibody at a dilution of 1:5000 was used as secondary
738 antibody. **Lower:** Semi-quantitative comparison of the pERK1/2 level. The data were mean ± s.e.m.
739 of densitometric measurements from three independent experiments and expressed as percent of
740 control without treatment (N=3). **(B)** Comparison of the ERK1/2 activity. The results shown are
741 obtained from three experiments using three samples. *$P<0.05$ for comparison with the no treatment
742 control. †$P<0.05$ for comparison with the corresponding control without PKA inhibitor. **(C)**
743 ZP-induced acrosome reaction(N=5). 1 μg/ml solubilized ZP was used to induce the acrosome
744 reation. $^{a\text{-}b,\ c\text{-}d,\ e\text{-}f,\ g\text{-}h}P<0.05$.
745
746 **Figure 4.** Effect of different concentrations of **(A)** PD98059 (0.1-100 μM) **(B)** U0126/U0124
747 (0.01-10 μM) and **(C)** raf kinase inhibitor (0.01-10 μM) on ZP-induced acrosome reaction (N=3). 1
748 μg/ml solubilized ZP was used to induce the acrosome reaction. *$P<0.05$ when compared with the
749 control without inhibitor treatment.
750
751 **Figure 5.** **(A)** Immuno-localization of Raf kinases in human spermatozoa. Capacitated spermatozoa
752 were collected on glass slides by cytospin (Shandon), fixed with 2% paraformaldehyde and
753 permeabilized by 0.2% Triton X-100 for 20 minutes before incubation with the 1:50 anti-A-Raf,
754 anti-B-Raf and anti-c-Raf antibodies for 24 hours in a humidified chamber at 4°C. Bound antibodies
755 were detected by Alexa-594-conjugated secondary antibody. Slides processed without the primary
756 antibodies were used as controls. Scale bar: 10 μm **(B)** Sperm protein extract (2×10^6/lane) were
757 resolved by SDS-PAGE, transferred to PVDF membrane and probed with anti-A-Raf, anti-B-Raf and
758 anti-c-Raf antibodies (1:1000). Horseradish peroxidase conjugated anti-rabbit antibody at a dilution
759 of 1:5000 was used as secondary antibody. Antibody preabsorbed with 1:100 blocking peptide was
760 used as controls. PA: Anti-c-Raf pre-absorpted with 1:100 blocking peptide (control). Arrow
761 indicates the 73 kDa c-Raf.
762
763 **Figure 6.** Time courses of solubilized ZP (1 μg/ml)-induced Ca^{2+} changes in the head of capacitated
764 human spermatozoa **(A)** without pretreatment, **(B)** with GdA pretreatment (30 nM) or **(C)** with

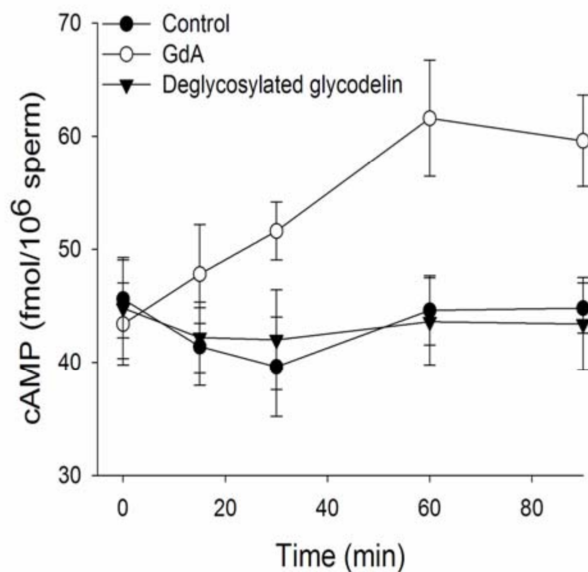
765 U0126 pretreatment (0.1 μM). The bar indicates the period of solubilized ZP exposure.
766 Representative responses of 5 cells (colored traces). Mean response of all the cells (N=20 from 5
767 sperm samples) in the experiment is also shown (black traces). Five greyscale images are included
768 showing the signal intensity of the same spermatozoon at the time points indicated. Similar results
769 were obtained in 4 other experiments. Scale bar: 4 μm
770
771 **Figure 7.** Effects of 30 nM GdA on the phosphorylation of IP3R1 in human spermatozoa (N=3).
772 Sperm protein extract (10×10^6/lane) were resolved by SDS-PAGE, transferred to PVDF membrane
773 and probed with anti-IP3R1 antibodies (0.26 μg/ml). Phosphorylation of IP3R1 was determined by
774 reprobing the membrane with antibody that recognized phosphorylated serine or threonine residue
775 adjacent to a proline (pS/TP), the consensus motif phosphorylated by ERK.
776
777 **Figure 8.** A proposed intracellular signaling network of the priming activity of glycodelin-A on
778 ZP-induced acrosome reaction. The cAMP/PKA-dependent suppression of GdA on sperm ERK
779 reduces the phosphorylation of IP3R1, thereby increasing its affinity to IP3 and enhancing the
780 ZP-induced calcium influx and acrosome reaction.

