REPRODUCTION

Induction of sperm acrosome reaction by perivitelline membrane glycoprotein ZP1 in Japanese quail (*Coturnix japonica*)

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Abstract

The extracellular matrix surrounding avian oocytes, called the perivitelline membrane (PL), consists of at least two major glycoproteins, ZP3 and ZP1. Our previous study using Japanese quail had demonstrated that the PL obtained from the preovulatory follicles was incubated *in vitro* with spermatozoa, and perforations were observed. This result indicated that the PL might contain a constituent that possesses activity to initiate the acrosome reaction (AR) in quail. In order to elaborate upon our previous findings, we evaluated the effects of ZP3 and ZP1 on the induction of sperm AR in Japanese quail. Ejaculated sperm were incubated with or without the purified PL glycoprotein, and their acrosome status was determined based on the presence or absence of the acrosome. Treatment of spermatozoa with increasing doses of the purified monomeric ZP1 led to a concentration-dependent stimulation of AR. The purified dimeric ZP1 had similar effect. Moreover, we found that the ZP1-induced AR was significantly blocked by the digestion of the PL protein with PNGaseF. In contrast, the addition of purified ZP3 failed to induce AR at any doses tested. These results indicate that *N*-linked glycans on ZP1 play an important role in triggering the AR in Japanese quail.

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Introduction

Fertilization is known as the process of combining two germ cells, egg and sperm, and is the consequence of precisely ordered multiple steps, including sperm–egg binding, induction of the acrosome reaction (AR) by sperm, and fusion of sperm and egg. It is well known that the zona pellucida (ZP), which is an extracellular matrix surrounding mammalian oocytes, plays an important role during fertilization because it possesses receptor molecules responsible for relatively species-specific sperm–egg binding, induces the sperm AR, as well as participates in the prevention of polyspermy achieved by its structural changes (Florman & Ducibella 2006). In mammals, the ZP family of glycoproteins has been identified as playing a key role in these events (Wassarman 1999). The components of this matrix include three glycoproteins (i.e. ZP1, ZP2, and ZP3, which are also known as ZPB1, ZPA, and ZPC respectively, according to Spargo & Hope (2003) and Smith *et al.* (2005)) in most mammalian species (Harris

et al. 1994) and four glycoproteins in humans (ZP1, ZP2, ZP3, and ZP4 (ZPB2); Lefievre *et al.* 2004).

In avian species, the inner layer of the vitelline membrane (also referred to as the perivitelline membrane (PL)), which is the egg envelope homologous to the ZP in mammals, is observed in follicles between granulosa cells and the ovum before ovulation (Wyburn *et al.* 1965). In birds, fertilization occurs within the infundibulum portion of the oviduct, and only the PL encloses the oocyte at the time of fertilization. At least two glycoproteins have been identified as constituents of the avian PL; ZP1 (ZPB1) and ZP3 (ZPC) in Japanese quail (Pan *et al.* 2001, Sasanami *et al.* 2003) and ZP1 (ZPB1), ZPB (ZPB2), ZP2 (ZPA), ZP3 (ZPC), and ZPD in chicken (Waclawek *et al.* 1998, Bausek *et al.* 2000, Okumura *et al.* 2004, Smith *et al.* 2005). We have previously cloned the cDNA encoding ZP3 (GenBank Accession no. AB012606) and ZP1 (GenBank Accession no. AB061520) of the quail PL.

Koyanagi *et al.* (1988) have demonstrated in the chicken that when the PL obtained from ovulated ova is

incubated with sperm *in vitro,* fragmentation of the PL occurred. Moreover, Howarth (1990) reported that solubilized PL contains one or more components that behave in a manner analogous to sperm receptor in mammalian species, since pretreatment of spermatozoa with solubilized PL eliminates their binding to and fragmentation of the PL in the chicken. In our previous study using Japanese quail, we observed perforations in PL obtained from preovulatory follicles after it was incubated *in vitro* with spermatozoa (Kuroki & Mori 1997). Similarly, Win *et al.* (2006) also confirmed the formation of holes in PL after the incubation with sperm in Japanese quail. These results indicate that the PL might contain a constituent that initiates the AR in quail. The aim of the present study was to determine which components in the PL possess the activity for the induction of sperm AR in Japanese quail. In order to achieve this goal, we established a method to discriminate acrosome-reacted from acrosome-intact sperm. By means of this method, we show that ZP1 of the PL possesses activity to induce the AR in Japanese quail. We also provide evidence that the *N*-linked oligosaccharide attached to the ZP1 plays an important role in triggering the AR.

