Cell Tissue Res (2016) 363:147–157
DOI 10.1007/s00441-015-2238-0

REVIEW

The acrosome of eutherian mammals

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Received: 30 March 2015 / Accepted: 25 May 2015 / Published online: 15 August 2015

Abstract The acrosome is not just a bag of enzymes, most of which, if not all, are singly non-essential for sperm–oocyte interaction. The Golgi-derived acrosomal cap reveals some extraordinary development and structure particularities. The acrosome of eutherian spermatozoa basically consists of two parts, the anterior and equatorial segments; the present review is devoted to the former, the initial actor in fertilization. Its occasional fanciful morphological changes during epididymal maturation are analyzed, together with its heterogeneous contents: enzymes, zona binding proteins, structural proteins (matrix) and yet to be chemically characterized crystalloids. The plasma and acrosomal membranes present stabilized ordered domains, whereas glycoprotein-free areas appear during capacitation and before fusion. Exocytosis, induced by the cumulus oophorus and/or the zona pellucida, may generally start proximally and progress anteriorly, resulting in the detachment of a hybrid membrane shroud, whose entity is probably maintained by the bound matrix. Immediately released soluble enzymes must be active during the first interactions of the gametes, whereas other lysins, bound to the matrix or stored as proenzymes, are only progressively released. Zona binding is probably achieved via the shroud and/or the IAM (depending on species). Penetration along an incurved slit through the stratified zona is allowed by the rigid and denuded head tip and flagellar hyperactivity, and assisted by the local proteolytic activity of proteasomes bound to the IAM, the unique essential zona lysin system.

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Keywords Acrosome · Anterior segment · Ordered structures · Zona binding · Zona penetration

Introduction

In the literature on secretory cell types, the germ-cells are not commonly given as an example. However, both male and female gametes generally contain vesicles or granules originating from the Golgi apparatus that include glycoproteins such as lytic enzymes, released by exocytosis, as described below. If we restrict our scope to eutherian mammals, the mature oocyte displays cortical granules under the oolemma, which are typical small secretory vesicles containing glycoproteins, as first shown at the light and electron microscope level by Yanagimachi and Chang (1961) and by Fléchon (1970), and enzymes (review by Liu 2011). Their exocytosis is induced by sperm–oocyte fusion and results in the block of polyspermy (Austin and Braden 1956), demonstrated by the fertilizability of parthenogenetically activated oocytes where cortical granules are discharged only subsequent to gamete fusion (Fléchon et al. 1975).

In male gametes, the acrosome is the only one secretory vesicle located on the anterior part of the cell. As early as the pachytene stage of meiosis, the Golgi apparatus of the spermatocyte produces secretory vesicles. Their contribution to the formation of the acrosomal cap was followed by histochemistry, such as the periodic acid–Schiff–PAS staining technique of the constitutive glycoproteins (Clermont and Leblond 1955). Similar to a lysosome, the acrosome was found to contain enzymes that were readily interpreted, by comparison with invertebrates, as agents of egg coat penetration (Yanagimachi 1994).

As recent reviews have already been devoted to evolutionary (Bedford 2014) and biochemical aspects (Buffone et al.

2014; Miles and Sutowsky 2014) of mammalian fertilization, the present synthesis will only deal with the structure of the acrosome, the distribution of its heterologous contents before and after exocytosis and their role during binding to and penetration of the zona pellucida. Also excluded are metabolic aspects such as sources of energy, role of ions, pH, etc.

General knowledge of the acrosome

Although the spermatozoa, sometimes considered one of the most specialized cell types, display common body plan and parts, defined by Fawcett (1975), they show, as illustrated by Retzius (1909), an extraordinary variety of shapes as a result of evolution. In ejaculated spermatozoa, the acrosome looks schematically like a flat cap enveloping the anterior part of the sperm head. It is limited by a single membrane called the outer acrosomal membrane (OAM) under the plasma membrane (PM) and the inner acrosomal membrane (IAM) around the nucleus, or more precisely the perinuclear theca and its anterior tip, the perforatorium, also named by analogy with invertebrates (Austin and Bishop 1958). The acrosome in eutherians basically consists in two parts, the big anterior segment (AS) and the smaller and thinner posterior segment, located in the median part of the sperm head and therefore called the equatorial segment (ES). Contrary to the cortical granules, the acrosome participates in the initial interaction of the gametes at fertilization. The acrosome reaction occurs at the start of the gamete interaction. It consists in the fusion of the PM with the OAM, initially described as a simple exocytosis of essentially proteolytic enzymes collectively termed egg coat lysins (Barros et al. 1967; Yanagimachi 1994).

