

# Acrosome Reaction Induced by Recombinant Human Zona Pellucida 3 Peptides rhuZP3a<sup>22~176</sup> and rhuZP3b<sup>177~348</sup> and Their Mechanism

YA NI,\*¶ KUN LI,\*¶ WANXIANG XU,‡ LIWEN SONG,‡ KANGSHOU YAO,§ XINZONG ZHANG,§ HEFENG HUANG,|| YANLING ZHANG,|| AND QI-XIAN SHI\*

From the \*Department of Reproductive Physiology, Zhejiang Academy of Medical Sciences, Hangzhou, China; †Department of Laboratory Medicine, Wenzhou Medical College, Wenzhou, China; ‡Shanghai Institute of Planned Parenthood Research, Shanghai, China; §Zhejiang Provincial Institute of Planned Parenthood Research, Hangzhou, China; and the ||Women's Hospital, School of Medicine, Zhejiang University, Hangzhou, China.

**ABSTRACT:** To explore the biological characteristics of the recombinant zona pellucida 3 (ZP3) peptides of rhuZP3a<sup>22~176</sup> and rhuZP3b<sup>177~348</sup>, we examined whether rhuZP3a<sup>22~176</sup> or rhuZP3b<sup>177~348</sup> trigger the acrosome reaction (AR) of human spermatozoa and we investigated the underlying mechanism. The assessment of AR was performed using chlortetracycline staining. The intracellular free calcium concentration ([Ca<sup>2+</sup>]<sub>i</sub>) in Fura-2/AM-loaded human sperm was monitored with a spectrofluorophotometer. We found that rhuZP3a<sup>22~176</sup> and rhuZP3b<sup>177~348</sup> were capable of eliciting AR at different concentrations. With the addition of either peptide, the [Ca<sup>2+</sup>]<sub>i</sub> level was raised to a peak with or without

a plateau. The AR could be inhibited by pertussis toxin (PTX), EGTA, and pimozide (a T-type calcium channel blocker), whereas verapamil was less effective in this regard. The results of the present study suggest that peptides rhuZP3a<sup>22~176</sup> and rhuZP3b<sup>177~348</sup> have a role similar to human ZP3, and that the mechanism of the response to the peptides involves influx of calcium, the G protein pathway, and a T-type calcium channel.

**Key words:** Human sperm, pertussis toxin, intracellular calcium, G protein, calcium channel, chlortetracycline.

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In the process of mammalian fertilization, sperm penetrate the cumulus mass and then bind to the zona pellucida (ZP), a thick and highly glycosylated protein matrix that surrounds the oocyte. ZP3, which is one of four proteins of the human ZP (ZP1-4), is a major secretory and structural glycoprotein of oocytes. The critical role that ZP3 plays as an inducer of the acrosome reaction (AR) makes it a prime candidate for the studies of gamete recognition and adhesion, as well as studies of receptor-mediated signal transduction during mammalian fertilization. ZP3 has been used to diagnose male infertility and to study immune contraceptives.

ZP3 binds to the ZP3 receptor and induces AR via a pertussis toxin (PTX)-sensitive GTP-binding protein (Bastiaan et al, 1999; Bray et al, 2002; Schuffner et al, 2002). With the activation of G proteins, intracellular Ca<sup>2+</sup> increases due to influx through voltage-operated calcium channels (VOCC) (Baldi et al, 1996). The increase in intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) is required for ZP-initiated acrosomal exocytosis (Babcock and Pfeiffer, 1987; Florman et al, 1992, 1994; Arnoult et al, 1996b). Although the exact nature of the VOCC has not been identified, a T-type VOCC (VOCC<sub>T</sub>) or the low voltage-activated calcium channel family may be involved (Arnoult et al, 1996a).

Due to the limited availability of native human ZP, recombinant human ZP3s (rhuZP3s) are being developed as alternative sources. Various expression systems have been used to prepare rhuZP3, including Chinese Hamster ovary (CHO) cells (van Duin et al, 1994; Bray et al, 2002), *Escherichia coli* (*E coli*) (Chapman et al, 1998), human ovarian teratocarcinoma (PA-1) cells (Dong et al, 2001), human embryonic kidney 293T cells (Martic et al, 2004), baculovirus (Chakravarty et al, 2005), and *Spodoptera frugiperda sf9* insect ovary cells (Caballero-Campo et al, 2006). We have developed a high-yield *E coli* thermoinducible

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¶ Y.N. and K.L. contributed equally to this work.

Correspondence to: Qi-xian Shi, Department of Reproductive Physiology, Zhejiang Academy of Medical Sciences, 182 Tian Mu Shan Road, Hangzhou, Zhejiang 310013, China (e-mail: qxshi@mail.hz.zj.cn).