Materials and Methods

Animals and tissue preparation

Male and female Japanese quail, *Coturnix japonica,* 15–30 weeks of age (Tokai-Yuki, Toyohashi, Japan), were caged individually under a lighting schedule of 14 h light (0500–1900 h) and 10 h darkness, and the animals were provided with tap water and a commercial diet (Tokai-Kigyo, Toyohashi, Japan) *ad libitum*. The female animals were decapitated and the largest preovulatory follicles were dissected. The granulosa layer from the largest preovulatory follicles was isolated as a sheet of granulosa cells sandwiched between the PL and the basal laminae, as previously described (Gilbert *et al.* 1977). A proctodeal gland secretion was obtained manually from male quail as meringue-like foam. This form was then centrifuged at 10 000 *g* for 10 min at 4 °C. After centrifugation, supernatants were collected and were stored as proctodeal gland secretion at −80 °C until use. All experimental procedures for the use and the care of animals in the present study were approved by the Animal Care Committee of the Faculty of Agriculture, Shizuoka University.

Semen collection and preparation

Ejaculated semen was obtained from male quail prior to mating according to the procedure of Kuroki & Mori (1997). Semen obtained from two to three males was suspended in Hanks' balanced salt solution (HBSS) containing 1.25 mmol/l of $CaCl_2$ and 0.1% (v/v) of

proctodeal gland secretion. We decided to include $CaCl_2$ in the sperm extender because it is reported that the induction of AR by homogenized PL was almost negligible in the absence of Ca^{2+} (Ashizawa *et al.* 2006). We added proctodeal gland secretion into the incubation mixture since it inhibits an agglutination of quail spermatozoa. The concentrations of spermatozoa were measured with a hemocytometer and the sperm viabilities were determined using LIVE/DEAD sperm viability kit according to the manufacturer's instructions (Molecular Probes, Eugene, OR, USA). In all experiments, sperm were incubated in a water bath adjusted to 39 °C.

Purification of PL glycoproteins

The PL was isolated according to a procedure described by Sasanami *et al.* (2002). The PL was then dissolved in 1% SDS (w/v) buffered at pH 6.8 with 70 mmol/l Tris–HCl overnight at room temperature. After centrifugation at 10 000 *g* for 10 min, the supernatants served as a PL lysate and the protein concentrations of the samples were measured using a BCA protein assay kit (Pierce, Rockford, IL, USA). The PL lysate was separated on one-dimensional SDS-PAGE, performed as described by Laemmli (1970) under non-reducing conditions on 12% (w/v) polyacrylamide for separating gel. The samples (750 µg of protein per gel) were applied to 5% (w/v) stacking gel without comb for lane casting. After electrophoresis, the gel was stained with Copper Stain (Bio-Rad Laboratories), and 175 kDa (dimeric ZP1), 97 kDa (monomeric ZP1), and 35 kDa (ZP3) bands were excised. The individual proteins were eluted by incubating the gel slices with 0.1% SDS (w/v) buffered at pH 8.0 with 100 mmol/ml Tris–HCl overnight at 25 °C with constant shaking. The eluent was then extensively dialyzed against water, lyophilized, and dissolved in 20 mmol/ml Tris–HCl (pH 8.0). The protein concentrations of the samples were measured as described above.

PNGase F digestion

The PL lysate was digested with PNGase F, an amidase that cleaves between the innermost *N*-acetylglucosamine and asparagines residues of *N*-linked glycoproteins (E.C.3.5.1.52; New England Biolabs, Beverly, MA, USA), according to the manufacturer's instructions. Briefly, the PL lysate (1 mg of protein) was mixed with 1/10 volume of 10×G7 buffer (0.5 M sodium phosphate, pH 7.5) and 10% (v/v) NP-40. The mixture was incubated in the presence or absence of 50 000 U of PNGase F at 37 °C for 20 h. It was then separated on one-dimensional SDS-PAGE and the dimeric ZP1 or monomeric ZP1 was purified as described above. For the control experiments, (−PNGase F in panels A and B of Figs 1 and 2), the PL lysate was treated in the same manner except for the
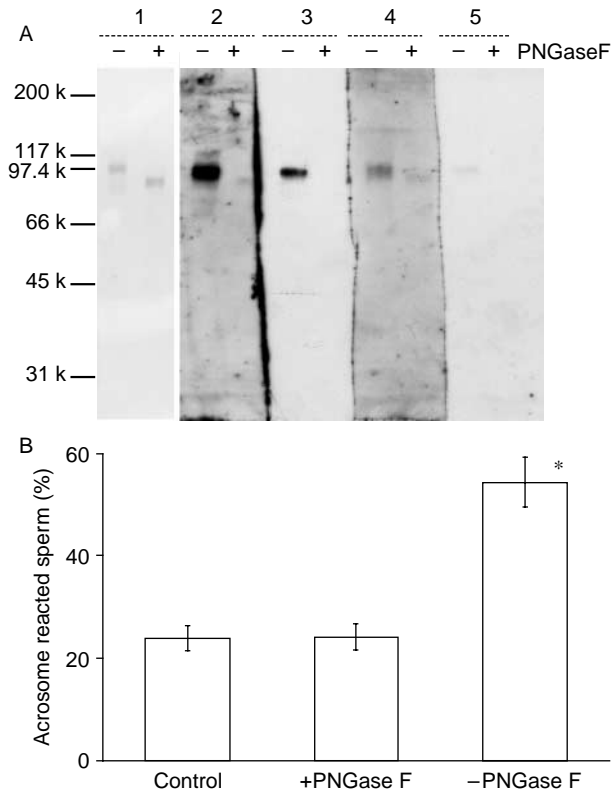
Figure 1 Effects of PNGase F treatment on the ability of monomeric ZP1 to induce quail sperm AR. (A) Lectin blot analysis of purified monomeric ZP1. The PL lysates were digested with ($+$) or without ($-$) PNGase F as described in Materials and methods. They were then separated on SDS-PAGE (0.1 μg per lane), transblotted onto PVDF membranes, and incubated with RCA$_{120}$ (panel 2, 1:10 000), PHA-E$_4$ (panel 3, 1:8000), WGA (panel 4, 1:2000) or LCA (panel 5, 1:5000). In panel 1, banding of the same sample stained with silver is shown. The panels of silver staining and lectin blot reflect one representative experiment out of a total of three independent experiments. (B) Ejaculated sperm were incubated for 10 min with vehicle alone (control), 1 μg/ml of the purified monomeric ZP1 digested with PNGase F ($+$PNGase F), or 1 μg/ml of the purified monomeric ZP1 treated without PNGase F ($-$PNGase F). The percentage of spermatozoa without acrosome was calculated. Values are shown as mean±s.d. of three independent experiments. Values with superscripts (*$P<0.01$) are significantly different from the control.