Acrosomal modifications during epididymal sperm maturation

Morphological changes

Among modifications occurring in spermatozoa during epididymal transit, the reshaping of the acrosome is sometimes one of the most obvious, with exceptions. In man, where the acrosome is very thin and closely wraps the nucleus, or in *Muridae* where it is falciform, there is not much change. In paddle-shaped acrosomes of *Artiodactyla, Perissodactyla* and *Lagomorpha*, the shape of the anterior margin is modified and becomes asymmetrically bulbous; this anterior rim (AR) is clearly evident in rabbit (Bedford and Nicander 1971). The most spectacular change is epitomized by spermatozoa of the guinea pig (Fawcett and Hollenberg 1963), the musk shrew (Cooper and Bedford 1976; Phillips and Bedford 1985) and the squirrel (Breed et al. 2011). Head elongation may be the result of an evolution related to sperm competition (Tourmente et al. 2011). In cauda epididymis, guinea pig sperm heads are stacked in "rouleaux" by their large apical segments bound by unique junctional zones (paracrystalline glycocalix) and the PM and OAM are bound on the ventral side of this segment (Friend and Fawcett 1974). There is no notable morphological change during ejaculation, storage and capacitation in the female genital tract.

Segregation of intra-acrosomal material

The acrosomal vesicle, partly derived from and containing the components of, the spermatid proacrosomal granule, is already heterogeneous at the secretory phase (review by Buffone et al. 2008). During epididymal transit, a dense body (DB) appears in the ventral face of the thickened AR in paddle-shaped sperm heads (Nicander and Bane 1966). Hamster spermatozoa show a different electron density of dorsal and ventral contents of the AS (Olson et al. 1998). In the large AS of mature guinea pig spermatozoa, several compartments are visible (Fawcett 1975). The heterogeneous aspect of the materials in the AS may reflect different degrees of protein glycosylation. If we rely on phosphotungstic acid (PTA) staining at a very low pH of thin sections in a hydrophilic embedding medium, glycoproteins appear preferentially located at a short distance from the OAM and not in the area of the DBs, e.g., in rabbit sperm (Fléchon 1979). Glycoprotein staining in superficial intra-acrosomal material, using labeled lectins, was effectively observed and used as a test of acrosome reaction, on spermatozoa of several species, as described and reviewed by Marti et al. (2000). Some of these glycoproteins were indeed characterized (see below). The DBs are essentially proteinaceous material, readily extracted by pronase treatment of thin methacrylate sections (Fléchon 1975).

The origin of the ES has been followed in man from the acrosomal vesicle of round spermatids, where a segment-specific protein was detected, up to the fully differentiated spermatozoon step of spermiogenesis (Wolkowicz et al. 2003). With the PTA technique, the contents of the ES of mature rabbit sperm are no longer stained (Fléchon 1979). The thin layer of material in the ES appears striated in transverse sections, evocating a septate junction and this ordered structure is supposed to tightly bind the OAM to the IAM (Moore and Bedford 1978, Russel et al. 1980). Accordingly, these membranes display a hexagonal pattern of intramembrane particles (Fléchon et al. 1986).

Neglected aspects of acrosome structure: paracrystalline components

The significance of ordered structures in spermatozoa is generally overlooked; they are found, if we restrict the focus to the

acrosome region, in the PM, the OAM and IAM and inside the acrosomal material. They first appear during epididymal maturation, a phase of sperm cell condensation.

Plasma and acrosomal membranes: fusion problematics

The changes in the fine structure of sperm membranes were studied during in vivo or in vitro capacitation, a necessary step before gamete interaction (Yanagimachi 1994). In epididymal and ejaculated spermatozoa, an ordered distribution of intramembrane particles (IMPs) was revealed by freeze-fracture in some areas of the sperm PM, OAM and IAM in several species. Arrays of IMPs were observed in the PM overlying the AR of ram spermatozoa (Fléchon et al. 1986), the acrosome of monkey (Reger et al. 1985) and rat spermatozoa (Toyama and Nagano 1988). A localized ordered distribution of IMP was also found in several species in the OAM (Friend and Fawcett 1974; Yanagimachi and Suzuki 1985; Aguas and Pinto Da Silva 1989) and in the IAM (Koehler 1975; Huang and Yanagimachi 1985; Fléchon 1985; Olson and Winfrey 1985a).

The IMPs of the PM were characterized as transmembrane glycoproteins by freeze-fracture labeling (Aguas and Pinto Da Silva 1983). Using this technique again, the same authors (1985) found on the contrary that the OAM was relatively poor in glycoproteins. Various labeled lectins and antibody conjugates were used to respectively localize PM glucidic surface residues and follow their intramembranous glycoprotein fluidity (discussed by Yoshida et al. 2010). However, no more precise relationship with the IMP was found and all surface markers (antibodies, etc.) may also have detected binding and/or loss of molecules during the transit from epididymis to the ampulla.