781 **Figure 1.**.



782 **Figure 2.**

A



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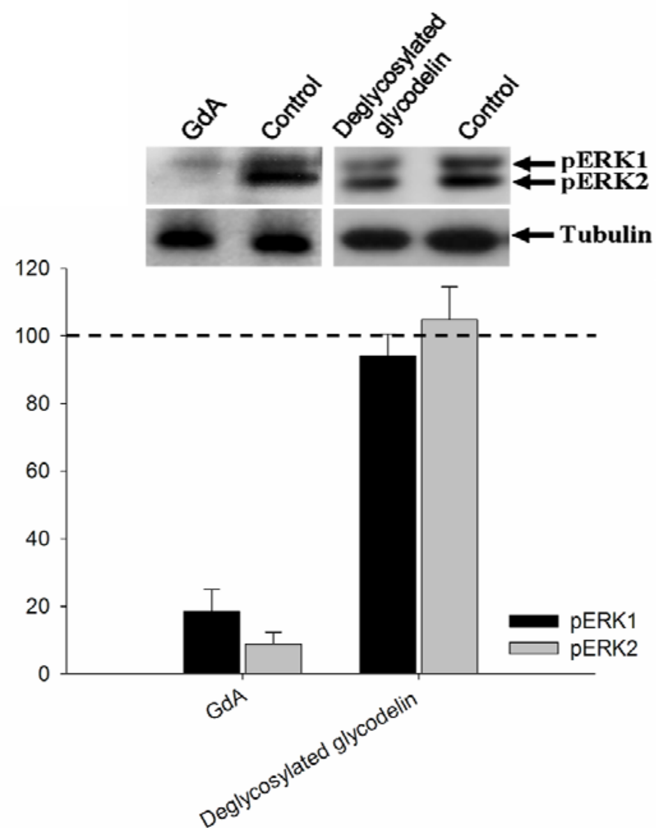

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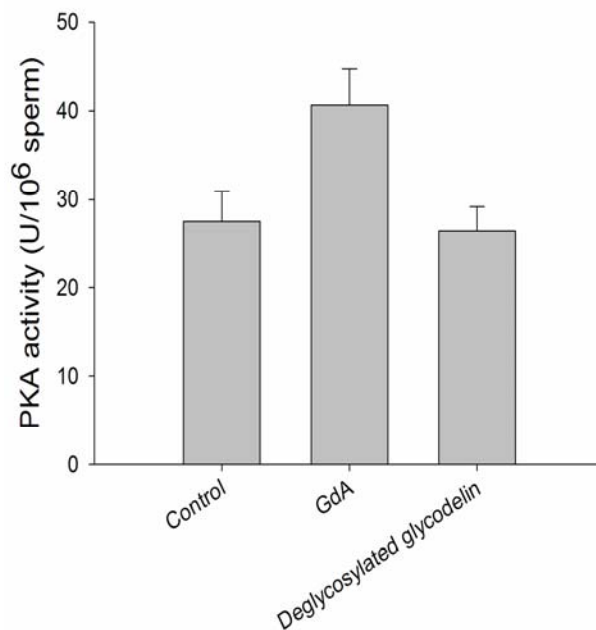