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system for the preparation of rhuZP3, and we have obtained two rhuZP3 polypeptides, rhuZP3a<sup>22~176</sup> and rhuZP3b<sup>177~348</sup>, the molecular weights of which are about 22 kDa and 19 kDa, respectively (He et al, 2005). The yields of rhuZP3a and rhuZP3b products, as assessed on SDS-PAGE gel, were approximately 10% of the total cell proteins (He et al, 2005).

To explore the biological characteristics of rhuZP3a<sup>22~176</sup> and rhuZP3b<sup>177~348</sup>, we investigated the effects of the two polypeptides on the [Ca<sup>2+</sup>]<sub>i</sub> of capacitated human sperm and the induction of AR, and explored the possible mechanism using PTX, EGTA, and pimozide (a T-type calcium channel blocker).

## Materials and Methods

### Chemicals

Acetoxymethyl ester of Fura-2 (Fura-2/AM) was obtained from Calbiochem (Darmstadt, Germany). Bovine serum albumin (BSA), sodium pyruvate, sodium lactate, HEPES, DMSO, chlortetracycline, N,N,N',N'tetraacetic acid (EGTA), verapamil, PTX, and pimozide were purchased from Sigma (St Louis, Mo), and Percoll was obtained from Amersham Bioscience (Uppsala, Sweden). Reagents of analytical grade were purchased from Shanghai Chemical Reagents (Shanghai, China). RhuZP3a<sup>22~176</sup> and rhuZP3b<sup>177~348</sup> were prepared using a previously described method (He et al, 2005). Human ZP was obtained from IVF with patient consent for donation and approval by the local ethics committee of the Women's Hospital at Zhejiang University.

### Media

Human tubule fluid (HTF), with some modifications, was constituted as follows: 90 mM NaCl, 5.06 mM KCl, 25.3 mM NaHCO<sub>3</sub>, 1.8 mM CaCl<sub>2</sub>, 1.17 mM KH<sub>2</sub>PO<sub>4</sub>, 1.01 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.27 mM sodium pyruvate, 21.6 mM sodium lactate, 5.56 mM glucose, 20 mM Hepes, 60 mg/L penicillin and 4 g/L BSA. This medium was maintained in equilibrium with 5% CO<sub>2</sub> in air, had pH of 7.5 at 25°C and an osmolality of 300 mOsmol/kg.

### Renaturation of rhuZP3a<sup>22~176</sup> and rhuZP3b<sup>177~348</sup>

RhuZP3a<sup>22~176</sup> and rhuZP3b<sup>177~348</sup> were separated and purified using an improved method of preparative gel polyacrylamide gel electrophoresis. Each of the peptides was dissolved for renaturation in a buffer that contained 8 mM urea, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl, 2 mM GSH, 1 mM GSSG, 10 mM DTT, and 0.1 mM PMSF. After adjustment to pH 10.7 with 10 mM KOH, the pH was readjusted to 8.0 with HCl. The solutions were placed at room temperature for 30 minutes before centrifuging at 12 000 × g for 15 minutes. The supernatants were placed in dialyzable bags to dialyze for 5 hours in PBS (pH 7.4) that contained 2 mM GSH and 1 mM GSSG. After the PBS was replaced with a larger volume of PBS (pH 7.4), the solutions

were dialyzed overnight and centrifuged at 12 000 × g for 15 min. The supernatants were identified by 15% SDS-PAGE electrophoresis and frozen to dryness.

### Human Sperm Preparation

Human semen samples were obtained by masturbation from 24 healthy donors through the Zhejiang Provincial Institute of Planned Parenthood Research. All samples were examined according to World Health Organization (WHO, 1999) criteria. Only samples with motility equal to or greater than 70%, viability equal to or greater than 85%, and sperm concentration equal to or greater than 20 × 10<sup>6</sup> cells/mL were used. The sperm were washed with an equal volume of HTF by centrifugation at 500 × g for 15 min. The pellets were resuspended in 1 mL fresh HTF before centrifugation at 600 × g for 15 minutes through a discontinuous Percoll gradient, which contained 90% and 45% Percoll. The pellets were resuspended in approximately 10 volumes of HTF and centrifuged at 300 × g for 5 min. The supernatants were discarded and resuspended in fresh HTF. The sperm concentration was adjusted to 2~3 × 10<sup>7</sup> sperm/mL. At this stage, 95% of the sperm were viable. Then sperm were incubated at 37°C for 5 hours for capacitation.

### Preparation of Solubilized ZP

ZP was obtained from IVF following appropriate patient consent for donation and approval by the local ethics committee of the Women's Hospital at Zhejiang University. The ZP were stored in PBS buffer (pH 7.4) at -20°C. On the day of the experiment, the ZP were thawed and solubilized by incubation at 60°C for 1 hour. The preparation was centrifuged at 13 000 × g for 8 minutes at 4°C to remove particulate debris, and the supernatant was used for the experiment.