Before and/or during induced acrosome reaction in several species, a redistribution of IMPs was observed in the PM overlying the acrosome, resulting in the formation of areas empty of particles (Fléchon 1985; Fléchon et al. 1986; Yanagimachi and Suzuki 1985; Aguas and Pinto Da Silva 1989). If this is not a freezing artifact, it may be related to the mobilization of glycoproteins and glycolipids (lipid rafts) already occurring during capacitation (Van Gestel et al. 2005; Khalil et al. 2006). Does the local ordered distribution of IMPs mirror the presence of crystalloids inside the acrosome (see below) or is it a sign of the probably less fusogenic ability of these domains? The PM and underlying OAM were observed to bind together in boar sperm AR with SNARE (attachment protein receptor) proteins before any fusion during in vitro capacitation (Tsai et al. 2010), although trans-SNARE complexes were found everywhere in the PM. These complexes result from loss of cholesterol, increased membrane fluidity and aggregation of proteins involved in membrane fusion (Ramalho-Santos et al. 2000). Filipin-labeled sterols are abundant in the PM over the acrosome in epididymal and ejaculated spermatozoa of the golden hamster (Toshimori et al. 1987). Glycolipids are migrating from the AR to the ES domain of pig spermatozoa before the acrosome reaction (Gadella et al. 1995), while an increase of bound sterols labeled by polymyxin B (and again formation of empty patches) was observed in the PM overlying the AS of in vitro capacitated human spermatozoa and similar results obtained in other species were discussed by Tesarik and Fléchon (1986). Many molecules were proposed to individually play a role in membrane fusion (Yoshida et al. 2010); however, only the synergy of several of them may be efficient (Jones et al. 2007). These authors also found that large lipid domains, contrary to single lipids, were not able to move outside the intra-acrosomal domain. Such a "molecular filter" between the acrosomal and postacrosomal regions evokes the limit between the basal and apical domains in epithelial secretory cells.

The initiation of membrane fusion is said to occur at multiple points but there is yet to be agreement as to where it starts and how it progresses, as it may depend on the species and the mode of induction. Natural inducers, although analyzed in vitro, are considered to be the cumulus oophorus, as described in early studies on a few but varied species, analyzed and reviewed by Siiteri et al. (1988), confirmed by Hiroashi et al. (2011) and Jin et al. (2011) and underlined by Bedford (2011) and/or the zona pellucida (Wassarman 2005), perhaps a two-step security system. In the golden hamster, the acrosome reaction is completed on the zona in a higher percentage of spermatozoa when the cumulus is present (Cherr et al. 1986). In contrast, it is well known that in livestock species the cumulus is lost soon after ovulation. In both cases, the acrosome exocytosis may be induced by the same mucopolysaccharides and proteoglycans either constituting the cumulus matrix or displayed on the surface of the zona. The deposition of this mucous layer during oocyte maturation was described and previous observations were reviewed by Fléchon et al. (2003).

In the peculiar case of the human spermatozoa, the fusion of PM and OAM was described as beginning at the anterior tip of the very thin acrosome, after induction with follicular fluid (Yudin et al. 1988) and an ionophore (Harper et al. 2008). The acrosome reaction in mouse spermatozoa was observed in vitro by confocal microscopy to start proximally when induced by tubal fluid (Yoshida et al. 2010), which is not a natural inducer of acrosomal exocytosis and distally when induced by the zona pellucida (Satouh et al. 2012). Similarly, exocytosis started randomly in mouse spermatozoa when induced by an ionophore and distally when induced by solubilized zona in vitro (Buffone et al. 2009b). In the guinea pig spermatozoa, the induced fusion starts in the huge anterior segment and progresses across the principal segment, along the branching arrays of hybrid membrane tubules, as revealed by transmission electron microscopy (Flaherty and Olson 1991). The Ca2+-ionophore treatment of ram spermatozoa initiates the acrosome reaction at the posterior limit of the

AS as observed after freeze-fracture (Fléchon 1985, Fléchon et al. 1986); fusion proceeds towards the AR as branching tubules following hexagonal patterns similar to that of the IMPs in the IAM. Ionophore-induced membrane fusion in the boar spermatozoa starts in the same place and progresses in the same direction as shown by the electron-micrographs of Topfer-Pedersen et al. (1985). In their work on induced acrosome reaction in the boar, Aguas and Pinto Da Silva (1989) conceded that it is difficult to capture the onset of fusion by freeze-fracture and they gave almost no evidence of membrane fusion into the AS. Interestingly, in these studies on ram and boar spermatozoa, fusion did not progress in the ES, or at least to a posterior part of it, particularly in boar spermatozoa and was stopped along similar arborescent tubules. The existence of a mini-ES, called the lunula, was first observed in ram spermatozoa by scanning electron microscopy (Schulte-Wrede and Wetzstein 1972); moreover, atomic force microscopy of spermatozoa undergoing epididymal maturation in *Artiodactyla* detected the appearance of a rough semi-circular surface area in the posterior part of the ES, called the equatorial subsegment (Ellis et al. 2002). After induced acrosome reaction, the subsegment persisted and exhibited anterior finger-like projections in ram spermatozoa, reminiscent of the aforementioned freeze-fracture images. The subequatorial segment is probably a general feature in eutherian spermatozoa; it may appear during epididymal maturation and becomes more evident after capacitation. The plasma and acrosomal membranes display there a high fusion ability (Jones et al. 2008).

The posterior initiation model fits well with the observed detachment, along a line of dehiscence, of the reacted AS from the head, looking like a perforated glove finger, habitually called the shroud or ghost. Along this line is also maintained the cell integrity by the fusion of the PM and OAM of the ES (Bedford et al. 1979) and the initial site of sperm–oocyte fusion following zona penetration (Yanagimachi 1994).