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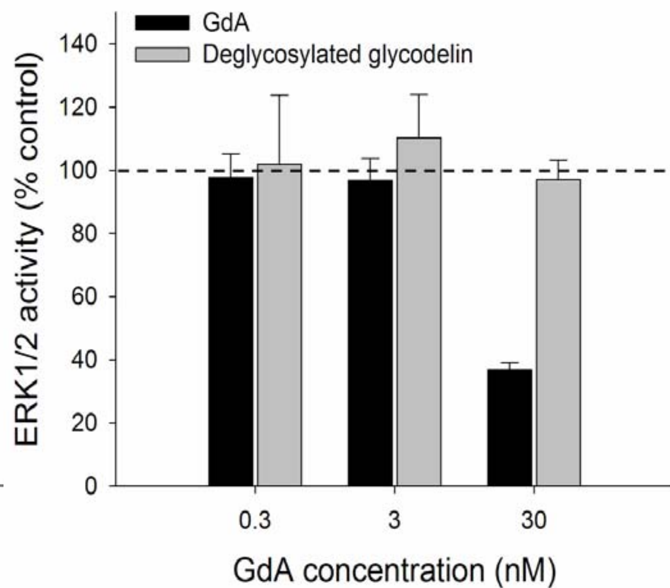
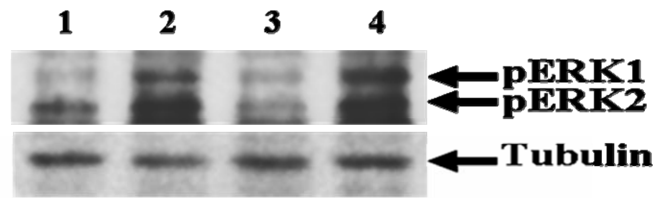
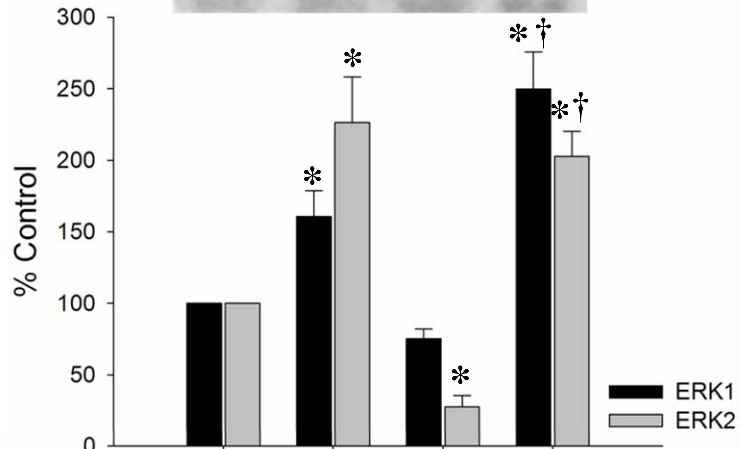


783 **Figure 3.**

A





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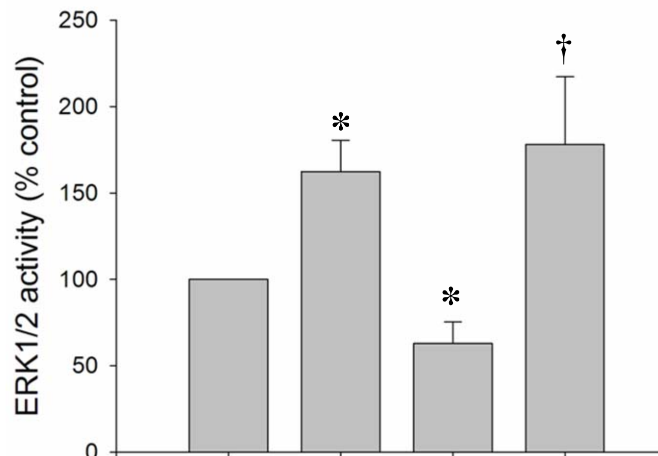
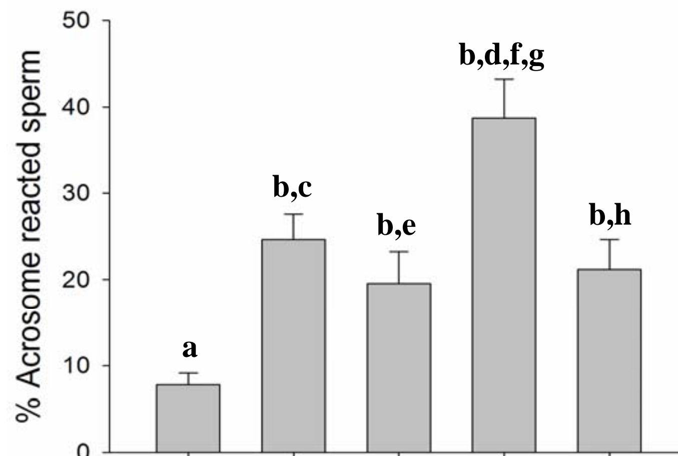


