ABSTRACT The aim of this study was to determine the site of enzyme release from the acrosome and the fate of the acrosomal cap during the process of acrosome reaction (AR) in fowl sperm. Gelatin substrate coverslips with halos were subjected to scanning electron microscopy to determine the site from which acrosomal proteolytic enzyme was released to form a halo around the acrosome of individual sperm. Aliquots of sperm treated with solubilized inner perivitelline layer (IPL) containing 5 mmol CaCl₂ were simultaneously subjected to fluorescent staining with fluorescein isothiocyanate-labeled peanut agglutinin and scanning electron microscopy to evaluate AR of sperm and to examine the status of the acrosomal region, respectively. Inside the halos, a gelatin-free (proteolyzed gelatin) layer was found extending some distance around the acrosome of sperm. All of the sperm showing the formation of halos on gelatin had a single circular opening around their subacrosomal rod at the base of the acrosomal cap. Interaction of sperm with solubilized IPL in the presence of 5 mmol CaCl₂ resulted in 41.4 ± 1.8% of the sperm to undergo AR, as evaluated by fluorescein isothiocyanate-labeled peanut agglutinin. Similarly, as observed using scanning electron microscopy, 38.2 ± 2.3% of the sperm treated with solubilized IPL plus 5 mmol CaCl₂ had exposed subacrosomal rod. In all sperm examined, no sign of disruption of the acrosomal membrane was found in the apical region of the acrosome. Rather, the acrosomal caps were found intact detached from the acrosomal region of the sperm, indicating that AR of fowl sperm resulted in the intact removal of the acrosomal cap. Based on these experimental observations, we suggest that the process of AR in fowl sperm is unique; the release of the acrosomal proteolytic enzyme may occur through a single circular opening formed at the base of the acrosomal cap and the acrosomal cap is detached in intact form from the posterior acrosomal region of the sperm.

Key words: acrosomal proteolytic activity, acrosome reaction, acrosomal cap

INTRODUCTION

It is well documented that when fowl sperm approach the freshly ovulated ovum, they first encounter its overlying inner perivitelline layer (IPL) to undergo acrosome reaction (AR), which aid sperm in reaching inside the ovum through penetration of the IPL (Anderson and Navara, 2011; Lemoine et al., 2011). In general, AR is characterized by the fusion between the outer acrosomal and overlying plasma membrane of sperm, followed by subsequent vesiculation of these membranes and exocytotic release of enzyme from the acrosomal matrix (Okamura and Nishiyama, 1978; Yanagimachi, 1994). In mammals, it is well recognized that fusion and subsequent vesiculation of the outer acrosomal and overlying plasma membrane occur at multiple points in the apical region of the acrosome, which results in the formation of several perforations through which acrosomal content is released followed by loss of the acrosomal cap from the head of sperm (Barros et al., 1967; Yanagimachi and Noda, 1970). Although there have been studies involving the examination of the mechanism of AR, and acrosomal status
of fowl sperm after interaction with IPL via electron microscopy, there is no precise indication for the site of enzyme release from the acrosome and the fate of the acrosomal cap during AR.

The first investigation to explore the mechanism of fowl sperm AR was undertaken by Okamura and Nishiyama (1978), who used hypothetical diagrams to propose that the vesiculation of fowl sperm outer acrosomal and overlying plasma membrane causes the fused membranes to burst at the acrosomal apical region, resulting in the formation of an opening through which acrosomal content is released and subsequent disappearance of the apical region of the acrosome. However, there is no direct experimental evidence to support this speculation because their observation on morphological changes of acrosome-reacted sperm was after the completion of penetration by sperm into the IPL of an ovulated ovum. It is well known that the spermatozoon with an exposed subacrosomal rod penetrate into the egg investment, whereas the acrosomal cap and its enzyme do not accompany the spermatozoon (Yanagimachi and Phillips, 1984; Shalgi et al., 1989). Thus, it remains unclear if the release of enzyme has occurred from the acrosomal apical region and acrosomal cap is completely fragmented during AR. Moreover, the transmission electron micrograph of the acrosome-reacted spermatozoon in their study showed no sign of perforation at the apical core of the acrosomal region. Rather, there were 2 very short remnants of the fused membranes at the junction between the anterior and equatorial segment of the acrosome, indicating that the anterior part of the acrosome might have been torn off from the equatorial segment at the junction site to liberate the content from the acrosomal matrix.