Acrosome crystalloids

The first electron microscope observations of an ordered structure inside the acrosome were made in rabbit and ram spermatozoa (Fléchon 1975; Courtens et al. 1976). These paracrystalline bodies (PBs), located in the AR, showed parallel striations in two crossing directions on thin sections; PBs were posteriorly apposed to the IAM. It was possible to observe the crescent form of a PB in a section parallel to the flat ram sperm head. In fact, the PBs correspond exactly to the dense bodies when observed at a sufficient resolution and their granular structure was already observed after freeze-fracture (Fléchon 1974). A periodic structure was also described in the AR of bovine spermatozoa (Olson and Winfrey 1985a, b). There is only one dense body (PB) on the ventral side of the AR of bovine spermatozoa and two, dissymmetrical ones, the larger also in the ventral side of a thicker AR and the other in the dorsal side of the rabbit spermatozoa (diagram in Fig. 1). After Triton X100 treatment of rabbit spermatozoa, the crystalloids remained associated with the acrosomal lamina, a peripheral layer of the acrosomal material (Olson and Winfrey

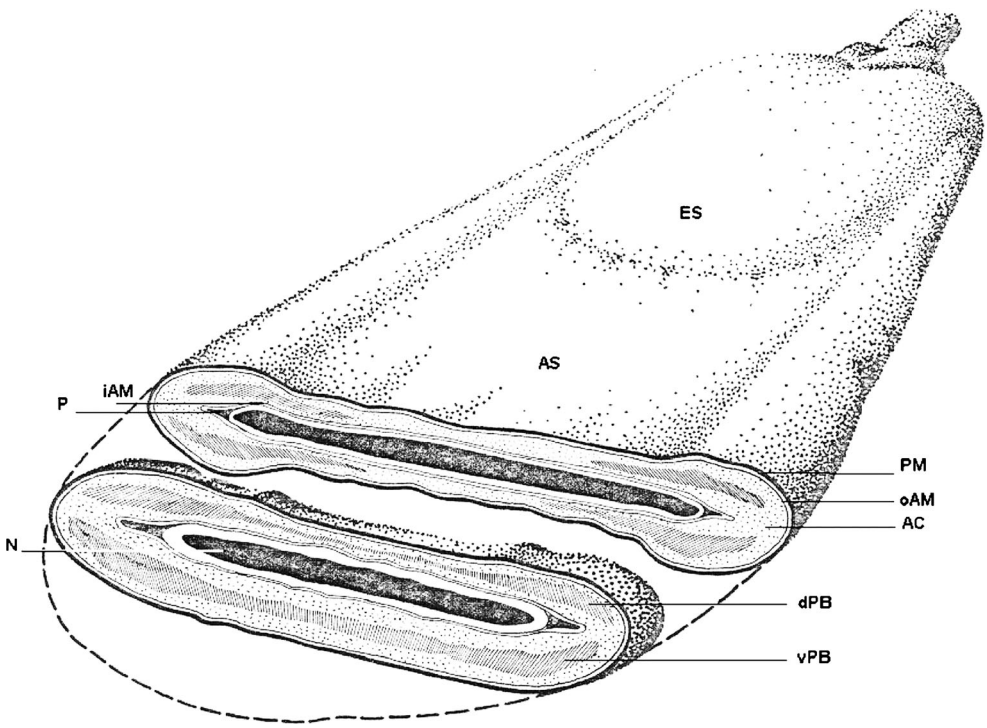

Fig. 1 Diagram of a rabbit sperm head sectioned twice transversally through the apical ridge. The nuclear envelope and the acrosomal lamina are not represented. Only one direction of striations is drawn in the PBs. *AC* acrosomal content, *AS* anterior segment, *ES* equatorial segment, *iAM* inner acrosomal membrane, *oAM* outer acrosomal membrane, *N* nucleus, *P* perforatorium, *dPB* dorsal paracrystalline body, *vPB* ventral paracrystalline body, *PM* plasma membrane

1994) and appeared as two horseshoes located on both sides of the head tip after negative staining. They were fragmented, either in their normal state or as a result of extraction. Crystalloids may also be present in falciform acrosomes (*Muridae*), as shown by a periodic structure observed in the superficial layer of the AS in rat spermatozoa (Phillips 1972).

The ordered structures may be built by condensation of acrosomal materials during epididymal maturation, as we could not detect them earlier than in the corpus epididymis of the rabbit (Fig. 2a, b). As the dense bodies are bound to the acrosomal matrix and so persist in the shrouds of acrosome-reacted spermatozoa around the zona pellucida (Kopecny and Fléchon 1987), it is suggested that PBs are aggregates of structural proteins rather than enzymes.

