Interactions of Sperm Perinuclear Theca With the Oocyte: Implications for Oocyte Activation, Anti-Polyspermy Defense, and Assisted Reproduction

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ABSTRACT Perinuclear theca (PT) is the cytoskeletal coat of mammalian sperm nucleus that is removed from the sperm head at fertilization. PT harbors the sperm borne, oocyte-activating factor (SOAF), a yet-to-be-characterized substance responsible for triggering the signaling cascade of oocyte activation, thought to be dependent on intra-oocyte calcium release. The present article reviews the current knowledge on the biogenesis and molecular composition of sperm PT. Possible functions of sperm PT during natural and assisted fertilization, and in the initiation of embryonic development are discussed. Furthermore, evidence is provided that SOAF is transferred from the sperm PT to oocyte cytoplasm through the internalization and rapid solubilization of the post-acrosomal PT. It is shown that during natural fertilization the sperm PT dissolves in the oocyte cytoplasm concomitantly with sperm nuclear decondensation and the initiation of pronuclear development. SOAF activity is preserved in the differentially extracted sperm heads only if the integrity of PT is maintained. After intracytoplasmic sperm injection (ICSI), activation occurs only in those oocytes in which the injected spermatozoon displays complete or partial dissolution of PT. In the latter case, the residual PT of the sub-acrosomal and/or post-acrosomal sperm region may persist on the apical surface of the sperm nucleus/male pronucleus and may cause a delay or arrest of zygotic development. We propose that the sperm PT harbors SOAF in the post-acrosomal sheath, as this is the first part of the sperm cytosol to enter the oocyte cytoplasm and its disassembly appears sufficient to initiate the early events of oocyte activation. Dissolution of the sub-acrosomal part of the PT, on the other hand, appears necessary to insure complete DNA decondensation in the internalized sperm nucleus and initiate DNA synthesis of both pronuclei. The release of the SOAF from the sperm head into oocyte cytoplasm at fertilization ultimately leads to the activation of oocyte mechanism including the completion of the meiotic cell cycle, pronuclear development and anti-polyspermy defense. *Microsc. Res. Tech. 61:362–378, 2003.*

However, for the semen to exert its effect on the eggs, it must enter their little bodies, as it seems unlikely that it could animate them by the mere contact with their skin.
—L. Spalanzani: Dissertazioni di Fisica Animale e Vegetabile, Vol. II, Modena, 1780 (as cited by Monroy, 1965)

INTRODUCTION

The early observations of Abbé Spalanzani (1780) withstood the test of time: Modern developmental biology embraces an opinion that animal fertilization involves complex interactions between the components of the spermatozoon and the oocyte that reach beyond the initial binding and fusion of their respective plasma membranes. Despite the ancient wisdom and modern knowledge, popular science propels the myth that the only function of the spermatozoon during fertilization is to provide a vehicle for the paternal half of the zygotic chromosomes. This misconception is embodied by an excerpt from *The Evolution of Life* (edited by Gamlin and Vines, 1987), affirming that "*in the majority of sexual species, only the female contributes energy and resources to the young,*" while "*the males rarely contribute more than the minimum, a tiny sperm carrying genes, but devoid of other resources.*" Modern developmental studies contradict this overly generalized notion of "inferior paternal contribution." Paternally contributed zygotic centrosome was first observed by Boveri (1901), and later confirmed by fertilization studies in mammals and invertebrates (e.g., Schatten et al., 1986). Paternal, sperm mitochondria are eliminated at fertilization in mammals, including humans

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(Sutovsky et al., 1999), yet a recent case (Schwartz and Vissing, 2002) indicates that they could be propagated under pathological conditions. The sperm head capsule, perinuclear theca (PT), which is the subject of this hybrid research/review article, dissolves in the oocyte cytoplasm at fertilization (Sutovsky et al., 1997a), but at the same time contributes important signaling molecules that trigger oocyte activation and embryo development (Kimura et al., 1998; Perry et al., 1999b, 2000). The purpose of the present study is to review the evidence that supports the existence of such molecules in the sperm PT, with particular emphasis on their ability to trigger oocyte activation and anti-polyspermy defense. In addition, original, unpublished data are presented in support of the diverse roles of PT in spermatogenesis, fertilization, and early embryo development.

STRUCTURE OF THE MAMMALIAN SPERM PERINUCLEAR THECA

Perinuclear theca, also called perinuclear substance or perinuclear matrix (Bellvé et al., 1992; Courtens et al., 1976; Lalli and Clermont, 1981; Oko and Clermont, 1988), is an untraditional cytoskeletal capsule that conceals the sperm nucleus. PT is assembled during spermiogenesis between the plasma membrane (PM) and the nuclear envelope (NE), effectively filling in most of the available cytoplasmic space in the sperm head. Structurally continuous but distinct segments of the PT occupy three major regions of mammalian sperm head: (1) The PT of the sub-acrosomal region (SAR), intercalated between the inner acrosomal membrane (IAM) and the nuclear envelope. (2) The PT overlying the equatorial segment (ES), between the PM and outer acrosomal membrane (OAM). (3) The PT of the post-acrosomal sheath (PAS) region extending distally from ES, between the PM and NE. Ultrastructural analysis of epididymal sperm or spermatids, perfusion fixed in situ, shows a close contact between the PT and sperm membranes it contacts. Sonnication-disruption studies show the IAM is especially tightly bound to the PT (Oko and Maravei, 1995). It has been suggested that a structural interaction exists between sperm nuclear lamina and PT (Moss et al., 1993) but this has never been experimentally substantiated. As discussed below, PT participates in assembling the acrosome and shaping of the sperm head during spermiogenesis. It provides a rigid protective shield for the sperm nucleus during sperm maturation and fertilization and contributes signaling molecules that may be important for the triggering of anti-polyspermy defense, oocyte activation, and early embryonic development.