### Assessment of AR

To estimate the AR of human sperm in response to peptides rhuZP3a<sup>22~176</sup> and rhuZP3b<sup>177~348</sup>, spermatozoa were stained with the supervital stain Hoechst 33258, which does not stain cells with intact plasma membranes, and with the CTC stain, as previously described (Ward and Storey, 1984; DasGupta et al, 1993; Shi et al, 1997). Briefly, a stock solution of 100 mg/mL Hoechst 33258 was prepared by dissolving the dye in triple-distilled water. The stock solution was stored in a foil-wrapped bottle at 4°C. The final concentration of dye when added to the sperm suspension was 1 µg/mL. Spermatozoa were stained for 10 minutes and then washed through 45% and 90% Percoll by centrifugation at 600 × g for 10 minutes to remove free dye. The pellet was resuspended in HTF and the spermatozoa were then stained with CTC. In a 0.5-mL Eppendorf tube, 200 µL of CTC solution (500–700 µM CTC, 130 mM NaCl, 5 mM cysteine, 20 mM Tris-Cl, [pH 7.8]) was added to 200 µL of sperm suspension and mixed well by gently aspiration with a cut pipette tip. The mixture was immediately added to 70 µL of the relevant fixative (10% formalin in 2.5 M Tris Base [1:1]), mixed for a while, and stored at 4°C in the dark until observation. Each sample was

scored for more than 250 spermatozoa under a fluorescence microscope (Nikon Eclipse 80i; Nikon Inc, Tokyo, Japan). Three patterns can be observed with CTC staining (Lee et al, 1987; Shi et al, 1997): pattern F (also referred to as pattern A), which consists of fluorescence over the entire head and equatorial region, is characteristic of uncapacitated acrosome-intact sperm; pattern B, which consists of a fluorescence-free band in the postacrosomal region, represents capacitated acrosome-intact sperm; and pattern AR, which consists of very low fluorescence over the head, corresponds to sperm that have undergone acrosomal exocytosis. The number of Hoechst-positive (dead) spermatozoa corresponded closely to the number of immotile cells (~5%), which indicates that rhuZP3 peptide-induced AR is not due to dead sperm that have lost their acrosome after the addition of the peptides and other reagents.

### Measurement of $[Ca^{2+}]_i$

After incubation at 37°C for 4 hours, the sperm suspension was loaded with 2  $\mu$ M Fura-2/AM for 30 minutes at 37°C in the dark, and the sperm suspension was centrifuged in HTF culture media 3 times at  $300 \times g$  for 5 minutes, in order to wash away the extracellular free Fura-2/AM. Sperm were finally resuspended to a concentration of  $10 \times 10^6$  cells/mL in HTF medium. After loading with Fura-2/AM, the sperm were subjected to alternating determinations at the excitation wavelengths of 340 nm and 380 nm (emission 510 nm), at a speed of once every 40 seconds, using the Shimadzu RF-540 spectrofluorometer (Shimadzu Co, Kyoto, Japan). In these processes, the cells were treated with either rhuZP3a<sup>22~176</sup> or rhuZP3b<sup>177~348</sup>. The  $[Ca^{2+}]_i$  is expressed as the ratio of the fluorescence intensities ( $F_{340}/F_{380}$ ) at the 2 excitation wavelengths (Dominguez et al, 1992).

### Statistical Analysis

Statistical analyses were carried out using the SPSS 13.0 software. All the AR percentages are transformed into  $[\arcsin[\sqrt{\% \text{ acrosome reactions}/100}]$ . The results are presented as the mean  $\pm$  SE and were analyzed by 1-way ANOVA. When the test of homogeneity of variances was significant ( $P < .05$ ), the data were analyzed by the Duncett C test. Otherwise, the LSD test was used.  $P$  values less than .05 were considered to be significant.

## Results

### The rhuZP3a<sup>22~176</sup> and rhuZP3b<sup>177~348</sup> Peptides Induce Sperm AR

We found that the 2 polypeptides were able to trigger human sperm AR by increasing  $[Ca^{2+}]_i$ . To examine the effects of rhuZP3a<sup>22~176</sup> or rhuZP3b<sup>177~348</sup> on human sperm AR, sperm were incubated with HTF for 5 hours and then stimulated with 5~50  $\mu$ g/mL rhuZP3a<sup>22~176</sup> or rhuZP3b<sup>177~348</sup> for 15 minutes before assaying for AR. In a preliminary study, spermatozoa were preincubated

