Membrane Specialization in the Inner Acrosomal Membrane of Abalone Sperm Studied by Freeze–Substitution and Quick–Freeze Deep–Etch Electron Microscopy

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Abstract

The ultrastructure of the inner acrosomal membrane of abalone sperm was examined before and during the acrosome reaction using quick freeze technique followed by either freeze–substitution or deep–etch electron microscopy. Freeze–substituted preparation revealed the presence of granules closely aligned along the inner acrosomal membrane in the funnel–shaped region at the posterior side of the acrosome. In quick–freeze, deep–etch replica images, these granules were found to be associated with the amorphous material surrounding the actin filament bundle which is interconnected by short strands. It is suggested that these granules may be relevant to the synchronous extension of the actin filament bundle and the inner acrosomal membrane that transforms into the acrosomal process membrane.

Furthermore, numerous intramembrane particles regularly arranged in a lattice–like pattern were shown to be distributed on the P face of the inner acrosomal membrane in the funnel–shaped region. Upon the formation of the acrosomal process, this dense lattice–like pattern of intramembrane particles on the P face of the acrosomal process membrane changed into a random or lattice–like pattern in patches. These observations using quick–freeze methods suggest that the lattice–like arrangement of intramembrane particles on the inner acrosomal membrane plays a potential role in the transformation of the inner acrosomal membrane into the acrosomal process membrane, which is closely coupled with the rapid elongation of the actin filament bundle enclosed by the inner acrosomal membrane.
Introduction

In many marine invertebrates, sperm undergo the acrosome reaction before they fertilize the eggs (1-3). The acrosome reaction involves the fusion of the sperm plasma membrane with the outer acrosomal membrane to open the acrosomal apex, which releases the acrosomal contents. This exocytotic event is accompanied by the rapid extension of the acrosomal process covered by the acrosomal process membrane, which is destined to fuse with the egg plasma membrane at fertilization. In close relation to these dramatic structural changes in the acrosome, two specialized domains in the acrosomal membrane have been documented. One is the apical region of the acrosomal vesicle, termed the 'trigger region', where the outer acrosomal membrane is in close contact with the plasma membrane (1,3-7) or with a layer of osmiophilic substance (8,9). The other is the adnuclear side of the acrosomal membrane (1,4,5,10-14), which is undercoated by some layered materials in some species (2,3,15), and has been suggested to serve as a precursor of the acrosomal process membrane.

In abalone sperm the adnuclear side of the acrosomal membrane is deeply invaginated by actin filament bundle termed the axial rod in the center of the adnuclear surface of the acrosomal vesicle (6,16,17), which is referred to as the inner acrosomal membrane. It is this membrane that rapidly extends and transforms into the acrosomal process membrane during the acrosome reaction (16,17,18). In view of the vital function performed by this acrosomal process membrane to fuse with the egg plasma membrane at fertilization, the present study was focused on the inner acrosomal membrane. To visualize the characterization of this inner acrosomal membrane and its critical transformation into the acrosomal process membrane, we used the quick-freeze technique combined with freeze-substitution and deep-etch, rotary shadow electron microscopy.

Materials and Methods

Mature shells of abalone, Haliotis discus, were provided by the Chiba Prefectural Fisheries Experimental Station, Chikura Japan and gametes were obtained by the method previously described (16).

The acrosome reaction was induced by exposing the sperm to artificial sea water at
pH 8.3 containing ionophore A 23187 (Calbiochem-Behring Co., La Jolla, Ca., 24μg/ml) and 40 mM CaCl₂.

For thin sectioning, specimens were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), postfixed with 1% osmium tetroxide in the same buffer, dehydrated, and embedded in Epon 812. Thin sections were cut, stained with uranyl acetate and lead citrate, and examined with a Hitachi HU-12A electron microscope.

For quick-freezing, fresh sperm were quick-frozen on a liquid helium-cooled copper block using a quick-freezing device (Slammer/QF 5000, Meiwa Co., Osaka, Japan).