MOLECULAR COMPOSITION OF THE PT

A group of distinct SDS-insoluble PT proteins making up the major portion of the PT have been isolated by differential extraction of the sperm head and resolved by SDS-PAGE (Fig. 1). Such PT extracts were composed of six prominent proteins of 15, 25, 28, 32, 36, and 60 kDa, all of which were previously immunolocalized to the PT (Oko and Maravei, 1994). Many other less prominent bands were observed, also presumably belonging to the PT because successive boosts of rabbit polyclonal immune serum raised against the whole isolated PT fraction (Fig 1B) exclusively labeled

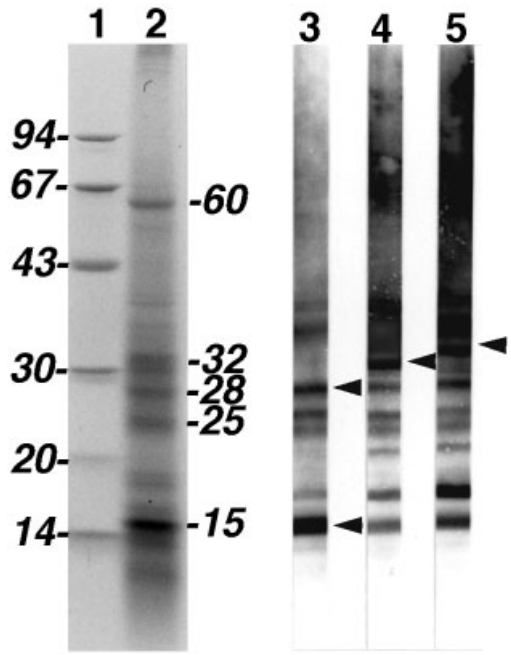

Fig. 1. Composition of bull PT extracts. SDS-PAGE of bull sperm PT extract shows the major SDS-insoluble PT polypeptides stained with Coomassie blue (**lane 2**) and compared to molecular mass standards (**lane 1**). Note the predominant bands of 15, 25, 28, 32, and 60 kDa. Preparative western blot strips (**lanes 3–5**) of electrophoresis-separated PT extract incubated with three successive immunization boosts the of PT-specific antibody pAb 427. Immune serum from the first boost (lane 3) stains predominantly PT 15 (SubH2Bv) and related PT 28. Second boost (lane 4) displays high affinity to PT 32 and third boost (lane 5) stains strongly, amongst other bands, PT 36.

the PT in sperm prepared for immunocytochemistry (Oko and Maravei, 1994; Sutovsky et. al., 1997a). The transferred proteins immuno-reacted with varying intensities depending on which boost of this immune serum was used. With the first, second, and third boosts the 15- and 28-, 32- and 36-kDa bands, respectively, were the most reactive of the major PT proteins (Fig. 1). The most prominent PT polypeptides as well as several less prominent ones have been sequence identified and are being screened for their biological activities pertinent to oocyte activation, triggering of anti-polyspermy-defense mechanisms and early embryonic development. Three classes of proteins can be distinguished in the mammalian PT so far. They include structural, histone, and signaling proteins.

The structural proteins of the mature sperm PT have on the whole been found to be sperm-specific and very basic (PI >10) and include calicin (Longo et al., 1987; Olson and Winfrey, 1988; Von Bulow et al., 1995), and cylicin I and II (Hess et al., 1993, 1995). Calicin has three consecutive repeating units, called kelch elements, that were first described in the 74-kDa Drosophila kelch-protein and found to associate with actin-rich intracellular bridges connecting the oocyte and the nurse cells (Xue and Cooley, 1993). This sequence similarity led to studies showing that calicin interacts with F-actin in both the developing sub-acrosomal and post-acrosomal regions, suggesting that F-actin mediated

targeting of calicin, which forms polymers in vitro, provides rigidity to the PT (Lecuyer et. al., 2000). The developmental localization of calicin within these two regions of the PT agrees with the localization studies of Oko and Maravei (1994) and Oko et al. (2001), which found calicin (PT60) to be localized throughout the PT of mature spermatozoa, and not just confined to the "calyx" or post-acrosomal sheath as initially shown (Longo et al., 1987). It is important to note that F-actin is depolymerized in the PT region at the end of spermiogenesis and is no longer a prominent component of the mature PT. Additional actin-related proteins Arp-T1 and Arp-T2 were recently described in sub-acrosomal PT (Heid et al., 2002).

Histone-like proteins include the sub-acrosomal histone H2B variant (SubH2Bv; Aul and Oko, 2002), designated as PT15 in earlier work (Oko and Maravei, 1995), and a group of core somatic histones found concentrated in the PT (Tovich and Oko, 2001). These histones can be considered structural proteins, yet at the same time, they could provide a rapid source of histone molecules for the early stages of male pronuclear development after fertilization (Sutovsky and Oko, unpublished data). PERF15, related to the superfamily of lipophilic transport proteins, is the major protein of the sub-acrosomal PT in falciform-shaped sperm of murids and probably plays a filler role in the greatly expanded sub-acrosomal region (Oko and Morales, 1994; Pouresmaeili et al., 1997; Breed et al., 2000). Unlike, PERF 15, SubH2Bv is present in both falciform- and spatulate-shaped sperm heads and, therefore, is likely a more fundamental component of mammalian sperm (Aul and Oko, 2002).