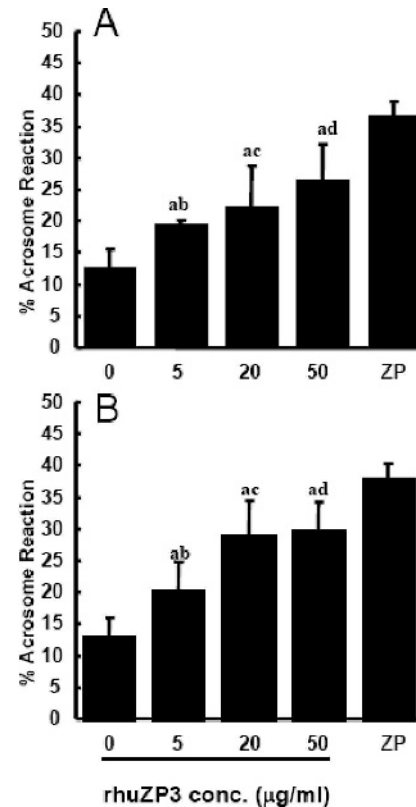


Figure 1. AR induced by different concentrations of rhuZP3a<sup>22~176</sup> (A) and rhuZP3b<sup>177~348</sup> (B). Capacitated sperm ( $2\text{--}3 \times 10^7$  cells/mL) were incubated for 15 minutes with different concentrations of rhuZP3a<sup>22~176</sup> or rhuZP3b<sup>177~348</sup>. The AR assay was performed as described in the Materials and Methods section. The data are represented as the mean  $\pm$  SEM ( $n \geq 6$ , except for ZP, where  $n = 2$ ). For each graph, the letters above the bars indicate statistically significant differences. (A) a compared to the negative control,  $P < .05$ ; c compared to b and d,  $P > .05$ ; d compared to b,  $P < .05$ . (B) a compared to the negative control,  $P < .05$ ; c compared to d,  $P > .05$ ; d and c compared to b,  $P < .05$ .

for 5 hours and then challenged with the peptides at different times. Some spermatozoa became responsive after exposure to peptides for 5 minutes, with a considerable proportion of the spermatozoa becoming responsive after 10 minutes. The proportion of spermatozoa that was capable of undergoing AR in response to peptides increased as the exposure time was extended, reaching maximum values after exposure for 15–20 minutes. For this reason, the subsequent experiments in the present study were carried out with exposure of the spermatozoa for 15 minutes after preincubation for 5 hours. PBS or the vehicle for rhuZP3a<sup>22~176</sup> and rhuZP3b<sup>177~348</sup> served as the negative control. Soluble native human ZP (1 ZP/ $\mu$ L), in agreement with the results of Chapman et al (1998), was used as a positive control. The amount of ZP3 resident in a human ZP has been estimated at ~5 ng/zona (Van Duin et al 1994). As shown in Figure 1A, rhuZP3a<sup>22~176</sup> induced human

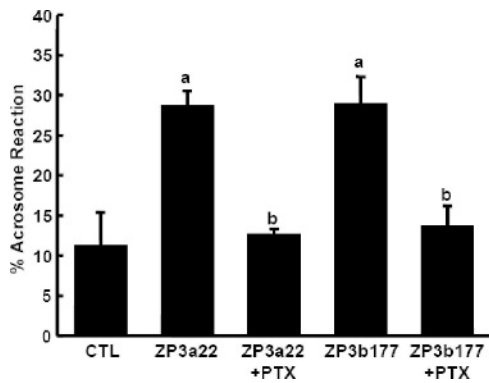


Figure 2. Inhibition by PTX of AR initiated by rhuZP3a<sup>22~176</sup> or rhuZP3b<sup>177~348</sup>. Capacitated sperm ( $2\sim 3 \times 10^7$  cells/mL) were preincubated for 10 minutes without or with PTX (2  $\mu$ g/mL), followed by further incubations with 50  $\mu$ g/mL rhuZP3a<sup>22~176</sup> or rhuZP3b<sup>177~348</sup> for 15 minutes. AR assays were performed as described in the Materials and Methods section. CTL, control; ZP3a<sup>22</sup>, rhuZP3a<sup>22~176</sup>; ZP3b<sup>177</sup>, rhuZP3b<sup>177~348</sup>. The data are represented as the mean  $\pm$  SEM of 3 different experiments. There is significant difference between a and b ( $P < .05$ ).

sperm AR in a concentration-dependent manner. RhuZP3b<sup>177~348</sup> also induced sperm AR to some extent (Figure 1B). These data suggest that rhuZP3a<sup>22~176</sup> and rhuZP3b<sup>177~348</sup> stimulate sperm AR in a manner similar to natural human ZP3.

#### PTX Inhibits rhuZP3-Mediated AR

We tested whether the rhuZP3-induced AR was regulated by a G protein. The capacitated sperm were pretreated with 2  $\mu$ g/mL PTX (a G protein inhibitor) for 10 minutes, before the addition of 50  $\mu$ g/mL rhuZP3a<sup>22~176</sup> or rhuZP3b<sup>177~348</sup>. After incubation with rhuZP3 for 15 minutes, CTC staining was performed. PTX completely abolished AR induced by rhuZP3a<sup>22~176</sup> and rhuZP3b<sup>177~348</sup> (Figure 2). The results indicate that both rhuZP3a<sup>22~176</sup> and rhuZP3b<sup>177~348</sup> act via a G protein.