omission of PNGase F. The dimeric ZP1 or the monomeric ZP1 after the treatments was purified as described above.

Assessment of acrosomal status

Spermatozoa were diluted to a final concentration of 1×10^7 spermatozoa ml^{-1} in HBSS containing 0.1% (v/v) of proctodeal gland secretion. The AR was induced by incubating the cells with Ca^{2+} ionophore A23187 dissolved in dimethylsulfoxide (DMSO; 0, 0.3, 1.0, or 3.0 μmol/l; Sigma) for up to 30 min. The reaction was stopped by incubating the samples with formaldehyde (final concentration of 3.7% (w/v)). The percentage of
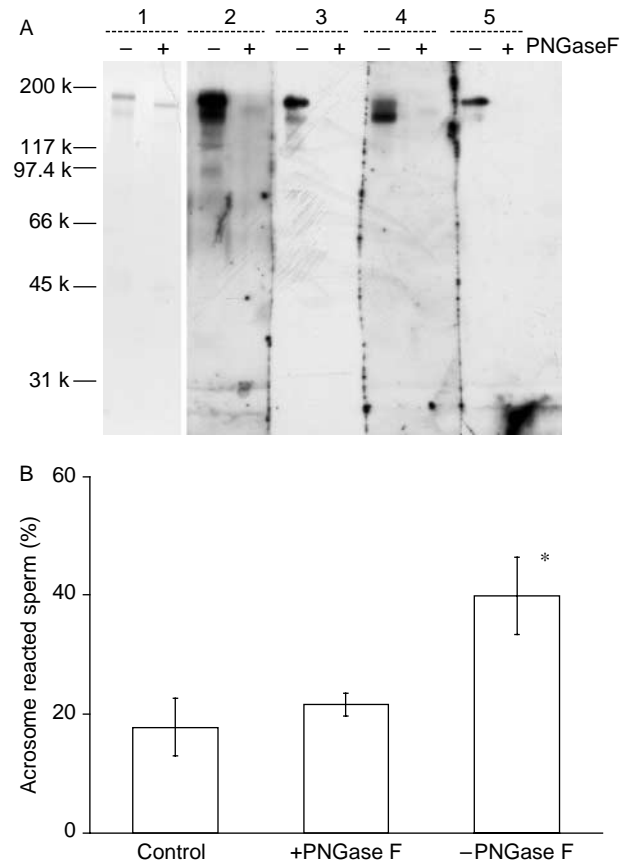

Figure 2 Effects of PNGase F treatment on the ability of dimeric ZP1 to induce quail sperm AR. (A) Lectin blot analysis of purified dimeric ZP1. The PL lysates were digested with ($+$) or without ($-$) PNGase F as described in Materials and methods, and were detected with RCA$_{120}$ (panel 2, 1:10 000), PHA-E$_4$ (panel 3, 1:8000), WGA (panel 4, 1:2000) or LCA (panel 5, 1:5000) as described in the legend for Fig. 1. In panel 1: banding of the same sample stained with silver. The panels of silver staining and lectin blot reflect one representative experiment out of a total of three independent experiments. (B) Ejaculated sperm were incubated for 10 min with vehicle alone (control), 1 μg/ml of the purified dimeric ZP1 digested with PNGase F ($+$PNGase F), or 1 μg/ml of the purified dimeric ZP1 treated without PNGase F ($-$PNGase F). The percentage of spermatozoa without acrosome was calculated. Values are shown as mean±s.d. of three independent experiments. Values with superscripts (*$P<0.01$) are significantly different from the control.