Signaling molecules detected in the sperm PT include transcription factor Stat 4 and two proteins from the signaling pathway of the Src-family tyrosine kinase c-Yes, including PT32 and c-Yes itself. Stat4 immunoreactivity can be detected in mouse spermatids and while it seems to disappear from fully differentiated spermatozoa, immuno-reactive protein can be isolated by DTT-extraction (Herrada and Wolgemuth, 1997). Stat4 could be contributed to the oocyte cytoplasm at fertilization and be utilized by the zygote to orchestrate early embryonic transcription (Herrada and Wolgemuth, 1997). Compelling evidence in support of such function has not yet been offered. PT 32 is a testis-specific gene product bearing close homology to WW-domain Binding Protein 2 (WBP2; Wu et al., 2001), a signal transduction protein involved in the c-Yes tyrosine-kinase activation pathway (Chen and Sudol, 1995). PT 32 is present exclusively in the PAS and inserted into PT at a late stage of spermiogenesis (Sutovsky et al., 2002), coinciding with the acquisition of oocyte activating ability by the elongating spermatids (Sofikitis et al., 1997). As discussed in the following, PT32 is a candidate component of the PT-harbored, sperm-borne, oocyte activating factor (SOAF; Kimura et al., 1998). Tyrosine kinase c-Yes, a putative target of PT32 after fertilization, is present in mammalian oocytes (Sutovsky and Oko, unpublished data). It was also found in the in the sperm head PT (Leclerc and Goupil, 2002), where it is separated from the PT32 by being restricted to the sub-acrosomal and outer peri-acrosomal regions, but not the post-acrosomal region where PT32 is found. Rather than being involved in oocyte activation, the sperm-borne c-Yes may participate in sperm capacitation (Leclerc and Goupil, 2002) and/or acrosome reaction (Sutovsky and Oko, unpublished data). Calmodulin binding protein p30 is associated with the bull sperm PT and may play a role in calcium signaling during sperm capacitiation (Leclerc and Goupil, 2000). D'Cruz et al. (2001) described several components of JAK/STAT kinase pathway in the apex of human sperm head, but did not provide definitive localization in the PT.

BIOGENESIS OF THE PT

The PT is thought to be a major contributor to the formation and shaping of the sperm head during spermiogenesis (Oko and Clermont, 1998) beginning with the biogenesis of the acrosomal granule. Immunogold-labelling of sperm at the ultrastructural level has revealed that PT proteins are assembled during spermiogenesis either in peripheral association with the developing acrosomic vesicle as it attaches to and caps the apical half of the spermatid nucleus to form the sub-acrosomal layer of the PT or in the wake of the descending microtubular manchette (during spermatid elongation) to form the post-acrosomal sheath of the PT (Oko, 1995, Oko and Maravei, 1995). Proteins that are found throughout the PT appear to utilize both patterns of assembly, with the result that the sub-acrosomal and post-acrosomal regions are structurally continuous with each other. PT15 or SubH2Bv is an example of a protein that is mainly sub-acrosomal (Aul and Oko, 2002) while PT32 is post-acrosomal (Wu et al., 2001). Calicin or PT60, on the other hand, appears to be shared between the two regions of the PT (Oko et al., 2001). No doubt, assembly of the post-acrosomal sheath involves an orchestrated interaction of PT proteins with the microtubule-based transporting machinery of the manchette (Longo and Cook, 1991; Kierszenbaum, 2002).

FATE OF THE SPERM PT AFTER FERTILIZATION

Although the PT plays a vital role in spermiogenesis (Oko and Clermont, 1998; Oko and Maravei, 1995), it would become an obstacle for normal pronuclear development after fertilization, when the sperm chromatin undergoes the transformation into a male pronucleus (PN; reviewed by Sutovsky and Schatten, 2000). This sperm-nuclear remodeling depends upon the activity of the oocyte-produced cytoplasmic factors including the histones (Ecklund and Levine, 1975; Kopecny and Pavlok, 1975) and glutathione (Perreault et al., 1984, 1988; Sutovsky and Schatten, 1997), and requires the unrestricted access of such oocyte-derived molecules to the sperm chromatin (reviewed by Poccia and Collas, 1996). Accordingly, the PT is removed from the sperm nucleus prior to the induction of nuclear swelling (Sutovsky et al., 1997a; this study). PT-removal is also likely to precede the reconstitution of a new nuclear envelope with functional nuclear pore complexes (NPC) on the surface of sperm chromatin, and the subsequent initiation of nucleo-cytoplasmic transport (Sutovsky et al., 1998). The first studies focusing on the role of sperm PT after spermiogenesis demonstrated that PT dissolves in the oocyte cytoplasm very early during fertilization, and that this dissolution coincides with

oocyte activation (Sutovsky et al., 1997a). The same study provided further support for the role of oocyte microvilli in sperm incorporation. These observations are consistent with previous studies showing that calicin and the post-acrosomal sheath (PAS) disappears from human sperm nuclei shortly after fertilization of the zona-free hamster eggs (Courtot and Lin-Tong, 1988; Paranko and Salonen, 1995). Depending upon the cell cycle stage of the oocytes, the appearance of the disintegrating PAS components is variable (Usui, 1996). The disappearance of the PT is immediately followed by the reconstitution of the nuclear envelope (Sutovsky et al., 1998; Usui et al., 1997). Further studies in mouse established the evidence that the sperm PT harbors the sperm borne, oocyte activating factor (SOAF; Kimura et al., 1998; Perry et al., 1999b, 2000).