#### RhuZP3 Requires Extracellular Ca<sup>2+</sup> for the Induction of AR

To assess whether the effects of these peptides on the induction of AR were related to Ca<sup>2+</sup> influx via Ca<sup>2+</sup> channels, the capacitated sperm were incubated with either 2 mM EGTA (a Ca<sup>2+</sup> chelator) or 100  $\mu$ M pimozide (a T-type calcium channel blocker) or 10  $\mu$ M verapamil (an L-type calcium channel blocker) for 15 minutes, before the addition of 50  $\mu$ g/mL rhuZP3a<sup>22~176</sup> or rhuZP3b<sup>177~348</sup>. At the same time, capacitated sperm without EGTA, pimoizide or verapamil were used as controls. EGTA significantly reduced rhuZP3-induced AR (Figure 3A). Pimoizide also abolished AR triggered by these two peptides (Figure 3B). Verapamil was less effective, whereas verapamil inhib-

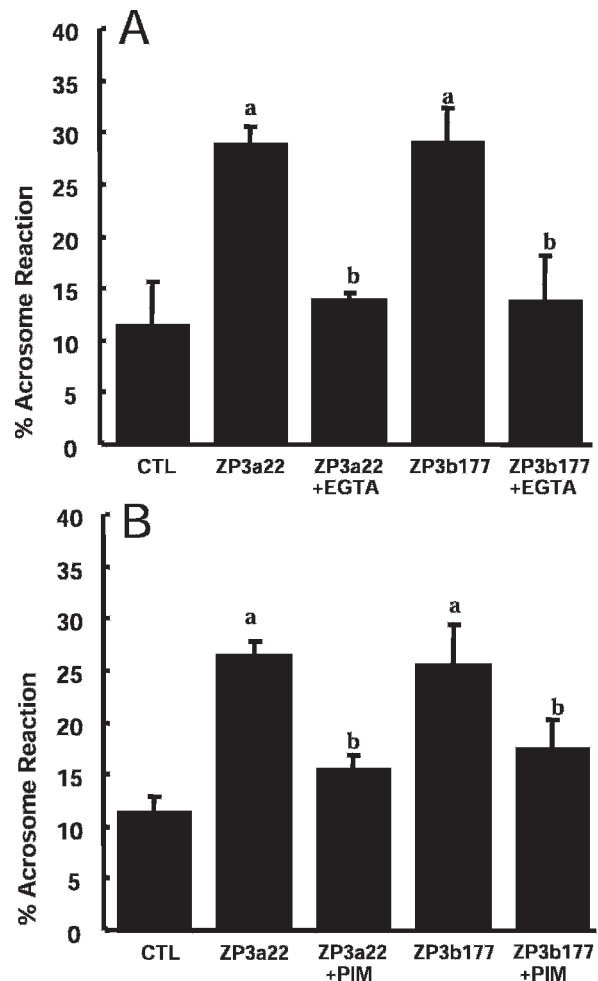


Figure 3. Extracellular Ca<sup>2+</sup> is required for AR induced by rhuZP3a<sup>22~176</sup> and rhuZP3b<sup>177~348</sup>. The effects of EGTA (A) or pimoizide (B) on the AR were recorded. Capacitated sperm ( $2\sim 3 \times 10^7$  cells/mL) were preincubated for 10 minutes without or with 2 mM EGTA or 100  $\mu$ M pimoizide, followed by a further incubation with 50  $\mu$ g/mL rhuZP3a<sup>22~176</sup> or rhuZP3b<sup>177~348</sup> for 15 minutes. The AR assay was performed as described in the Materials and Methods section. CTL, control; ZP3a<sup>22</sup>, rhuZP3a<sup>22~176</sup>; ZP3b<sup>177</sup>, rhuZP3b<sup>177~348</sup>; PIM, pimoizide. Each data group represents the mean  $\pm$  SEM of 3 different experiments. There is significant difference between a and b ( $P < .05$ ).

ited the AR induced by rhuZP3a<sup>22~176</sup> ( $23.5 \pm 2.8\%$ ) and rhuZP3b<sup>177~348</sup> ( $25.5 \pm 2.8\%$ ). These results suggest that the rhuZP3s induce AR via a major T-type calcium channel.