Olson and Winfrey (1994) described the rabbit crystalloids as made of globular subunits spaced of 7–8 nm center to center. Similarly, a repetitive interval of 6–7 nm was measured between the striations in rabbit spermatozoa in various physiological states, epididymal, ejaculated, or incubated in vitro (Fléchon, unpublished). As this interval may depend on the orientation of the crystalloids relative to the plane of the section, the dimension was examined again on micrographs obtained with a goniometer stage; the tilt was adjusted so that the electron beam was parallel successively to one and then the other crystal plane (Fig. 3a, b); the mean value obtained was around 6 nm. Optical diffraction of micrographs did not provide further information except that the angle formed by the two crystal planes was about 60°. These values may be useful to contribute to the elucidation of the composition of the PBs.

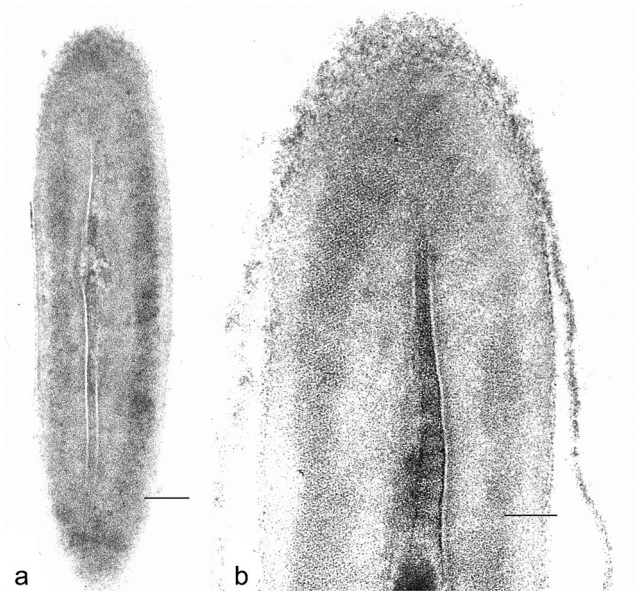

Fig. 2 PB appearance in corpus epididymal spermatozoa. **a** Regular striations of the two PBs in a transverse section of a sperm head. *Bar* 0.1 μm (×80,000). **b** High magnification of the striations in a part of a consecutive section of the same sperm head. Bar 0.1 μm (×140,000). *The PBs can be more easily observed by increasing the magnification on screen*

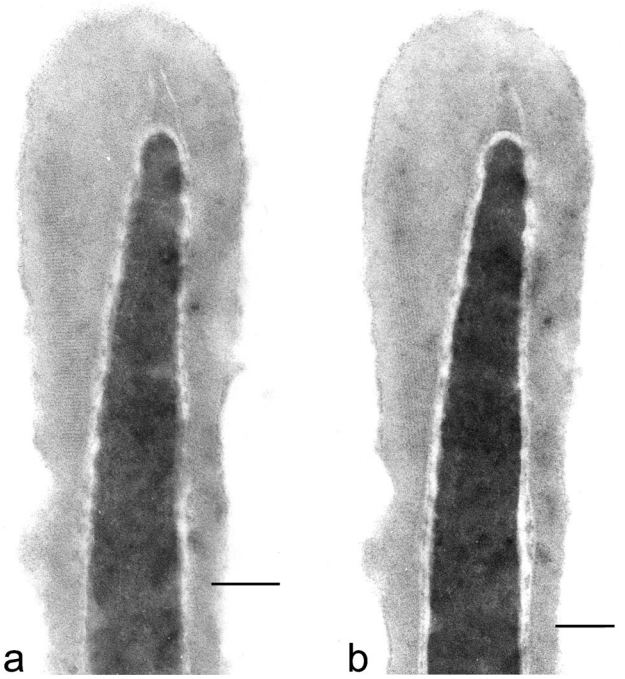

Fig. 3 Sagittal section of an ejaculated sperm head observed with the goniometer stage. *Bar* 0.1 μm (×106,000). **a** −15° inclination: alignments almost perpendicular to the sperm axis in the large PB. **b** +20° inclination: alignments almost parallel to the sperm axis in the large PB. *The PBs can be more easily observed by increasing the magnification on screen*

Is the presence of crystalloids in the AS of spermatozoa of eutherian mammals a general rule? There is a striking similarity of crescent morphology between the extracted rabbit PBs (Olson and Winfrey 1994), the isolated matrix of guinea pig sperm (Buffone et al. 2008) and the purified SDS resistant core of mouse spermatozoa (Guyonnet et al. 2014), although the acrosome shapes are different. Reciprocally, are the crystalloids part of the acrosomal matrix in the restricted meaning of structural proteins involved in the binding and stepwise release of enzymes? No zona lysin activity has been detected in the PBs thus far (see below); only binding (artifact?) of peroxidase-labeled antibodies against hyaluronidase and acrosin could be observed in images published by Gould and Bernstein (1975) and Huneau et al. (1984). A suggestion may be made about the composition of crystalloids, as amyloids would contribute to the "formation of the SDS resistant core" of the mouse spermatozoa (Guyonnet et al. 2014).

If crystalloids are composed purely of structural proteins, they may play a role in determining the shape of the acrosome, as proposed by Phillips (1972) for murine spermatozoa. The PBs may be backbones for the bulging ARs of paddle-shaped acrosomes during epididymal maturation. With the acrosomal matrix being considered as the scaffold of the AS and shroud (Buffone et al. 2008), the crystalloids may in turn be the backbone of the acrosomal matrix.