acrosome-reacted sperm was determined microscopically. Samples of the cells treated to induce the AR were smeared on poly-L-lysine coated microscope slides. After air drying, sperm smears were stained with 1 μg/ml propidium iodide (Sigma) for 10 min. The slides were washed between incubations by dipping them in PBS for 5 min. After washing, slides were mounted in glycerol and examined under a fluorescence microscope equipped with an interference-contrast apparatus with a 40× objective (BX 50, Olympus Optics, Tokyo, Japan) and photographed. At least five areas 0.237 mm^2 were randomly selected for enumeration. The acrosome status

was observed based on the presence (acrosome-intact sperm) or absence (acrosome-reacted sperm) of the acrosome, which is observed as a propidium iodide-negative structure located on the tip of the sperm nucleus. In order to investigate the effects of the purified PL glycoprotein on the AR, spermatozoa was incubated with or without dimeric, monomeric ZP1, or ZP3 for 10 min. After incubation, the acrosome status was observed using the same procedure as that used for A23187 treatment. To test the effects of pertussis toxin (PTX; Calbiochem, La Jolla, CA, USA), the membrane-penetrating ADP-ribosyltransferase inhibitor of G protein, on the A23187- or PL glycoprotein-initiated AR, sperm were incubated with the test substance in the presence or absence of PTX (2 μg/ml) for 10 min. Their acrosomal status was then observed. Appropriate solvent controls (DMSO, 20 mmol/l Tris–HCl (pH 8.0) or H_2O for A23187, purified glycoproteins or PTX respectively) were utilized in parallel in all experiments and the final concentration of each solvent was adjusted to 0.1% (v/v).

Lectin blot analysis and gel staining

For the lectin blot analysis, the purified ZP1 treated with or without PNGase F was separated on SDS-PAGE under non-reducing conditions (0.1 μg of protein per lane) and was transferred to PVDF membranes (Immobilon-P, Millipore, Bedford, MA, USA) (Matsudaira 1987). The membranes were incubated with three changes of saline buffered at pH 7.4 with 10 mmol/l Tris–HCl containing 0.1% (v/v) Tween 20 (TBS-T) for 10 min at room temperature in order to inhibit non-specific binding. The membrane was reacted with lectins, conjugated to horseradish peroxidase for 1 h at room temperature, and the reactive bands were detected as described previously (Pan *et al.* 2000). The lectins used were *Ricinus communis* agglutinin (RCA_{120}), which interacts with *N*-acetylglucosamine-containing glycopeptides (Baenziger & Fiete 1979), *Phaseolus vulgaris* agglutinin (PHA-E_4), which binds to *N*-acetylglucosamine linked to β1, 4 to the β-linked mannose residue in the core (Cummings & Kornfeld 1982), and *Triticum vulgare* agglutinin (WGA), specific for terminal *N*-acetylglucosamine, sialic acids (Debray *et al.* 1981), and *Lens culinaris* agglutinin (LCA), which recognizes α1,6-fucosylated *N*-glycans of a complex-type glycopeptide (Yamamoto *et al.* 1982) purchased from Honen Corp. (Tokyo, Japan).

To detect the protein of PL lysate or the purified glycoprotein, samples separated on SDS-PAGE under non-reducing conditions were detected with a silver staining kit (Wako Pure Chemicals, Tokyo, Japan).

Statistical analysis

All AR percentage data were transformed to the arcsine of their square root. The Duncan's multiple range test was used for a comparison of the group mean difference. Differences were considered statistically significant when $P < 0.05$.

Results

Analysis of AR of quail sperm

In order to establish a quantitative assay for quail sperm AR, we observed sperm that had been incubated with or without A23187. As shown in Fig. 3A, sperm incubated with vehicle alone had a propidium iodide-negative structure on the tip of the nucleus (arrowhead). The size of this structure ranged from 2 to 4 μm in length. In contrast, in the case of the spermatozoa treated with 3 μmol/ml of A23187, this structure was found to be missing (panel B). In order to validate this simple method for quail AR assay, we next incubated the sperm with various concentration of A23187 (Fig. 3C), and the percentage of sperm without acrosome was calculated. It was found that the percentage of sperm for which the acrosome had disappeared increased in a dose-dependent manner. In addition, a time-course study indicated that the acrosome-reacted
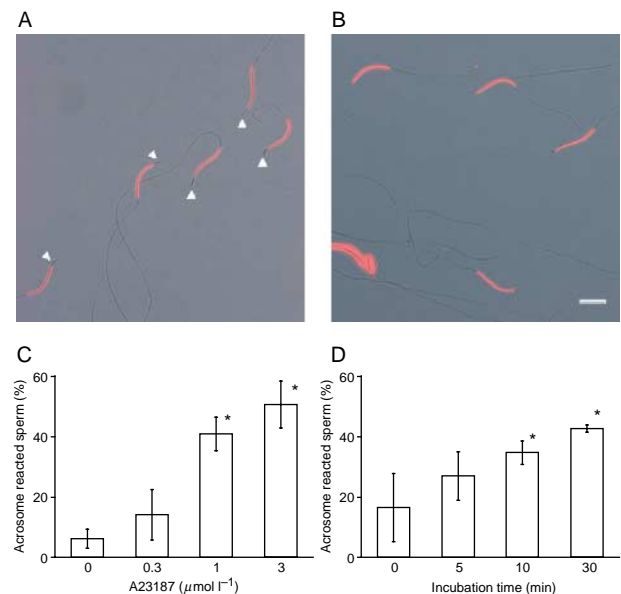