#### Rhuzp3a<sup>22~176</sup> or Rhuzp3b<sup>177~348</sup> Induces a Rise in Intracellular Calcium

The addition of rhuZP3a<sup>22~176</sup> to capacitated sperm loaded with Fura-2/AM caused a rapid rise in the intracellular Ca<sup>2+</sup> levels (peak) but almost no sustained elevation of intracellular Ca<sup>2+</sup>. The addition of pimoizide or EGTA before the addition of rhuZP3a<sup>22~176</sup> significantly reduced the peak values (Figure 4A). The

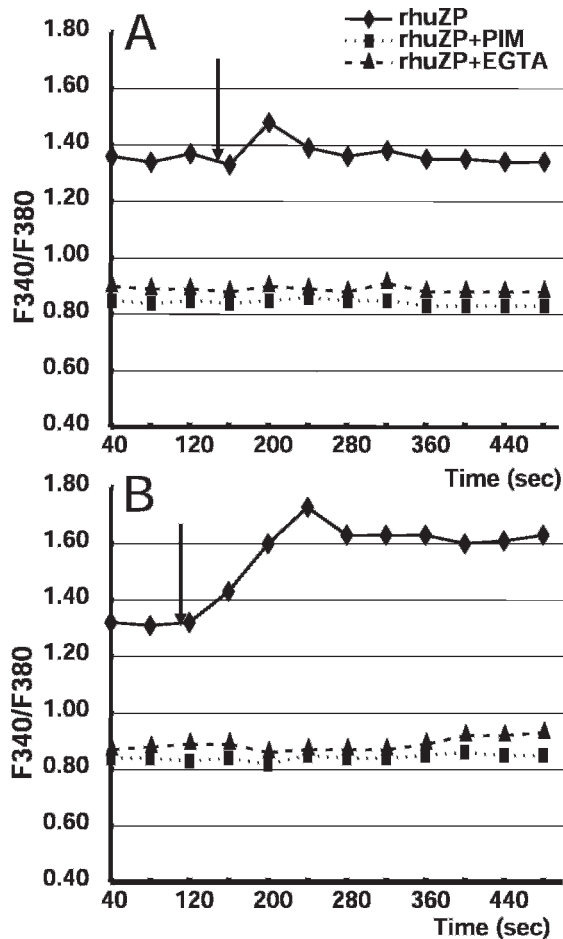


Figure 4. The  $[Ca^{2+}]_i$  in human sperm induced by rhuZP3a<sup>22~176</sup> (A) or rhuZP3b<sup>177~348</sup> (B).  $[Ca^{2+}]_i$  was calculated by the ratio of fluorescence intensity at the two excitation wavelengths (340 / 380 nm) (emission 510 nm). The arrow represents the time at which rhuZP3a<sup>22~176</sup> or rhuZP3b<sup>177~348</sup> was added. The solid line shows the Fura-2/AM-loaded sperm plus rhuZP3a<sup>22~176</sup> or rhuZP3b<sup>177~348</sup>. Pimozide (100  $\mu$ M) was added to Fura-2/AM-loaded sperm 10 minutes before the addition of rhuZP3a<sup>22~176</sup> or rhuZP3b<sup>177~348</sup> (dotted line). EGTA (2 mM) was added to Fura-2/AM loaded sperm 10 minutes before the addition of rhuZP3a<sup>22~176</sup> or rhuZP3b<sup>177~348</sup> (dashed line).

addition of ZP3b<sup>177~348</sup> to capacitated sperm loaded with Fura-2/AM caused a sudden rise in the intracellular  $Ca^{2+}$  levels (peak) and sustained elevation of intracellular  $Ca^{2+}$  (plateau). Pimozide or EGTA significantly reduced both the peak and the sustained values (Figure 4B). These results suggest that rhuZP3 induces sperm AR via increases in intracellular calcium levels.

## Discussion

ZP3 plays an important role in mammalian fertilization. As an inducer of sperm exocytosis (AR) and as a sperm receptor that is involved in carbohydrate-mediated

gamete recognition and adhesion (Wassarman, 1990), ZP3 serves as a tool for the diagnosis of male infertility. RhuZP3 has been developed because of the limited availability of native ZP3. It has been reported that rhuZP3s prepared from various express systems has biological activities, including the induction of sperm AR, stimulation of calcium influx, promotion of sperm-egg fusion, and stimulation of sperm mobility (Van Duin et al, 1994; Brewis et al, 1996; Dong et al, 2001; Bray et al, 2002; Caballero-Campo et al, 2006).