Distribution of components in the intact acrosome

The internal heterogeneous aspect of the AS corresponds to a segregation of its contents, not randomly mixed. Many compounds have been characterized by biochemical techniques (Ito et al. 2013), among them enzymes (Buffone et al. 2008; Ito et al. 2013) and zona binding proteins (Yu et al. 2006; Yoshida et al. 2010; Kim et al. 2011), some of them transported to the PM as aggregates by chaperones during capacitation (Kongmanas et al. 2014). Even if we limit the scope essentially to the quest for essential lysins, their partial characterization and localization result from a large amount of work.

Although initial studies of immunocytochemical studies suffered from lack of resolution, at least specific localization of enzymes was, as a rule, limited to the AS. Hyaluronidase, e.g., was detected in the AS in several livestock species (Fléchon and Dubois 1975). This location was confirmed by immunoelectron microscopy (Gould and Bernstein 1975). The same technique allowed precise localization of several proteins and enzymes (e.g., matrix proteins, proacrosin and a soluble enzyme) in different domains of the large AS of guinea pig spermatozoa (review by Buffone et al. 2008). As acrosin at the time was supposed to play a role in sperm–zona penetration, the sperm proteolytic activity was first analyzed by digestion of a gelatin substrate: lysis diffused around reacted acrosomes and disappeared after head denudation (Gaddum-Ross and Blandau 1977). Confirming earlier studies, Puigmule et al. (2011) observed by immunoelectron microscopy the proacrosin–acrosin complex and a metalloproteinase concentrated in the AR of in vitro capacitated boar spermatozoa, both originating from the proacrosomic granule; acrosin was also detected, almost uniformly, in the AS of epididymal bull spermatozoa, with the notable exception of the DBs, not pointed out by the authors (Ferrer et al. 2012). Equatorin, a glycoprotein bound to the acrosomal membrane of round spermatids, was later associated with the OAM (except in the apical region) and IAM on one side and the underlying acrosomal material on the other side in mouse spermatozoa (Ito et al. 2013); it is externalized during the acrosome reaction (Yoshida et al. 2010), however, its role in fertilization is yet to be determined. Other intra-acrosomal enzymes, analyzed upon exocytosis, are described below.

Acrosomal exocytosis

During acrosome reaction, some enzymes are readily released, such as hyaluronidase that is supposed to play a role during the passage through the hyaluronan-rich cumulus oophorus, although this may not be essential (Talbot et al. 1985) and the fact that this action may be affected by another (redundant) means (Nguyen et al. 2014). The mucous layer on the zona surface may be scoured by sperm hyaluronidase before binding, which is effectively inhibited by a monoclonal antibody to the cell surface hyaluronidase PH20 (Myles et al. 1987). Note that the latter (complementary) enzyme does not seem to be effective in the sperm progression through the cumulus (Baba et al. 2002).

The acrosome reaction is a peculiar exocytotic event, as it is not reversible. Moreover, although membrane fusion is almost instant (a few seconds according to Harper et al. 2008), the secretion of only part of its contents takes an unexpectedly long time (several minutes). A disulfide bond stabilized acrosomal matrix binds enzymes, e.g., proacrosin (NagDas et al. 1996). The distribution of soluble and insoluble components, as detected on micrographs of intact spermatozoa, was partially superposed (Hardy et al. 1991). The release of some enzymes is progressive as molecular changes must occur such as depolymerization or degradation of, e.g., proacrosin into acrosin and matrix proteins by acrosin itself (Kim et al. 2011; Buffone et al. 2014). Exocytosis may also depend on other enzymatic activities (Morales et al. 2003; Chakravarty et al. 2008).

An extracted acrosomal matrix was recovered with the OAM of the AS of rabbit, bull and hamster spermatozoa, termed acrosomal lamina (Olson and Winfrey 1994; Olson et al. 1997, 1998). This superficial layer of material may be composed of glycosylated proteins (Fléchon 1979). In hamster spermatozoa, Olson and Winfrey (1985b) observed a local association of ventral peripheral matrix material (acrosomal lamina), OAM areas containing regular arrays of IMPs and a sheet of longitudinal and parallel filaments of unknown nature applied on the cytoplasmic side of this membrane.

Matrix material was present in the shrouds of reacted acrosomes, both in vitro and in vivo and still contained incorporated labeled precursors of glycoproteins (Kopecny and Fléchon 1981, 1987); it is probably responsible for the integrity of the shroud, otherwise this ghost of perforated membranes would soon disintegrate. In hamster spermatozoa, two matrix proteins bound to hydrolases, one ventral and the other dorsal, appear on thin sections of different electron density and distinct from the acrosomal lamina; they derive from a common precursor in round spermatids (Olson et al. 1998). The acrosomal matrix was eventually described in spermatozoa of all species examined, including hamster (review by Olson et al. 1998), guinea pig (reviewed by Westbrook-Case et al. 1994; Buffone et al. 2008; Kim et al. 2011), mouse (Buffone et al. 2009a) and confirmed in bovine (epididymal) spermatozoa (NagDas et al. 2010). With mass spectrometry and proteomic techniques, it is now possible to identify an unexpected diversity of acrosomal proteins, although their precise location and roles may remain unknown (Byrne et al. 2012; Guyonnet et al. 2012). It was suggested that the acrosomal matrix includes several cytoskeletal components (review and own results by Zepeda-Bastida et al. 2011), although it is unusual for, e.g., F-actin to be located inside a secretory vesicle.