Later, Koyanagi et al. (1988), following the sperm-intact IPL interaction assay, induced AR of fowl sperm, and using scanning electron microscopy, demonstrated that the acrosome-reacted sperm show a complete lack of the acrosomal cap at their head region. However, they did not clarify whether the acrosomal cap was completely detached in intact form from the acrosome or fragmented to complete dispersal, and did not indicate the site from which the acrosomal enzyme was released from the acrosome.

In contrast, studies have shown that when sperm are smeared on the fixed gelatin and incubated to induce proteolytic activity of the sperm acrosome, the release of acrosomal enzyme is visible in the form of halo formation around the acrosome of individual sperm (Maeda et al., 1990), and when sperm are incubated in a solution containing solubilized IPL to induce physiological AR in fowl sperm (Howarth 1990), the loss of the acrosomal cap in any form can be recovered from the solution.

Therefore, in order for the determination of the site in the sperm acrosome from which exocytotic release of proteolytic enzyme occurs, and the fate of acrosomal cap during the process of AR, the present study was designed to examine the acrosomal status of fowl sperm using scanning electron microscopy after the formation of halos around the acrosome of sperm on gelatin as well as incubation of sperm with solubilized IPL.

**MATERIALS AND METHODS**

**Birds**

A total of 18 mature White Leghorn male chickens (*Gallus domesticus*) aged 43 to 45 wk with proven fertility were selected as donors of the semen in the present study. They were caged individually under a photoperiod of 14L:10D and fed a commercial breeder’s ration (19% CP and 2,800 kcal of ME/kg) ad libitum. The present study was conducted in accordance with the regulations for the care and use of experimental animals outlined by the Animal Care Committee of the University of the Ryukyus, Okinawa, Japan.

**Collection and Preparation of Sperm, and Collection of IPL**

Semen, devoid of transparent fluid, was collected twice weekly from those selected roosters using the massage method (Burrows and Quinn, 1935). Immediately after collection, the pooled semen was diluted 4 times with NaCl-TES (150 mmol of NaCl and 20 mmol of TES (N-[hydroxymethyl] methyl-2-amino-ethanesulfonic acid)) buffer, pH 7.4 and washed by centrifugation at 600 × g for 10 min to discard seminal plasma. The sperm pellets were resuspended in the same buffer. The number of sperm cells was counted using a Neubauer hemocytometer (American Optical Co., New York, NY).

The IPL was separated from the outer perivitelline layer by acid hydrolysis using 1-d-old oviposited chicken egg as described by Kido and Doi (1988).

**Induction of Acrosomal Proteolytic Activity of Sperm**

The gelatin substrate technique was used for inducing acrosomal proteolytic activity of sperm according to the method described previously (Maeda et al., 1990). Briefly, a volume of 40 µL of warm gelatin suspension was placed in the middle of a clean, grease-free coverslip (18 × 18 mm). A second coverslip was placed lengthwise on the first one to spread the gelatin suspension completely, and the coverslips were gently pulled apart along the horizontal axis. The gelatin substrate coverslips (GSC) were stored for 24 h in a moist chamber at 4°C and then fixed by immersing them in 0.05% (vol/vol) glutaraldehyde solution for 10 min; they were then stored at 4°C for 6 h. A 20 µL drop of sperm suspension containing 1 × 10⁷ cells/mL was placed on one end of the GSC, smeared uniformly, and incubated for 2 h at 37°C. After incubation, the GSC were examined with a phase-contrast microscope (Nikon Eclipse 50i, Tokyo,
Japan) for the formation of halo (Figure 1b). Sperm smeared on gelatin-free coverslips and subsequently subjected to this identical process of incubation were used as the control.

**Induction of Sperm AR**

Sperm-homogenized IPL interaction assay was accomplished according to the method of Horrocks et al. (2000) to induce AR of sperm. To obtain the homogenized solution of IPL, an intact sheet (2 × 2 cm) of IPL was dissolved in 1 mL of 5 mmol NaH2PO4, pH 2.5, and incubated at 40°C for 1 h. To induce AR of sperm, 100 µL of solubilized IPL was added to 900 µL of NaCl-TES (pH was adjusted to 7.4) with 5 mmol CaCl2 containing 1.25 × 107 sperm/mL and incubated at 40°C for 5 min. Sperm incubated under this identical condition only in NaCl-TES buffer (pH 7.4) served as the control.

**Evaluation of Sperm AR**

Samples of the solubilized IPL-treated sperm and the control sperm were examined for the induction of AR using fluorescent staining with 100 µg/mL fluorescein isothiocyanate (FITC)-labeled peanut agglutinin (PNA; Invitrogen, Burlingame, CA), as previously described by Horrocks et al. (2000).