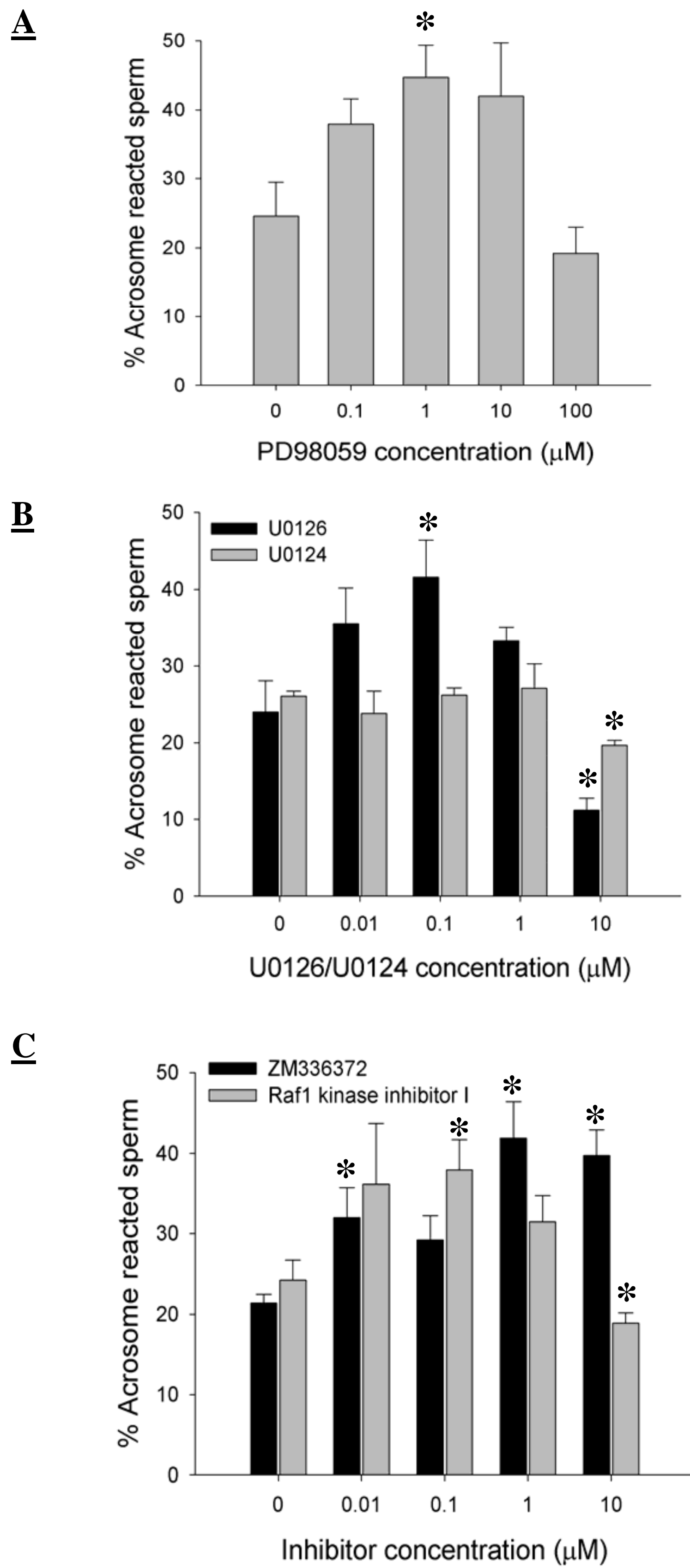
GdA (30 nM)	-	-	+	+
Rp-cAMPS (200 μM)	-	+	-	+