Figure 3 Effects of A23187 on quail sperm AR. (A and B) Ejaculated sperm were incubated with (panel B) or without (panel A) 3 μmol/l of A23187 for 10 min at 39 °C. Acrosomal status was evaluated based on the presence (acrosome-intact sperm, arrowhead in panel A) or absence (acrosome-reacted sperm, panel B) of their acrosome. Shown are representative results of repeated experiments. Bar = 10 μm. (C) Ejaculated sperm were incubated with 0, 0.3, 1.0, or 3.0 μmol/l of A23187 for 10 min, and the percentage of spermatozoa without acrosome was then calculated. Values were mean ± s.D. of seven independent experiments. Values with superscripts (*$P < 0.01$) are significantly different from the 0 μmol/l culture. (D) A time-course study of the induction of AR was performed. Sperm were incubated with 1.0 μmol/l of A23187 for 0, 5, 10, or 30 min. The percentage of spermatozoa without acrosome was calculated. Values are mean ± s.D. of five independent experiments. Values with superscripts (*$P < 0.01$) are significantly different from the 0-min culture.

sperm increased in a time-related manner and that 10 min of incubation led the sperm to induce AR significantly in the presence of 1 μmol/ml of A23187 (Fig. 3D). These results indicated that the sperm AR in quail could be simply analyzed by staining the sperm with a DNA-staining dye like propidium iodide.

Sperm AR is induced by ZP1 stimulation in qauil

In order to identify the proteins possessing activity to induce the AR, we purified two major glycoproteins, ZP3 and ZP1, from the PL lysate. As shown in Fig. 4A, rerun of the purified ZP3 (lane 2) as well as the dimeric (lane 3) or monomeric ZP1 (lane 4) produced a dominant band migrating at a molecular weight of 35, 175, and 97 kDa respectively. These results suggest that each glycoprotein purified by means of the methods in this study was practically pure. To test the ability of the individual glycoprotein to induce the AR, we incubated the sperm with various concentrations of the purified glyco-proteins. Treatment of spermatozoa with increasing doses of purified monomeric ZP1 for 10 min led to a concentration-dependent stimulation of the AR (Fig. 4C). The purified dimeric ZP1 had similar effects (Fig. 4D). On the other hand, the addition of purified ZP3 failed to induce the AR at any doses tested (Fig. 4B). Collectively, these results indicate that both monomeric and dimeric ZP1 possess activity to trigger the AR in Japanese quail.

Effects of PTX on ZP1- or the A23187-induced AR

In order to investigate the involvement of Gi protein in initiation of the AR, we evaluated whether the ZP1- or A23187-induced AR was affected by PTX, which is an inhibitor of the Gi protein function. As depicted in Fig. 5, incubation of the sperm with PTX completely inhibited the effects of ZP1 (Fig. 5A). It is not due to the toxic effect of PTX, because the motility and the viability of the sperm were not altered by the addition of PTX (data not shown). No significant difference was found between the percentage of acrosome-reacted sperm in the control and PTX culture. Similar results were obtained in the case of the A23187-induced AR, since the effects of A23187 on AR induction were significantly inhibited by the PTX treatment (Fig. 5B).

Effects of PNGase F digestion on the ZP1-stimulated AR

We next attempted to determine which region of the ZP1 molecule is involved in AR induction. We therefore investigated whether removal of N-glycans attached to the ZP1 molecule affect the ability to induce the AR. When PL lysate was treated with PNGase F, the band of monomeric ZP1 was shifted to migrate at a molecular weight of 90 kDa (Fig. 1A, panel 1, lane +). We next performed a lectin blot analysis of the proteins in order to