PT of the Sub-Acrosomal Region (SAR-PT)

Considering its composition (see above), the SAR region of PT appears to be more rigid than the post-acrosomal sheath. Accordingly, this region seems to remain intact for a relatively long time after fertilization. Early ultrastructural studies in rodents pointed out that the complex of inner acrosomal membrane and SAR-PT, then called "perforatorium," appeared to persist in oocyte cytoplasm for several cell cycles after fertilization (Szöllösi, 1976). The persistence of SAR-PT was illustrated by studies of primate zygotes conceived by intracytoplasmic sperm injection (ICSI), in some of which it seemed to interfere with male pronuclear development (Sutovsky et al., 1996) and coincided with the delayed replication of DNA, a developmental block synchronized between the male and female pronuclei (Hewitson et al., 1999; Ramalho-Santos et al., 2001).

Our new data (Fig. 2) provide a detailed account of SAR-PT fate after in vitro fertilization of bovine oocytes. Removal and incorporation of sperm PT at fertilization were monitored by the labeling of the fertilized oocytes with the PT-specific antibody pAb 427 (see Fig. 1.). Reconstitution of the nuclear envelopes (NE) on the developing male and female pronuclei was visualized by adding the nuclear pore complex (NPC) specific antibody mAb 414. DNA-specific UV dye DAPI, and the pre-insemination tagging of the sperm tails with MitoTracker Green FM (Sutovsky et al., 1996b) complemented the above detection tools and allowed to distinguish between the male and the female pronuclei. With the exception of DAPI-stained maternal chromosomes, the unfertilized, metaphase II-arrested oocytes displayed neither of the above immunofluorescence labelings (Fig. 2A). The intact PT with prominent SAR labeling was found on the surface of oocyte-bound spermatozoa at an early stage of fertilization (Fig. 2B) and the PT-labeling acquired a fuzzy appearance in the spermatozoa undergoing incorporation into the oocyte cytoplasm (Fig. 2C,D). Clumps of the PT-derived material were found next to the incorporated sperm nuclei when the new nuclear envelope, delineated by the mAb 414-positive NPCs, assembled on their surface (Fig. 2E). Such clumps of PT-material were seen near the male pronucleus throughout the initial stages of pronuclear development, during which the female chromatin completed the second meiotic division and the oocytes extruded the second polar bodies (Fig. 2F–H). Remnants of the PT were last seen in the zygotes in which the developing male and female pronuclei entered the process of pronuclear apposition (Fig. 2I). No PT-derived material was detected in the zygotes reaching full pronuclear size (Fig. 2J,K) and apposition (Fig. 2L,M), or in the spontaneously activated parthenogenetic oocytes (Fig. 2N). Such spontaneous parthenogenotes also displayed aberrant patterns of NPC assembly (Fig. 2N). Rapid solubilization of the PAS-PT protein species recognized by P427 antibody supports our hypothesis that PT harbors molecular precursors necessary for zygotic nuclear remodeling. It is yet to be seen whether the putative proteins of SAR-PT and/or PAS-PT are directly trans-located into the pronuclei and become incorporated into nuclear matrix.

PT of the Equatorial Segment (ES-PT)

Mammalian sperm-oocyte fusion is initiated at the equatorial region of the sperm head (Bedford et al., 1979; Yanagimachi, 1994). Structural integrity of the equatorial plasma membrane and its functional competency to participate in the fusion process may be dependent on the presence of the outer PT leaf in the equatorial region immediately underlying the plasma membrane. It has been shown that the PT of the ES may undergo structural changes during capacitation and acrosome reaction, which may enhance the fusibility of the overlying plasma membrane (de Lourdes Juarez-Mosqueda and Mujica, 1999; Ellis et al., 2002). Molecular changes and functional maturity of the equatorial plasma membrane after acrosome reaction and zona penetration are also reflected by the unmasking of the posterior acrosomal content, rich in MN9-equatorin, on the surface of the plasma membrane (Manandhar and Toshimori, 2001).

After zona penetration, the process of sperm incorporation occurs very rapidly and thus is seldom documented by electron microscopy. We succeeded to capture the interactions between ES-PT and the oocyte cortex/cytoplasm in several specimens. During sperm-oolemma binding, the oocyte microvilli seemed to bind to the sperm plasma membrane and eventually attach to the sperm PT (Fig. 3A–E). To further confirm the observations on PT-oolemma binding, bull spermatozoa were demembranated with 0.05% lysophosphatidilcholine (lysolecithin) to expose their PT and co-cultured for 4 hours with zona-free oocytes. This treatment resulted in a strong binding of oocyte microvilli to the exposed PT of such demembranated spermatozoa (Fig. 3F–H).

Binding of PT to oolemma was also observed during sperm incorporation into oocyte cytoplasm: The oocyte microvilli remained attached to the innermost leaf of PT in the ES, while the outer layers of ES-PT became detached from the sperm nucleus and partially dissolved in the cytoplasm (see Fig. 4).

PT of the Post-Acrosomal Sheath (PAS-PT)

After sperm-oocyte fusion, the post-acrosomal PT is exposed to the oocyte cytoplasm ahead of any other sperm structure. The inner acrosomal membrane and the sub-acrosomal PT are engulfed in a manner reminiscent of phagocytosis and enclosed within reverted oocyte plasma membrane (Bedford et al., 1979), creating a structure reminiscent of an endocytotic vacuole.