**Scanning Electron Microscopy**

To examine the acrosomal status of sperm, samples of GSC with halo formation, sperm treated with solubilized IPL, and control sperm were subjected to scanning electron microscopy. Suspensions of sperm were placed on glass coverslips (18 mm in diameter) for 5 min to adhere sperm to the coverslips. All the GSC and sperm samples were fixed with 2.5% (vol/vol) glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 in a light shield container, dehydrated through ascending concentrations of ethanol (50, 70, 80, 90, 95, and 99% for 30 min each) and in absolute ethanol (100%, 2 changes for 15 min each), and then dried by the t-butyl alcohol freeze-drying method (Inoué and Osatake, 1988). The freeze-dried specimens were mounted on stubs with double-sided carbon adhesive tabs (Nisshin EM Co. Ltd., Tokyo, Japan), ion-sputter coated with platinum at a thickness of 150 Å using an ion coater (IB-5, Eiko Engineering Co. Ltd., Ibaraki, Japan), and then examined with a scanning electron microscope (Hitachi S-2380N, Hitachi Ltd., Tokyo, Japan).

**Statistical Analysis**

Data from 4 replicate trials were expressed as the mean ± SEM. Percentage data were subjected to arcsine transformation before statistical analysis. All percentage data were analyzed with the chi-squared test using IBM SPSS Statistics version 19 (SPSS Inc., Chicago, IL). A probability of $P < 0.05$ was considered statistically significant, unless stated otherwise.

**RESULTS**

Acrosomes remained intact for the control sperm that had undergone incubation at 37°C for 2 h after smearing on a gelatin-free coverslip, as shown in Figure 1a. However, incubation of sperm smeared on the GSC under this identical condition resulted in the formation of halo (proteolyzed gelatin) around the acrosome (Figure 1b). When sperm with halo on gelatin were examined for acrosomal status using scanning electron microscopy, all of the sperm (Table 1) had a single circular opening around the subacrosomal rod at the base of the acrosomal cap (Figure 1c).
the evaluation of the induction of AR using FITC-PNA fluorescent staining, and for the status of their acrosome using scanning electron microscopy, it was found that a nonsignificant \((P > 0.05; \text{Table 2})\) number of sperm that were acrosome reacted had become free of their acrosomal cap (sperm with exposed subacrosomal rod). The acrosomal caps were found in intact and detached form (Figure 2b and c). However, acrosomal caps were not detached from the control sperm (Table 2 and Figures 1a and 2a).

**DISCUSSION**

In the present study, as a means of determining the site of enzyme release from the acrosome, the acrosomal proteolytic activity of sperm was induced using a gelatin substrate technique, which is an established method of assessing proteolytic activity of the sperm acrosome in humans and domestic animals, including fowl (Ficsor et al., 1983; Welker et al., 1988; Maeda et al., 1990). It is well documented that fowl sperm contains a trypsin-like proteolytic enzyme (Langford and Howarth, 1974) or acrosin (Ho and Meizel, 1975), which is localized in their acrosomal matrix. Studies have shown that the trypsin-like proteolytic enzyme liberated from the sperm acrosome is involved in the process of gelatin digestion during the formation of halo (Win et al., 2006). When sperm showing halo formation on the GSC were examined with scanning electron microscopy, the present study revealed for the first time the distinct feature of acrosomal enzyme release from fowl sperm during proteolysis of the gelatin. It was obvious that a single circular opening around the subacrosomal rod was formed at the base of the acrosomal cap, and a gelatin-free (proteolyzed gelatin) layer was appeared extending some distance around the acrosome of sperm. Therefore, it seems reasonable to suggest that the release of proteolytic enzyme might have occurred through the opening formed at the posterior region of the sperm acrosome.