confirm the absence of N-linked oligosaccharides in ZP1 digested with PNGase F. As shown in the figure, the purified monomeric ZP1 without treatment reacted with either RCA_{120} (panel 2, lane −), $PHA-E_4$ (panel 3, lane −), WGA (panel 4, lane −), or LCA (panel 5, lane −), which are known to recognize N-linked oligosaccharides, while the PNGase F digestion eliminated (panels 2, 3, and 5 lane +) or markedly attenuated (panel 4 lane +) the reactivity of ZP1 to the lectins used. These results suggested that our PNGase F treatment efficiently remove the N-glycans from the ZP1, with only trace amounts of N-glycans remaining on the ZP1 after PNGase F digestion. When the ejaculated sperm were incubated with ZP1, which were treated in the absence of PNGase F (−PNGase F), the percentage of acrosome-reacted sperm was significantly increased compared with that of the vehicle alone group (control). This result suggests that the course of treatments used for the PNGase F digestion itself did not result in an inactivation of ZP1 activity for the stimulation of AR. When the cells were incubated with N-glycan-deficient ZP1 (+PNGase F), the percentage of the acrosome-reacted sperm was significantly decreased compared with that in the − PNGase F group, and reached the levels equivalent to those in the control group. As shown in Fig. 2, similar results were obtained in the case of the experiments using purified dimeric ZP1. These results demonstrated that the N-linked oligosaccharides attached to both monomeric and dimeric ZP1s play an important role in triggering the AR in quail sperm.

Discussion

Our previous study demonstrated that the holes that are a result of hydrolysis of the PL are observed when PL obtained from preovulatory follicles is incubated with ejaculated sperm *in vitro* (Kuroki & Mori 1997). This result implied the presence of AR inducer in the PL of quail oocyte. Horrocks *et al.* (2000) previously developed a method to detect the AR of chicken spermatozoa based on the differential binding of FITC labeled PNA (FITC-PNA) to acrosome-intact but not acrosome-reacted spermatozoa. However, our pre-liminary experiments using their method indicated that the FITC-PNA does not bind to quail sperm irrespective of the treatment of A23187 (data not shown). In order to investigate the AR of quail sperm, we established a method for discriminating acrosome-reacted from acro-some-intact sperm. Since the acrosome in quail sperm is large enough to be visualized under a light microscope (Korn *et al.* 2000), we employed a simple method in which the sperm nucleus was stained with fluorescence DNA dye. As expected, we successfully differentiated between acrosome-reacted and acrosome-intact sperm based on the presence or absence of the acrosome (Fig. 3A and B).

By means of this method, the results in the present study clearly demonstrate for the first time that both monomeric and dimeric ZP1, one of the major constituents of the PL,
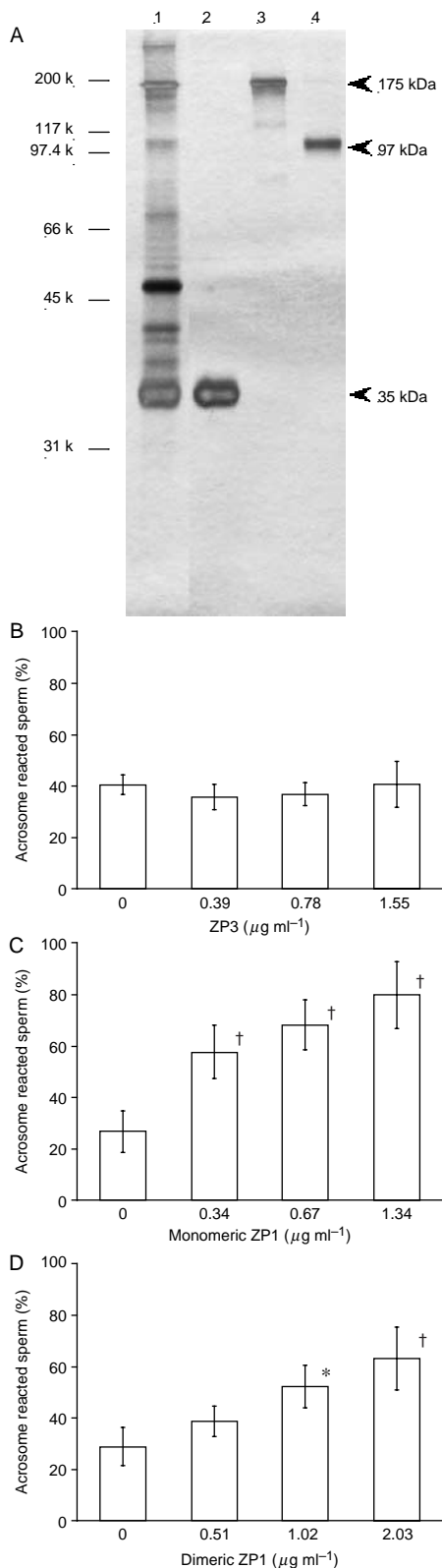
possess activity for stimulation of the AR in quail. In accordance with our findings, another recent study has demonstrated that dimeric ZP1 stimulates sperm AR in the chicken (Okumura *et al.* 2004). However, the results of this previous study also suggest that monomeric ZP1 has no effect on induction of the AR. They speculate that the inefficacy of monomeric ZP1 for AR induction was due to its secondary structure suggested by CD spectra measurement, i.e. the monomeric ZP1 is rich in unordered structure, whereas the dimeric one was estimated to have ordered secondary structure, which might contribute to the activity for AR induction. Although the reason for this discrepancy between chicken and quail is not investigated in this study, these authors used a gel filtration technique with buffer containing 6 mol/l urea, whereas we employed SDS-PAGE under non-reducing conditions for the purpose of ZP1 purification. On the other hand, Bausek *et al.* (2004) have demonstrated that both ZP3 purified from a culture supernatant of granulosa cells and ZP1 obtained from the serum of laying hens are bound to the acrosomal region of rooster sperm by means of immunofluorescent microscopy. In addition, they also showed that both ZP3 and ZP1 interact with a 180 kDa protein present in the lysate of rooster sperm. Since they did not analyze AR induction in the chicken, it is unknown why the acrosome still remained on the head of the rooster sperm after incubation with ZP1, an AR inducer in both chicken and quail. However, it should be noted that we purified ZP1 from the PL of the largest preovulatory follicle, just prior to fertilization, while they used ZP1 of serum origin.