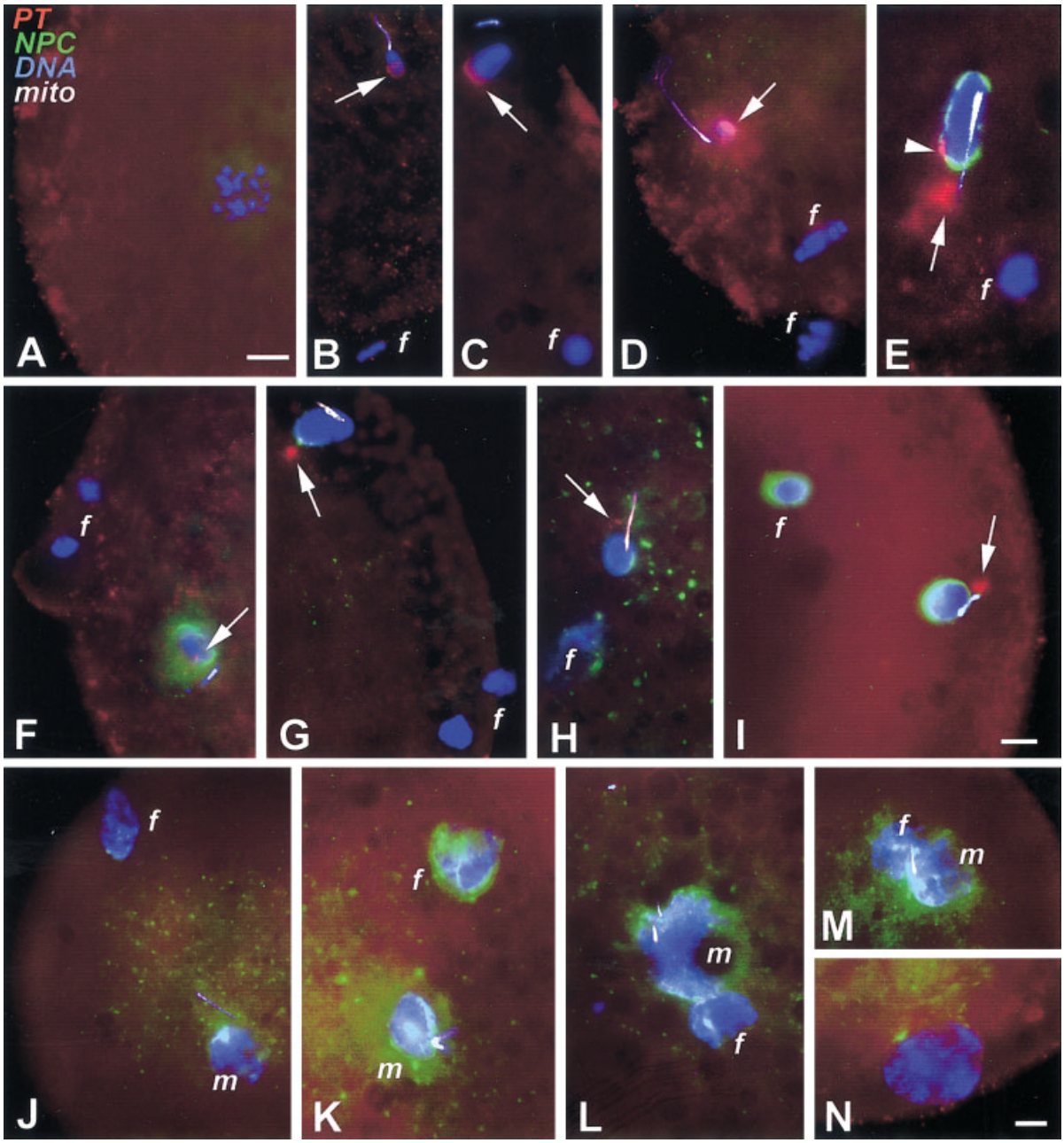

Fig. 2. Incorporation and dissolution of the sperm perinuclear theca in the cytoplasm of bovine oocytes at fertilization. Bovine oocytes were fertilized with the MitoTracker Green FM-tagged spermatozoa (white; sperm tail mitochondria) and processed for indirect immunofluorescence with a PT-specific antibody pAb 427 (red), the nuclear pore complex-specific antibody mAb 414 (green), and the DNA stain DAPI (blue). **A:** An unfertilized, metaphase-II-arrested oocyte shows no labeling with either antibody. **B–D:** The removal of sperm PT (arrows) from the surface of sperm nucleus during sperm incorporation into oocyte cytoplasm. Concomitantly with this process, the female chromosomes (*f*) complete the second meiosis. **E:** An early stage male pronucleus with a continuous ring of nuclear pores marking the presence of a newly reconstituted nuclear envelope, and a clump of PT-derived material in the adjacent cytoplasm (arrow). Note a small stretch of PT (arrowhead) still attached to the male pronucleus, and the nascent female pronucleus (*f*) that is still devoid of nuclear pores at this early stage of pronuclear development. **F:** Reconstitution of the nuclear envelope, complete with nuclear pore complexes, on the surface of a developing male pronucleus. This pronucleus overlaps with a clump of PT-derived material in the cytoplasm (arrow). Female chromatin completed second meiosis marked by the presence of the second polar body (*f;* bottom) and a small nascent female pronucleus (*f;* top). **G–I:** Dissolution of the PT (arrows) in the cytoplasm of fertilized bovine oocytes. Similar to their male counterparts, the female pronuclei (*f*) acquire the nuclear envelope and nuclear pore complexes at this stage of pronuclear development. The sperm PT is not detectable in the zygotes containing large, nonapposed (**J–K**) or apposed (**L,M**) male (*m*) and female (*f*) pronuclei. Note the abundance of the NPC-containing annulate lamellae (green) in the cytoplasm of these zygotes. **N:** A spontaneous parthenogenote displaying aberrant assembly of NPCs and AL on its nucleus and in its cytoplasm, respectively. Scale bars = 10 μm.