It is well recognized that the solubilized IPL plus 5 mmol CaCl₂ acts as an inducer of fowl sperm AR (Lemoine et al., 2008, 2011). In this study, using FITC-PNA staining, we have also observed that the solubilized IPL plus 5 mmol CaCl₂ induced AR of sperm. When samples of the sperm treated with the solubilized IPL plus 5 mmol CaCl₂ were observed using scanning electron microscopy, it was found that a similar number of sperm that were acrosome reacted had their acrosome free of the acrosomal cap, which was found intact, detached from the acrosome of the sperm, indicating that AR of fowl sperm resulted in the intact removal of the acrosomal cap. Our observations on the integrity of the acrosomal apical region are inconsistent with the hypothesis proposed by Okamura and Nishiyama (1978) that like in mammalian sperm, the fusion of fowl sperm membranes occurs at the apical region of the acrosome, which results in the complete disintegration of the anterior part of the acrosome during AR. In all the sperm examined in the current study, we did not find any spermatozoon showing the sign of disruption of the acrosomal membrane in the apical acrosomal region. Therefore, the process of AR in fowl sperm seems unlikely to the mammalian process; the present study is the first to show the unique evidence for acrosomal cap shedding in intact form, and the site of acrosomal enzyme release during the process of AR in fowl sperm using scanning electron microscopy. It is well recognized that a period of capacitation is a prerequisite for AR to occur in mammalian sperm (Austin, 1952; Suzuki et al., 2010). Studies have demonstrated that the capacitation involves subtle structural and biochemical changes of the sperm membrane such as increase in membrane permeability and destabilization that facilitates formation of multiple openings at the apical region of the acrosome (Witte and Schäfer-Somi, 2007; Suzuki et al., 2010). Conversely, fowl sperm have been shown to un-

<table>
<thead>
<tr>
<th>Type of sperm</th>
<th>No. of sperm examined</th>
<th>Sperm showing removal of acrosomal cap1 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without halo formation (control)</td>
<td>242</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>With halo formation</td>
<td>256</td>
<td>100.0 ± 0.0 **</td>
</tr>
</tbody>
</table>

1Values are expressed as mean ± SEM (n = 4).

**Values differed \((P < 0.001)\) compared with the control.

Table 2. Effects of incubating sperm in NaCl-TES (150 mmol of NaCl and 20 mmol of TES (N-[hydroxymethyl] methyl-2-aminoethanesulfonic acid)) buffer with or without solubilized inner perivitelline layer (IPL) plus 5 mmol CaCl₂ at 40°C for 5 min on induction of acrosome reaction of sperm and removal of acrosomal cap

<table>
<thead>
<tr>
<th>Type of sperm</th>
<th>No. of sperm examined</th>
<th>Acrosome-reacted sperm1 (%)</th>
<th>Acrosomal cap-free sperm1 (sperm with exposed SAR; %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treated with NaCl-TES buffer only (control)</td>
<td>226</td>
<td>0.0 ± 0.0 NS</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Treated with NaCl-TES buffer with solubilized IPL and 5 mmol CaCl₂</td>
<td>231</td>
<td>41.4 ± 1.8** NS</td>
<td>38.2 ± 2.6**</td>
</tr>
</tbody>
</table>

1Values are expressed as mean ± SEM (n = 4).

**Values differed \((P < 0.001)\) compared with the control. NS: Values did not differ \((P > 0.05)\) compared with the acrosomal cap-free sperm [sperm with exposed subacrosomal rod (SAR)].
Figure 2. Representative scanning electron photomicrographs of sperm showing: (a) intact acrosome after incubation at 40°C for 5 min in NaCl-TES buffer only (control); and (b and c) lack of acrosomal cap (AC), which remains detached from the acrosome in intact form in the vicinity of the spermatozoon with an exposed subacrosomal rod (SAR), following a 5-min incubation period at 40°C in NaCl-TES buffer containing solubilized inner perivitelline layer and 5 mmol CaCl₂ (b and c). Scale bar = 4 µm at 125,000× magnification (a); 5 µm at 100,000× magnification (b and c).

dergo AR, and to fertilize the ovum directly without requiring capacitation (Howarth, 1970).

The mechanism by which acrosomal enzyme is liberated from the acrosome of sperm is not completely understood at the present time. However, we proposed a tentative mechanism of cap removal and enzyme release from the acrosome of fowl sperm in a way that the pro-TLE or proacrosin in the acrosomal matrix may undergo activation at the initial stage of AR (De los Reyes and Barros, 2000; Cortes et al., 2006). Therefore, activation and subsequent conversion of pro-TLE or proacrosin into active TLE or acrosin, respectively, may create an internal pressure that may push forward the acrosomal cap, resulting in the intact detachment of the acrosomal cap from the posterior region of the sperm acrosome. Thus, the acrosomal enzyme may be liberated from the acrosome through the opening formed at the posterior region of the acrosome.

In conclusion, on the basis of our experimental evidence, we inferred that the AR in fowl sperm is a unique process by which the acrosomal cap over the head of the sperm cell is shed completely, retaining its integrity to release acrosomal proteolytic enzyme, which may occur at the posterior region of the acrosome.

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REFERENCES