Unique features of the IAM in the anterior segment

The IAM of the AS has its own peculiarities; it is bound to the perinuclear theca and resistant to extraction by detergents (Huang and Yanagimachi 1985). Although the IAM plays the role of PM in the AS region after acrosome reaction, it shows arrays of IMPs and lack of fluidity (inability to fuse with the oolemma): during gamete fusion, it is incorporated into a phagocytic vesicle inside the oocyte cytoplasm (Satouh et al. 2012). The perinuclear theca contains proteins linked by -S-S- bonds (Bedford and Calvin 1974; Courtens et al. 1976), a pool of cytoskeletal proteins according to Oko and Maravei (1994). It is closely packed around the nucleus made of proline and cystein-rich -S-S- crosslinked protamines (Bedford and Calvin 1974), forming stratified sheets visible after freeze-fracture (Fléchon 1974). Thus, the denuded thin and rigid anterior part of the sperm head constitutes an indispensable tool for zona penetration. This is the basis of the "hypothesis of mechanical penetration". In this concept, the thin and rigid sperm head functions as a scythe propelled laterally and forward by the hyperactivated flagellum (reviewed by Bedford 2014). The thrust for penetration of hamster spermatozoa may be increased by the presence of the cumulus material (Drobnis et al. 1988). In this species, the spermatozoon is maintained parallel to the zona surface by the bound shroud, transpierced at its tip and relegated posteriorly; around the latter, the sperm head is free to oscillate (Cummins and Yanagimachi 1982). It is perhaps not always the case (Yanagimachi and Phillips 1984) and not the rule in other species, as discussed by Baltz et al. (1988). If the penetration was purely mechanical, it could be explained by the curved trajectory of the slit first observed by Dziuk and Dickmann (1965); the head would be able to negotiate the spongious zona surface and then, step by step, the stratified (liquid crystal) inner texture of the zona (Fléchon et al. 2004). Interestingly, zona proteins themselves contain crystalline domains (Monné et al. 2008). The structure of the zona may be important for binding (Dean 2004) but also for penetration (Mugnier et al. 2009). The texture of the generally thick egg coat of eutherian mammals, comparable to that of a tennis ball, may explain why it is flexible but not easily perforated perpendicularly (Green 1987), as invertebrate spermatozoa do in their respective egg coats.

To demonstrate how acrosin or other proteases could contribute to sperm penetration through the zona, some contents of the AS should remain on or bind to the surface of the IAM after the acrosome reaction; however, no trace of radioactively labeled glycoproteins was found on denuded sperm heads in the perivitelline space of oocytes fertilized in vivo, except around the ES (Kopecny and Fléchon 1987). Thus, only a very low amount of labeled material, if any, may remain on the IAM. In spite of contradictory results, acrosin is probably not retained on the IAM after acrosome reaction (Schams-Borhan et al. 1979). Acrosin alone is not able to dissolve the zona (Dunbar et al. 1985) and *Acr* gene knockout did not prevent fertilization (Baba et al. 1994), whereas it may at least contribute to the disaggregation of the matrix (see above).

As no alternative protease with zona lysine ability was proposed at that time, the question is now what would be the indispensable enzyme for zona lysis if the mechanical zona penetration is necessary (Bedford 2014) but insufficient alone? The criteria for such a lysin would be:

- presence in (or on) the acrosome
- binding to the IAM after acrosomal exocytosis
- no zona binding ability per se and, adversely, aptitude to loosen spermatozoa bound to the zona
- ability to dissolve the zona or at least to break bonds between some zona glycoprotein molecules (this would explain why the slit cannot close again after sperm penetration)
- no diffusibility from the IAM, as the slit is typically not enlarged during and after sperm passage.

Most of these criteria have been met as a result of a long series of experiments, made essentially with the pig as a model, the mouse being a peculiar species in this field and others (Sutovsky 2011). The boar sperm acrosome carries active proteasomes on its surface (Sutovsky et al. 2004; Yi et al. 2007a, b) able to digest solubilized ubiquitinated sperm species-specific receptors of the zona; inversely, isolated sperm proteasomes loosen the zona and consequently detach bound spermatozoa (Zimmerman et al. 2011). After the acrosome reaction, at least some proteasomes remain bound to the IAM (Yi et al. 2010). The same mechanism exists in other species of eutherians where the egg coat penetration is also blocked by antibodies to proteasomes and by proteasomal inhibitors, and in Prochordates (e.g., Sawada et al. 2002), although their egg coats are very different (reviewed by Miles and Sutowsky 2014).