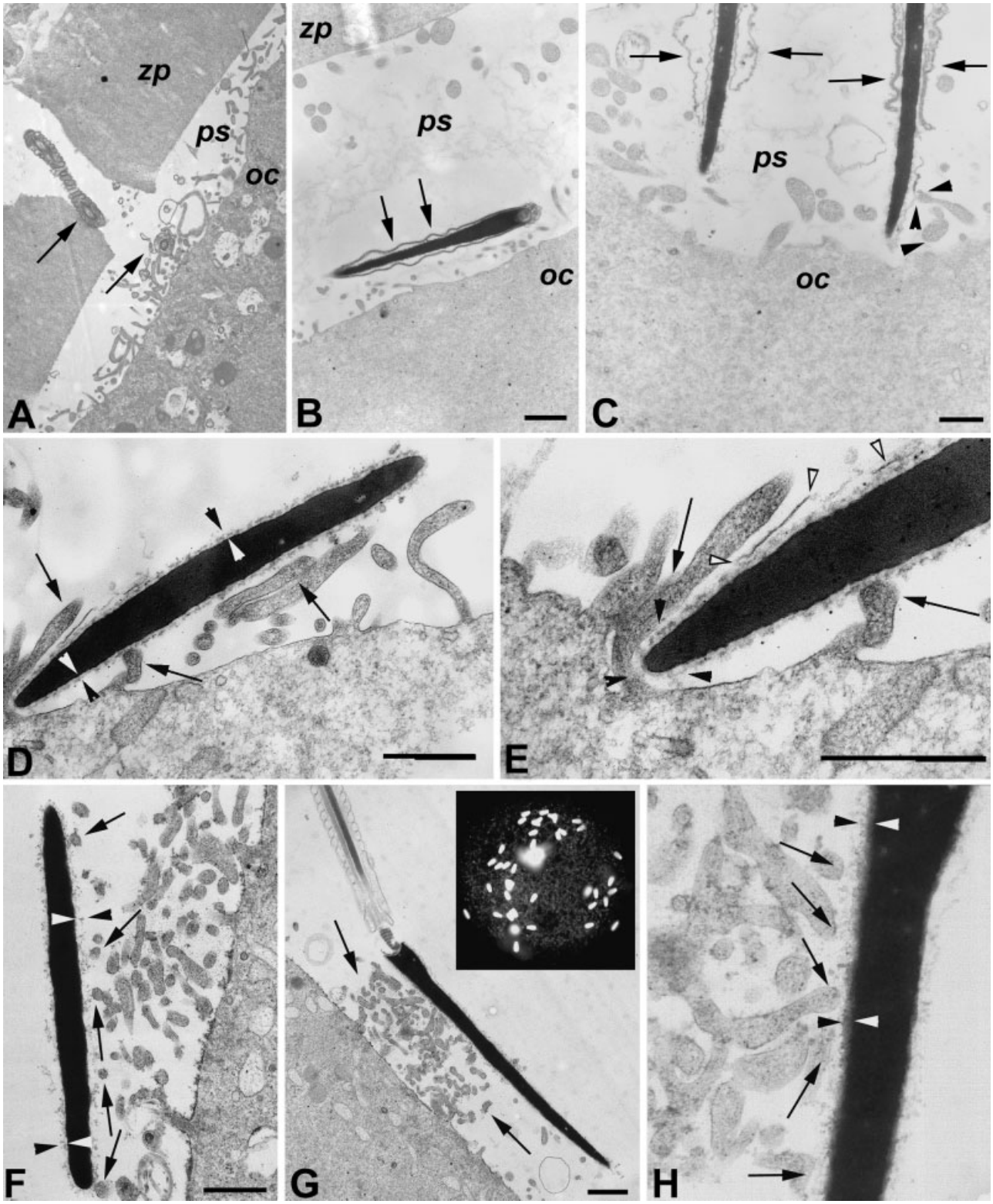

Fig. 3. Early interactions of sperm PT with the oocyte surface during bovine in vitro fertilization. **A:** The penetration of zona pellucida (*zp*) and cross-sections of sperm flagellum (arrows) are seen in the zona-penetration path and in the perivitelline space (*ps*), adjacent to oocyte cytoplasm (*oc*). **B:** A supernumerary spermatozoon, bound perpendicularly to the oolemma of a fertilized oocyte. Note the undulation of the sperm PT (arrows), probably caused by the changes of the PT resulting from acrosome reaction, zona penetration and oolemma binding. **C:** Two sperm heads in the perivitelline space, prevented from incorporation into oocyte cytoplasm by the anti-polyspermy block. Note the undulation and swelling of the equatorial segment (arrows). The spermatozoon on the right side has a more intact ES than the one on the left side. Oocyte microvilli (arrowheads) are attached to the inner acrosomal membrane adjacent to SAR-PT. Sperm-oolemma interactions are illustrated by a cross-section of the acrosomal part of an acrosome-reacted bull sperm head found in the perivitelline space. Several oocyte microvilli (arrows) are bound to the sub-acrosomal layer of PT (arrowheads). Continuous inner acrosomal plasma membrane is not discernible in this micrograph. **D,E:** Sperm head interactions with the oolema. Two oocyte microvilli (arrows) are anchored to the lateral edge of anterior sperm head. Note the inner acrosomal membrane (empty arrowheads) on the lateral face of this sperm head. **F–H:** Binding of the oocyte microvilli (arrows) to the sperm PT (arrowheads) of the lysolecithin-demembranated spermatozoa after their incubation with zona-free oocytes. Note the unusually