For freeze-substitution, quick-frozen samples were freeze-substituted for 2 days with absolute acetone containing 2% OsO₄ at −80°C. The samples were slowly warmed by transfer to −20°C for 2h, and then kept at 4°C for 1h. After being washed twice in absolute acetone at room temperature, the samples were washed in propylene oxide and embedded in Epon 812.

For quick-freeze, deep-etching, quick-frozen samples were fractured at −182°C in a Cressington CFE-50 freeze fracture apparatus and allowed to etch at −98°C for 10 min at a pressure below 2×10⁻⁷ mbar. The specimen was then rotary shadowed with platinum at an angle of 20°, followed by carbon at an angle of 90°. Replicas were cleaned in commercial bleach and mounted on Formvar-coated carbon-stabilized copper grids for electron microscopy. All quick-freeze, deep-etch figures presented in this paper were photographically reversed, thus, platinum deposits appear white.

**Results and Discussion**

Abalone sperm has been used for analyzing the mechanism of the acrosome reaction, since it has a large acrosome on the top of the cylindrical nucleus. From thin section images, the large bullet-shaped acrosome was demonstrated to contain a membrane-bounded acrosomal vesicle deeply invaginated at the adnuclear side (Fig. 1a).

Within this invagination, the axial rod that consists of a bundle of actin filaments was shown to be settled (6,16). The anterior surface of the acrosomal vesicle was surrounded by the outer acrosomal membrane and the posterior surface by the inner acrosomal membrane (6,7). The axial rod was localized between the acrosomal vesicle and the nucleus, protruding into the both sides. Therefore, the anterior half of the axial rod
Fig. 1. Diagrams of unreacted (a) and acrosome-reacted (b) abalone sperm head, as seen in longitudinal sections in the transmission electron microscope. a) The axial rod (AR) composed of actin filament bundle is interposed between the acrosomal vesicle (A) and the nucleus (N). b) Note that the acrosomal contents have been released and the acrosomal process (AP) elongated beyond the acrosomal apex, where a coiled filamentous structure termed truncated cone (TC) elongates into a cylinder and closely surrounds the acrosomal process (AP). VA, vacant acrosome.

appeared being closely covered by the inner acrosomal membrane, while the posterior half of the axial rod by the nuclear membrane. In the boundary region between the acrosomal vesicle and the nucleus, the axial rod was surrounded by subacrosomal amorphous material (6,7,16), where both the inner acrosomal and the nuclear membranes appear to be funnel-shaped (Fig. 1a, 2).

Thin section of the quick-frozen and freeze-substituted preparations in unreacted sperm revealed dense distribution of granules (7~10 nm in diameter) closely aligned along the rim of the inner acrosomal membrane in the funnel-shaped region (Fig. 2), which were not observed in the conventional thin section. This region having the closely
Fig. 2. Longitudinal section through the adnuclear side of the inner acrosomal membrane prepared by freeze-substitution. Inner acrosomal membrane is shown to be coated with a string of granules (arrows) in the funnel-shaped region. Note that these granules aligned along the inner acrosomal membrane are associated with the subacrosomal amorphous material. No such granules are detected in the shaft region (arrowhead). Actin filaments regularly arranged parallel inside the axial rod (AR) are identified. Characteristic striations of actin filament are also seen. ×105,000.
aligned granules corresponds to the specialized region where the inner acrosomal membrane was shown to be thick in thin section images (6, 7, 16). This thickening of the inner acrosomal membrane was no longer seen after the acrosome reaction. Some of these aligned granules appeared to be pointed towards the subacrosomal amorphous material. In contrast, no such granules were detected in the deeply invaginated shaft region of the inner acrosomal membrane (Fig. 2), where this membrane tightly encloses the axial rod composed of actin filament bundle.