Although most individually knocked out zona lysins seem dispensible alone, Redgrove et al. (2011) described complexes of zona binding proteins on the sperm surface, prominently featuring proteasomes and chaperones for externalization that may participate in the binding to and local lysis of the zona surface (reviewed by Miles and Sutowsky 2014). As already suggested by Yanagimachi (1994), bound sperm heads may effectively appear trapped, embedded in the spongiform superficial layer of the egg coat (Yanagimachi and Phillips 1984; Jedlicki and Barros 1985; Fléchon 1987) or ploughing their own bed (Sutovsky et al. 2004). The proteasomes may degrade the zona binding proteins eventually exposed to the IAM (detected on isolated AS of rat spermatozoa by Yi et al. 2007a, b) before zona penetration to allow free progression of the sperm. Another possibility is the removal of sugars from the ligand zona glycoproteins (Sutovsky P, personal

communication). In fact, the already mentioned mucous matrix deposited on the zona pellucida surface may be another neglected piece of gamete interaction; it may be the superficial layer where ubiquitinated proteins are located (Sutovsky et al. 2004), or it is the peripheral stratum of the zona, also secreted by the cumulus cells (Kolle et al. 1996). The role of the cumulus may be confirmed by the detection of ubiquitin in the follicular fluid (Einspanier et al. 1993). Finally, a unique proteasomal subunit may recognize mannose-rich sugar residues (Yoshida et al. 2002) and binding of a mannose ligand on the zona by a human sperm surface specific receptor (Benoff et al. 1997) may be one more mechanism of induced acrosome reaction.

Finally, what about spermatozoa recovered from the perivitelline space and able to cross the zona again (Kuzan et al. 1984; Inoue et al. 2011)? Do they maintain the necessary complement of enzymes on the IAM or ES? Both of those sperm head elements appear paradoxically inert, whereas they may in fact play essential roles respectively in zona penetration and gamete fusion.

Conclusion

Contradictory ideas sometimes last about the structure of the acrosome and its role in gamete interaction of eutherian mammals. The present review provides an opportunity to underline a few personal views of old overlooked and new breaking-through facts on the subject.

The acrosomal membranes and the overlying PM present regionally ordered arrays of IMPs, which may correspond to relatively stable domains, whereas the appearance of IMP free areas during capacitation is probably related to the formation of lipid rafts preliminary to exocytosis.

The acrosome reaction may be induced by the cumulus oophorus and ultimately by the zona pellucida, depending on the species. Cumulus-enclosed oocytes may in some cases give a better rate of in vitro fertilization. Membrane fusion may start, with exceptions, at the posterior limit of the AS and progresses anteriorly along hybrid membrane tubules, branching in directions following, at least under favorable conditions of observation, the hexagonal IMP arrays of acrosomal membranes.

The perforated acrosomal ghost detaches from the ES, is pierced by the perforatorium in rodents and may remain around a posterior part of the sperm head, bound to the zona. This way, the sperm head is maintained parallel to the zona surface. Consecutively or alternatively, when the ghost is lost, the sperm head may be tethered to the egg coat by its IAM and in this case binding proteins have to be removed before penetration. In both cases, the sperm head is digging a small bed in the superficial layer of the zona, made of mucopolysaccharides and glycoproteins.

The disulfide bonds in sperm cells constitute a general system to reinforce the cohesion and rigidity of elements as different as the nuclear material, the perinuclear theca and the acrosomal matrix; the last is a part of the acrosomal material, not readily soluble after exocytosis. It contains various enzymes and structural proteins bound to and giving its cohesion, via a distinct acrosomal lamina, to the hybrid membranes of the reacted acrosome.

The ultimately well-designated perforatorium and other hardened head parts are necessary to perforate, along a curved parabolic slit, the successive layers of the thick stratified zona. It seems that the primitive mode of perforation of the egg coat with zona lysins, operating in lower class animals, survived in eutherians in order to ease the mechanical penetration. Although most of the acrosomal proteins (enzymes, zona adhesins, etc.) may not appear essential according to single gene knock-out studies in mouse, proteasomes bound to the non fusogenic IAM would do the job.

Protein crystalloids of an as yet unknown nature were observed in the AS of spermatozoa in several species, assembled not later than during epididymal maturation; they may be the backbone of the acrosomal matrix scaffold.

Seemingly redundant and/or alternative mechanisms are at work at each step of sperm–oocyte interplay (way through the cumulus oophorus, initial interaction with the mucous layer and/or the surface of the zona, sperm binding to the egg coat) in order to increase the chances of fertilization. Sometimes, opposite views are revealed to be complementary.

The ES, due to the paracrystalline structure of its contents and of its membranes, is not involved in acrosomal exocytosis. Nevertheless, its hybrid membrane domain (PM/OAM) is most probably the site of gamete fusion but this is out of our scope.

Acknowledgments I thank Mike Bedford for his initial help and encouragement, Peter Sutovsky for inviting me to contribute to this special issue and for advice in the elaboration of the manuscript and Ms Kathy Craighead for manuscript editing.

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