high number of oocyte microvilli bound to these sperm heads, as compared to the spermatozoa with intact plasma membrane in A–E. The demembranated spermatozoa bind to the oolemma but are not incorporated in the oocyte cytoplasm. **Inset** (G)**:** Multiple, demembranated spermatozoa bound to a single, zona-free oocyte, as visualized by DAPI labeling. These spermatozoa were not removed by repeated, vigorous washing in culture medium. Scale bars = 500 nm.

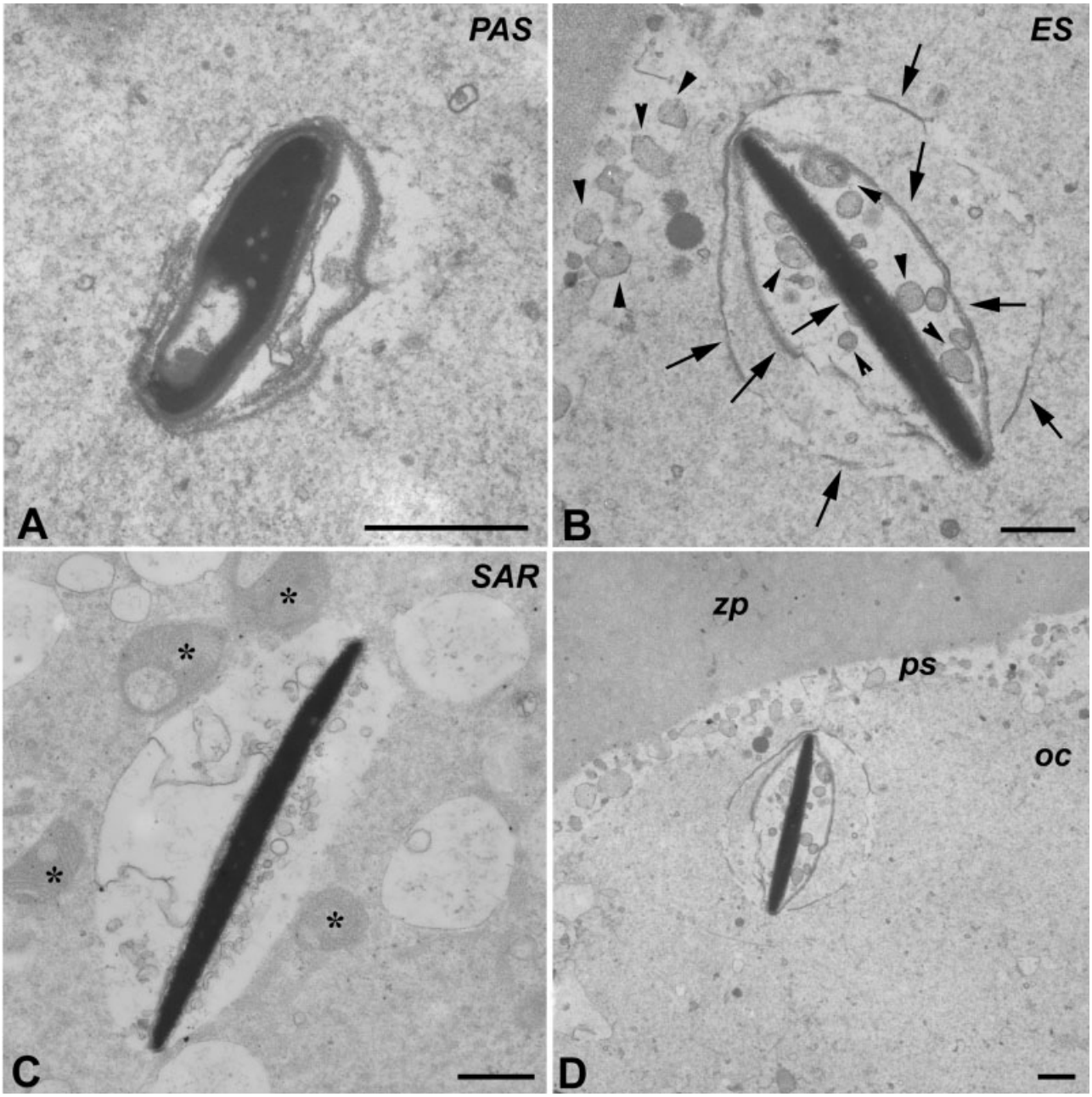

Fig. 4. Removal of the bull sperm PT at an early stage of incorporation into oocyte cytoplasm. Consecutive serial sections show the distal end of the post-acrosomal segment (**A**, *PAS*), the equatorial segment (**B**, *ES*), and the sub-acrosomal layer (**C**, *SAR*) engulfed by the oocyte cytoplasm. All parts of this sperm head are inside the cytoplasm of the oocyte fertilization cone. Oocyte mitochondria (asterisks) are seen around the sperm head SAR in C. Note the layers (B; arrows) detaching from the equatorial segment region of this sperm head, and the oocyte microvilli (B, arrowheads) bound to the sub-acrosomal PT layer and also present in the perivitelline space. The outermost layer seen in B is most likely the plasma membrane combined with the outer peri-acrosomal layer of the PT. The next, double-layer, is the equatorial segment and the innermost layer, to which the microvilli (arrowheads) are attached, is the sub-acrosomal layer of the PT. The individual PT layers appear to be dissolving in the oocyte cytoplasm. Low magnification view of this spermatozoon is shown in **D**, depicting the relative position of zona pellucida (*zp*), perivitelline space (*ps*), and oocyte cytoplasm (*oc*). Scale bars = 500 nm.