It is quite interesting that sperm binding to the PL in chicken appears to be mediated by both ZP1 and ZP3 (Bausek *et al.* 2004). Although both ZP3 and ZP1 bind to the acrosomal region of rooster sperm, only ZP1 possesses the activity for AR induction (Okumura *et al.* 2004 and in this study). These results implied the following two possibilities: (1) there are multiple ligands on the sperm surface, and one of which is responsible for the specific binding to ZP3, while the other one contributes to ZP1 binding and AR induction or (2) ZP3 and ZP1 share a common ligand with different

Figure 4 Effects of purified PL glycoproteins on the induction of AR in Japanese quail. (A) The purified ZP3, (lane 2, 0.1 µg per lane), the purified dimeric ZP1 (lane 3, 0.1 µg per lane), or the purified monomeric ZP1 (lane 4, 0.1 µg per lane) were separated on SDS-PAGE under non-reducing conditions, and were detected with silver staining. The PL lysate (1 µg per lane) was also separated on SDS-PAGE under non-reducing conditions (lanes 1) as a reference. Shown are representative results of repeated experiments. (B, C and D) Ejaculated sperm were incubated with ZP3 (0, 0.39, 0.78, or 1.55 µg/ml, panel A), monomeric (0, 0.34, 0.67, or 1.34 µg/ml, panel B) or dimeric ZP1 (0, 0.51, 1.02, or 2.03 µg/ml, panel C) for 10 min. The percentage of spermatozoa without acrosome was calculated. Values of each panel are shown as mean $\pm$ s.d. of four independent experiments. Values with superscripts (*$P < 0.05$, †$P < 0.01$ respectively) are significantly different from the respective control culture (0 µg/ml).
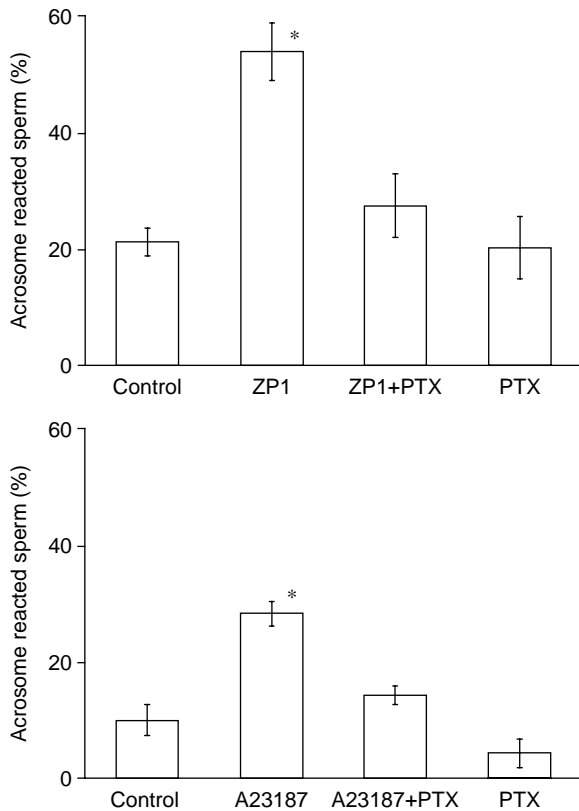
Figure 5 Effects of pertussis toxin on ZP1- or A23187-initiated quail sperm AR. (A) Ejaculated sperm were incubated with vehicle alone (control), 1 µg/ml of purified ZP1 alone (ZP1), ZP1 with 2 µg/ml pertussis toxin (ZP1 + PTX), or pertussis toxin alone (PTX) for 10 min. The percentage of spermatozoa without acrosome was calculated. Values are shown as mean ± S.D. of four independent experiments. Values with superscripts (*$P < 0.01$) are significantly different from the control. (B) Ejaculated sperm were incubated with vehicle alone (control), 10 µmol/l of A23187 alone (A23187), A23187 with 2 µg/ml pertussis toxin (A23187 + PTX), or pertusis toxin alone (PTX) for 10 min. The percentage of spermatozoa without acrosome was calculated. Values are shown as mean ± S.D. of three independent experiments. Values with superscripts (*$P < 0.01$) are significantly different from the control.

affinity, and that the signal transduction that mediates the AR in quail spermatozoa does not proceed after binding with ZP3. Although these are only hypotheses, efforts are presently underway to identify the specific sperm ligand(s) for egg envelope glycoproteins, ZP3, and ZP1.