C

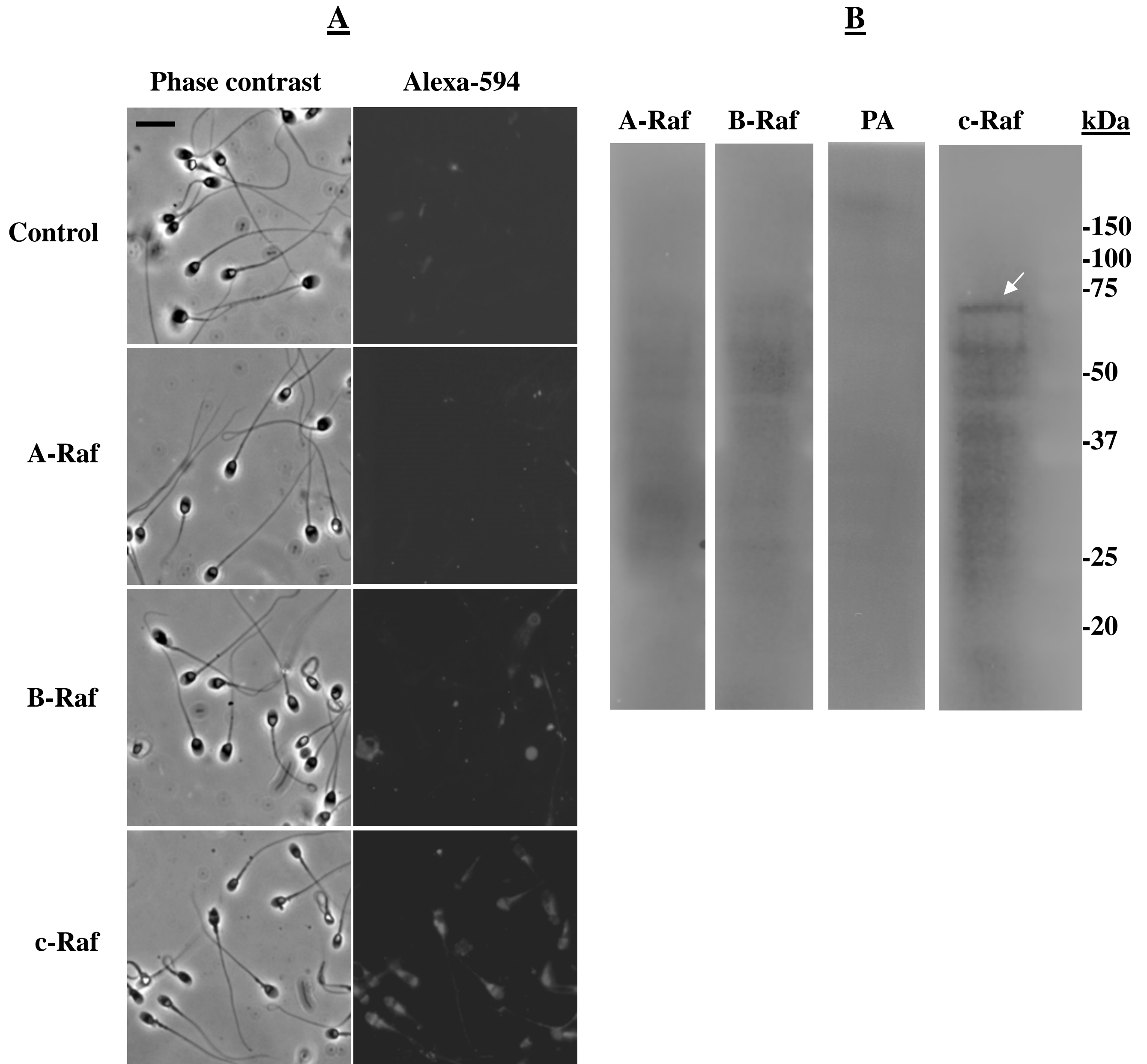


Solubilized ZP (1 μg/ml)	-	+	+	+	+
GdA (30 nM)	-	-	-	+	+
Rp-cAMPS (200 μM)	-	-	+	-	+