The construction, expression, and purification of rhuZP3a<sup>22~176</sup> and rhuZP3b<sup>177~348</sup> have been described in our previous study (He et al, 2005). Both peptides are able to generate higher antibody titers in rabbits. Each antiserum specifically recognizes or binds to each target peptide expressed in *E coli* and the native human ZP3 in vitro. The antisera also inhibit sperm-egg binding in the competitive hemizona assay (HZA) (Song et al, 2005). To study further whether rhuZP3a<sup>22~176</sup> and rhuZP3b<sup>177~348</sup> are capable of triggering the AR, the effects of different concentrations of these 2 peptides have been analyzed in the present study. Our results show that 5  $\mu$ g/mL rhuZP3a<sup>22~176</sup> incubated with capacitated human sperm for 15 minutes induces the AR. The concentrations of rhuZP3a<sup>22~176</sup> and rhuZP3b<sup>177~348</sup> that induced AR ranged from 5 to 50  $\mu$ g/mL (Figure 1A and B). These results show that the abilities of rhuZP3a<sup>22~176</sup> and rhuZP3b<sup>177~348</sup> to initiate the AR are not identical. This is probably due to the different lengths of the amino acid sequences of the two peptides, since rhuZP3b<sup>177~348</sup> has a longer amino acid chain than rhuZP3a<sup>22~176</sup>. Compared to rhuZP3 expressed in CHO cells (Bray et al, 2002), rhuZP3b<sup>177~348</sup> appears to be more potent. The rhuZP3 from CHO cells induced about 30% of AR at 100  $\mu$ g/mL, whereas this rate could be achieved by rhuZP3b<sup>22~176</sup> at 50  $\mu$ g/mL.

*E coli*-expressed, intact ZP3-induced AR in human spermatozoa, as reported by Chapman et al (1998), requires 18 hours, whereas rhuZP3a<sup>22~176</sup> and rhuZP3b<sup>177~348</sup> induced-AR required 15 minutes. These different results raise four major possibilities. First, the purified recombinant human ZP3 used by Chapman et al (1998) was expressed in *E coli* as a C-terminal fusion to the dimeric GST from *S japonicum*. The peptide fragments and lengths are different from those used in the present work. Alternatively, some differences exist in the domains of the peptide fragment. Second, the method of isolation of motile spermatozoa is also different. Chapman et al (1998) used a direct swim-up technique, which ensures a longer time for sperm capacitation than washing the spermatozoa by centrifugation through a 3-step Percoll gradient (Ni et al, unpublished data). Third, in a previous study, we

demonstrated that the antisera against rhuZP3<sup>22~176</sup> and rhuZP3<sup>176~384</sup> blocked human sperm-egg binding using a competitive hemizona assay (HZA), which indicates that these peptides are similar to the domain of the native ZP3 (Song et al, 2005). Finally, the AR depends on the sperm capacitation status. Due to a lack of agreement on how female-derived factors affect the process of capacitation, it is not clear whether the time required for completion of the capacitation process is different in vitro. The time required for sperm capacitation in the human is a topic for debate (Jaiswal and Eisenbach, 2002).

It is generally accepted that the carbohydrate domain displayed by the ZP3 glycoprotein plays a central role in sperm-egg binding (Wassasman, 1990). However, although both native human ZP and purified, glycosylated rhuZP3 secreted by CHO cells have been shown to stimulate AR in human spermatozoa, it has yet to be firmly established whether the ability to initiate AR resides in the carbohydrate moieties of the ZP3 glycoprotein alone, the polypeptide backbone of ZP3 or elements of both (Chapman et al, 1998). Although glycosylation of ZP3 is required for the induction of acrosomal exocytosis (Gahlay and Gupta, 2003), the significance of the polypeptide backbone should still be considered. Different recombinant ZP3s expressed from *E coli* are free of carbohydrate modifications but provide diverse results (Chapman et al, 1998; Gahlay and Gupta, 2003). This phenomenon may be caused by the different polypeptide backbones of recombinant ZP3, since one is effective (Chapman et al, 1998) and another is ineffective (Gahlay and Gupta, 2003). Furthermore, our results from the present study are distinct from the results of these previous studies. The different polypeptide backbones may explain why these results are discordant.

In the present study, PTX was shown to inhibit the AR induced by both rhuZP3a<sup>22~176</sup> and rhuZP3b<sup>177~348</sup> (Figure 2), suggesting that a pertussis toxin-sensitive G protein is involved in the signal pathways. Our results are consistent with the observations obtained using other rhuZP3s in different laboratories (Dong et al, 2001; Bray et al, 2002). To test the effects of PTX on peptide-initiated AR, sperm were preincubated without PTX under capacitation conditions for 5 hours and then exposed to PTX at various concentrations for 10 minutes before the addition of rhuZP. After incubation, the sperm were challenged with rhuZP3 for 15 minutes, stained, and fixed. According to the inhibition test of concentration response, 0.5~2.0 µg/mL PTX caused a significant dose-dependent inhibition of rhuZP3-stimulated AR, with maximal effects seen with 1.0~2.0 µg/mL of PTX. There were no effects on sperm motility or integrity. It is generally believed that the

peptides are sensitive to pertussis toxin. Although the magnitude of the PTX concentration (0.5~2.0 µg/mL) used in the present study are slightly higher than those used by Bray et al (2002) and Dong et al (2001), who reported that PTX inhibited the AR induced by recombinant human ZP3 at a concentration of 100 ng/mL. They pretreated the spermatozoa with PTX (100 ng/mL) for 3 hours before stimulating with rhuZP3. This is a longer time period than we used. In other words, this difference is probably the result of spermatozoa exposure time to PTX. Therefore, we consider that peptide-induced AR is mediated by a transmembrane signaling pathway that involves the activation of PTX-sensitive G proteins.