The role of PL *N*-glycans in sperm–egg interactions have been demonstrated in chicken because *N*-glycans released from the PL by PNGase F as well as the materials bound to WGA-conjugated agarose can induce the AR (Horrocks *et al.* 2000). Moreover, the addition of galactose to terminal *N*-acetylglucosamine residues of PNGase F-released *N*-glycans by galactosyl transferase suppresses the AR-inducing capacity of the oligosaccharide preparation, suggesting the involvement of *N*-linked glycans with a terminal *N*-acetyl-D-glucosamine residue on AR induction (Horrocks *et al.* 2000). However, the identification of which carrier of *N*-glycans could be

involved in AR induction remained to be accomplished. In our results, the removal of *N*-glycans from ZP1, which was confirmed by the absence of the reactivity to lectins, had no effect on induction of the AR (Figs 1B and 2B), demonstrating for the first time the important role of *N*-glycans of both monomeric and dimeric ZP1 in the induction of AR in Japanese quail. Although it appears that there is participation by *N*-linked oligosaccharides, we did not investigate the involvement of *o*-glycans of ZP1 in the induction of the AR in quail. However, Robertson *et al.* (2000) have demonstrated that the pretreatment of chicken PL with *o*-glycanase does not reduce the number of holes after incubation with ejaculated spermatozoa *in vitro*. A determination of the carbohydrate structure of ZP1 required for AR induction in quail must await future analysis, however, our lectin blot analysis indicated the presence of *N*-acetylglucosamine in the sugar moiety of ZP1 protein, which is recognized with RCA_{120} and $PHA-E_4$. It is of interest to note that the sperm hydrolysis of the PL in chicken was inhibited when *N*-acetylglucosamine was included in the incubation mixture (Robertson *et al.* 2000).

A common element of the ZP-initiated AR mechanism in eutherian mammals is involvement of the Gi protein, and PTX, which is an inhibitor of Gi protein function, is known to inhibit the ZP-initiated AR in mammalian sperm (Florman & Ducibella 2006). Interestingly, it has been reported that PTX does not inhibit the progesterone-induced AR of human and mouse sperm (Tesarik *et al.* 1993, Murase & Roldan 1996). In addition, PTX also does not inhibit the acetylcholine-stimulated AR in the mouse, suggesting the involvement of a PTX-insensitive receptor like the nicotinic acetylcholine receptor in AR induction (Son & Meizel 2003). These data also suggest that different physiological stimuli may utilize different signal transduction pathways to induce the sperm AR. Our results demonstrate that both ZP1- and A23187-induced AR are significantly inhibited when PTX is included in the incubation mixture. These results also indicate that the ZP1 or A23187 might be acting through a Gi protein-mediated mechanism similar to that in the zona-initiated AR in mammalian sperm.

A number of important issues regarding quail fertilization remain to be addressed. Specifically, it is not yet known how acrosome-reacted sperm could remain on the surface of the PL and then penetrate the membrane. In an analogous situation, it has been suggested that the adhesion between the acrosome-reacted sperm and the ZP may be mediated by the binding sites on the sperm inner acrosomal membrane that interact with ZP2 in several mammalian species (Bleil *et al.* 1988, Mortillo & Wassarman 1991, Tsubamoto *et al.* 1999). It remains to be resolved whether or not similar ZP2-mediated sperm retention on the PL could function in the case of the quail fertilization. It should be noted that our recent observation using electron microscopy has demonstrated that calcium-coated sperm-associated bodies (SAB) were

accompanied with the hole in the PL of quail fertile egg, and suggested that the SAB assist fertile spermatozoa in binding to the PL, making holes in the membrane and passing through it (Rabbani *et al.* 2006). Since the sperm bind with the SAB via the posterior portion of the flagella, it appears possible that the SAB could control sperm motility and might facilitate PL penetration by amplifying the forward thrust from the flagellum in quail. In support of this idea, it has been suggested that sperm penetration of the eutherian ZP is achieved by lysis of the egg coat by acrosomal enzymes in conjunction with forward thrust from the sperm-tail oscillation (Bedford 1998).

In conclusion, our results indicate that *N*-linked glycans on ZP1 play an essential role in triggering the AR in Japanese quail. Additional studies will be needed to identify the machinery mediating the specific binding of spermatozoa with the PL as well as the organization of the three-dimensional structure of the PL, including the targeting of ZP1 and ZP3 to the PL from the bloodstream, and from the granulosa cells respectively.

Acknowledgements

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