784 **Figure 4.**



785 **Figure 5.**



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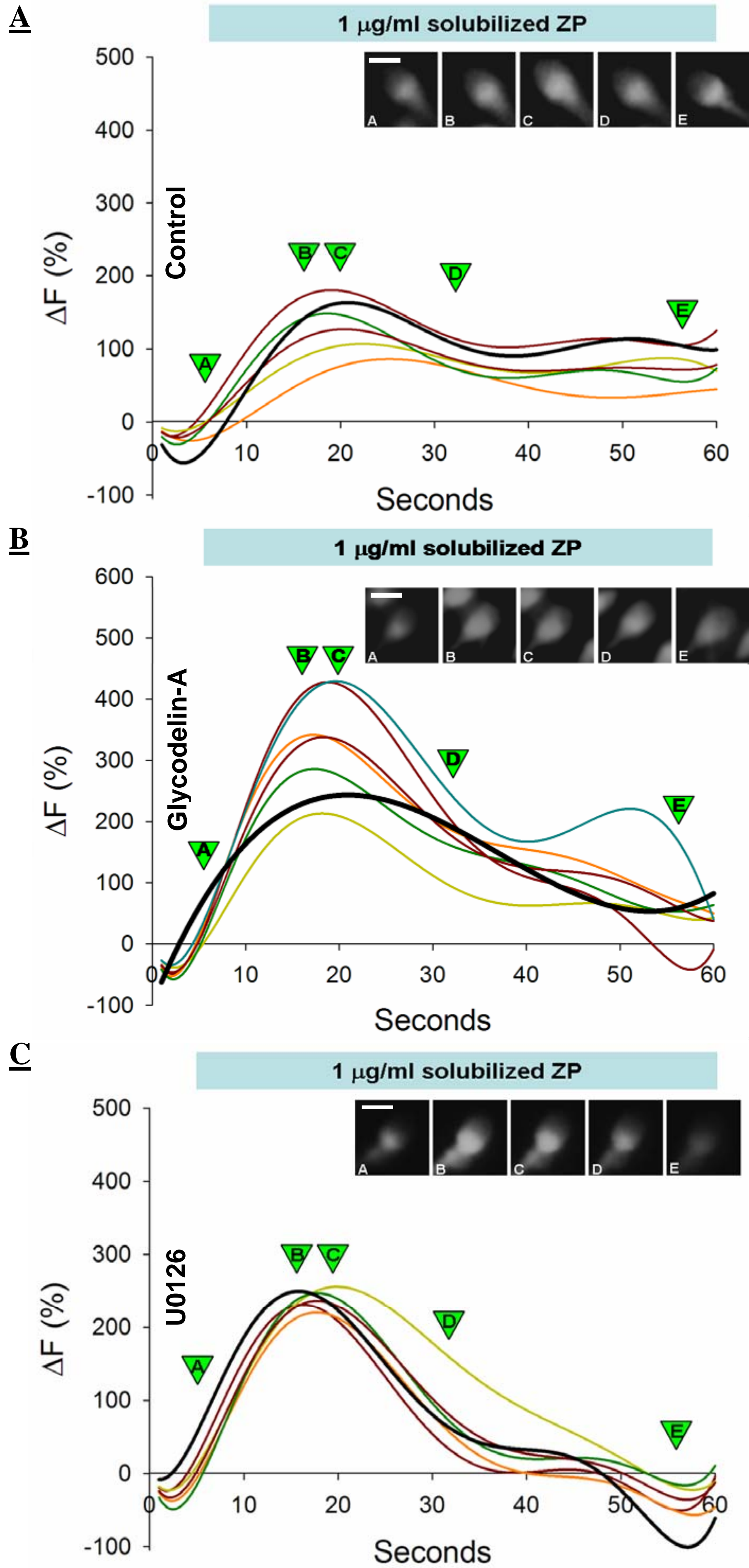
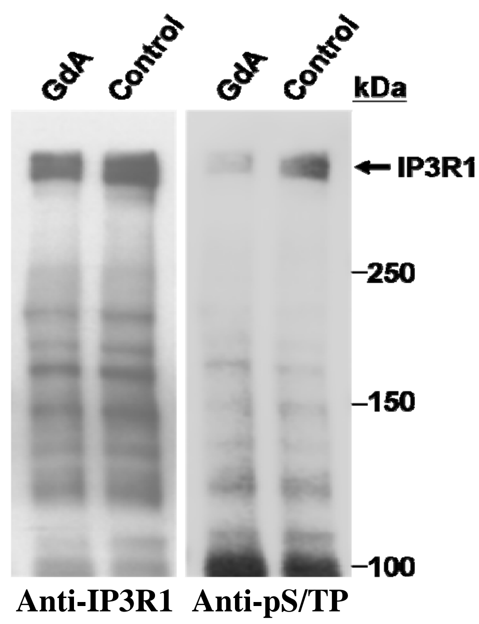


787 **Figure 7.**



788 **Figure 8.**