The removal of extracellular Ca<sup>2+</sup> from the medium by the addition of EGTA significantly inhibited the AR induced by rhuZP3a<sup>22~176</sup> or rhuZP3b<sup>177~348</sup>, which suggests that extracellular Ca<sup>2+</sup> is required for the signal pathway. Pretreatment with EGTA did not inhibit the basal or spontaneous acrosomal exocytosis, which suggests that intracellular sources of calcium may be sufficient for sustaining basal levels of AR (Schuffner et al, 2002).

The AR stimulated by rhuZP3a<sup>22~176</sup> or rhuZP3b<sup>177~348</sup> was possibly mediated by a VOCC<sub>T</sub>. It has been demonstrated that T-type current, but not L-type current, is involved in mouse sperm AR induced by ZP (Arnoult et al, 1996a). We demonstrated that the AR induced by these 2 peptides was blocked by the VOCC<sub>T</sub> inhibitor pimozide but less effectively by verapamil. However, this does not rule out the potential influence of phospholipase A<sub>2</sub> (Thakkar et al, 1984). It is well known that verapamil is an L-type calcium channel blocker that inhibits calcium influx, resulting in the inhibition of sperm AR. However, it is still not known how verapamil inhibits phospholipase A<sub>2</sub> activity.

[Ca<sup>2+</sup>]<sub>i</sub> elevation was initiated by rhuZP3a<sup>22~176</sup> and rhuZP3b<sup>177~348</sup> (Figure 4A and 4B). The elevation of [Ca<sup>2+</sup>]<sub>i</sub> induced by ZP3 has been demonstrated to proceed via two signaling pathways in sperm (Felix, 2005). ZP3 activates a heterotrimeric GTP-binding protein and PLC, thus elevating [Ca<sup>2+</sup>]<sub>i</sub>. After binding to the same receptor(s), ZP3 can also stimulate a transient influx of calcium through T-type channels. The influx of Ca<sup>2+</sup> through T-type Ca<sup>2+</sup> channels is transient (500 ms) (Darszon et al, 2001; Jagannathan et al, 2002). Furthermore, the initial response to ZP is a large, transient [Ca<sup>2+</sup>]<sub>i</sub> spike with kinetics comparable to those of T currents (Arnoult et al, 1999). This initial Ca<sup>2+</sup> entry induces a second, sustained Ca<sup>2+</sup> influx. Recent evidence indicates that the sustained component of Ca<sup>2+</sup> influx is mediated primarily by store-operated channels (SOCs) that are activated after the depletion of a small Ca<sup>2+</sup> store (O'Toole et al, 2000; Jungnickel et al,

2001), probably in the acrosome. When sperm were treated with rhuZP3a<sup>22~176</sup> or rhuZP3b<sup>177~348</sup>, the ratio of fluorescence intensity (F<sub>340</sub>/F<sub>380</sub>) rose, indicating the elevation of [Ca<sup>2+</sup>]<sub>i</sub>. Both rhuZP3a<sup>22~176</sup> and rhuZP3b<sup>177~348</sup> enable the capacitated sperm to increase the [Ca<sup>2+</sup>]<sub>i</sub>, which indicates that they have biological activities similar to those of the native human ZP3. To our knowledge, this is the first report that rhuZP3 peptides generated from a prokaryotic cell induce changes in the intracellular calcium levels of human spermatozoa. It has been reported that native ZP3 causes a spike followed by activation of a sustained influx of [Ca<sup>2+</sup>]<sub>i</sub> (Baldi et al, 1996). We found that rhuZP3a<sup>22~176</sup> caused a spike without plateau elevation of [Ca<sup>2+</sup>]<sub>i</sub>, while rhuZP3b<sup>177~348</sup> caused a spike followed by activation of a sustained influx of [Ca<sup>2+</sup>]<sub>i</sub>. This suggests that ZP3b<sup>177~348</sup> is similar to natural ZP3 and can act as an alternative for native human ZP3.

In conclusion, rhuZP3a<sup>22~176</sup> and rhuZP3b<sup>177~348</sup> obtained from *E coli* were capable of inducing AR at different concentrations. With the addition of each of these 2 peptides, the [Ca<sup>2+</sup>]<sub>i</sub> was raised, although the patterns differed. The rhuZP3-induced AR was inhibited by PTX, EGTA, and pimozone. The present study demonstrates that rhuZP3a<sup>22~176</sup> and rhuZP3b<sup>177~348</sup> have the same efficiency as natural human ZP3 to induce human AR, and that they induce AR via the activation of a G protein and the induction of extracellular calcium influx through a T-type calcium channel.

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