Quick-freeze, deep-etch analysis provided the three-dimensional architecture of the membrane specialization displayed on the funnel-shaped region. In the replica fractured obliquely through the axial rod in the funnel-shaped region, short strands were identified projecting from the granules aligned along the inner acrosomal membrane (Fig. 3). These strands were found to contact with the amorphous material surrounding the axial rod. In quick-freeze, deep-etch preparation, the exposed P face of the inner acrosomal

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**Fig. 3.** Quick-freeze, deep-etch replica through the axial rod in the funnel-shaped region. Note that aligned granules (small arrows) along the inner acrosomal membrane are cross-linked to the amorphous material by short strands (arrowheads). Inner acrosomal membrane and actin filament bundle composing the axial rod are also cross-bridged by short linkages (large arrows). \( \times 140,000 \).

**Fig. 4.** Quick-freeze, deep-etch replica fractured through the P face of the inner acrosomal membrane at the funnel-shaped region. Numerous intramembrane particles are densely distributed in lattice-like or concentric circular pattern on the fractured P face (PF) of the inner acrosomal membrane. AM, amorphous material surrounding the axial rod; NM, nuclear membrane enclosing the posterior half of the axial rod. \( \times 100,000 \).

**Fig. 6.** Quick-freeze, deep-etch replica of the acrosome-reacted sperm fractured at the apical region of the acrosome. Acrosomal process (AP) is elongating through the truncated cone (TC) at the opening of the acrosomal apex. VA, vacant acrosome. \( \times 90,000 \).

**Fig. 7.** Quick-freeze, deep-etch replica of the P face of the acrosomal process membrane protruding from the opening of the vacant acrosome, showing lattice-like distribution of particles with particle-free areas. TC, truncated cone; VA, vacant acrosome. \( \times 105,000 \).
membrane revealed numerous particles about 10 nm in diameter regularly arranged in a lattice-like or a concentric circular pattern in the funnel-shaped region (Fig. 4, 5). It should be noted that this densely packed intramembrane particles were found to be distributed specifically throughout the funnel-shaped region. What is characteristic is that such membrane specialization is restricted in the funnel-shaped region of the inner

Fig. 5. Quick-freeze, deep-etch replica of the inner acrosomal membrane enclosing the anterior half of the axial rod. True surface of the inner acrosomal membrane in the shaft region, and fractured P face with numerous intramembrane particles in the funnel-shaped region are seen. ×100,000.
acrosomal membrane in unreacted sperm. From our present quick-freeze, deep-etch images, it could be speculated that the presence of the short strands interconnected with the amorphous material in the funnel-shaped region may be involved in restricting or anchoring the intramembrane particles in order to maintain regionalized distribution. In addition, the aligned granules along the inner acrosomal membrane in the funnel-shaped region may play some roles in the synchronization between the rapid elongation of the actin filament bundle and the simultaneous extension of the acrosomal process membrane, cooperating with the amorphous material.

As observed on thin section, the apex of the acrosome opens and the acrosomal contents are discharged during the acrosome reaction (6,16). Through this opening the rapid extension of the axial rod i.e. the rapid elongation of the actin filament bundle (16) occurs to form the acrosomal process (Fig. 1b), which is closely limited by the acrosomal process membrane derived from the inner acrosomal membrane.

The acrosomal process protruding from the opening of the vacant acrosome simultaneously becomes surrounded closely by a coiled filamentous structure (19,20,21), termed the truncated cone, at the apex of the vacant acrosome (Fig. 1b, 6). In quick-freeze, deep-etch replica of the acrosomal process membrane extending through the truncated cone, the distribution pattern of the intramembrane particles showed loose lattice-like or random pattern in patches (Fig. 7), as previously observed by freeze-fracture analysis (18). In some cases, patches of particle-free areas emerged in this loose lattice-like arrangement of the intramembrane particles. While the precise role of these densely packed particles in the inner acrosomal membrane remains to be elucidated, our observations using quick-freeze method suggest that these particles may be involved in the rapid extension of the acrosomal process membrane during the acrosome reaction. It should be mentioned that the acrosomal process membrane is formed simultaneously with the rapid elongation of the actin filament bundle, suggesting that the actin filament bundle may participate in regulating the membrane events.

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References


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