The PAS is rapidly dispersed during the sperm head entry, an event probably related to the initiation of the oocyte activation cascade (Sutovsky et al., 1997a; Yanagimachi, 1994). Among the sub-domains of PAS, the mAb MN13 recognizes the outermost layer (Toshimori et al., 1991, 1998) that disperses rapidly in the oocyte cytoplasm before the sperm nucleus begins its decondensation (Manandhar and Toshimori, 2002). In the activated oocytes, the dissociated residual MN13p immuno-reactivity is translocated to the polar regions of the mid-zone spindles while in inactive oocytes; it forms rings around the spindle pole-regions of the

metaphase II plate and around the sperm head whenever premature chromatin condensations occurs in it. It is interesting to note that several cell cycle regulating proteins reside in the spindle polar regions (Hinchcliffe et al. 1999; Vehrlac et al., 1993; Wianny et al., 1998). The putative residual MN13p disappears completely at the pronuclear stage.

We observed the solubilization of the sperm PT in the oocyte cytoplasm in spermatozoa undergoing incorporation at the early stages of fertilization (Fig. 5A–H). It is likely that the observed oocyte was already activated at this stage of partial incorporation and PAS-PT solubilization, because its cytoplasm contained one, presumably female pronucleus (not shown). The distal portion of the PAS of this spermatozoon, protruding into the perivitelline space, was not exposed to oocyte cytoplasm and contained an intact PT (Fig. 5G). In contrast, the incorporated proximal portion of PAS of this sperm head contained no PT and displayed partially decondensed chromatin surrounded by a newly formed nuclear envelope (Fig. 5H).

Our previous studies (Sutovsky et al., 1996b) demonstrated that the addition into fertilization medium of cytochalasin B, a drug causing the depolymerization of F-actin and the retraction of the oocyte-surface microvilli, prevented the completion of the sperm incorporation into oocyte cytoplasm during bovine fertilization. However, even the relatively small region of PAS that became exposed to oocyte cytoplasm after sperm-oolemma fusion in such trials (Fig. 6A,B), released an amount of SOAF sufficient for oocyte activation (Fig. 6C,D). Such conclusions were recently supported by observations in hamster (Sanchez-Gutierez et al., 2002) and mouse (McAvey et al., 2002), and the present data show a similar pattern of partial PAS-PT solubilization at an early stage of bovine fertilization with (Fig. 6A–D) or without (Fig. 5) cytochalasin treatment. In addition to the effect of F-actin disruptors on the oocyte microvilli (Sutovsky et al., 1996b), the block of actin polymerization in sperm SAR may contribute to incomplete sperm incorporation (Sanches-Gutierez et al., 2002). McAvey et al. (2002) also suggested that the oocyte F-actin cytoskeleton is involved in regulating the mechanisms of anti-polyspermy. Altogether, the above studies provide evidence that SOAF most likely originates in the PAS rather than in the SAR or ES. Less than one sperm equivalent of PT proteins/SOAF is sufficient to induce oocyte activation, as shown by cytochalasin B studies, in which only part of the PT dissolves in the oocyte cytoplasm and caused full activation (Sutovsky et al., 1996b; present data). Further support to this hypothesis has been provided by a recent ICSI experiment showing the loss of oocyte activating function of the sperm heads when their PAS was labeled with MN13 antibody before microinjection (Manandhar and Toshimori, 2002). Of the hypothesized multimeric components of SOAF (Perry et al., 2000), mn13p antigen seemingly comprises an insoluble fraction, since the antibody labels sperm heads strongly after the soluble fraction(s) have been removed by DTT or heat extractions (Manandhar and Toshimori, 2002).

INTERACTIONS OF SPERM PT WITH THE OOLEMMA AND OOCYTE MICROVILLI

The interaction between the sperm plasma membrane protein fertilin, or PH-30, and the oolemma's integrin $\alpha 6\beta 1$ may be the major, but not the exclusive gamete binding mechanism in mammals, as the knockout of fertilin β-gene in mice failed to completely eliminate sperm-oolemma binding and fertilization (Cho et al., 1998). The sperm plasma membrane partner of the recently discovered tetraspanin/CD9, a putative sperm receptor on the mammalian oolemma (Chen et al., 1999; Le Naour et al., 2000), is yet to be found (Primakoff and Myles, 2002). Furthermore, there is little evidence suggesting that integrin-fertilin interactions trigger oocyte activation in mammals (Fenichel and Durand-Clement, 1998). Ultrastructural observations presented in this study complement our earlier data on the binding of the oocyte microvilli to the sperm PT (Sutovsky et al., 1997a), further supported by the binding of plasma membrane-free spermatozoa to the oolemma of the zona-free oocytes (this study). Despite enormous sperm binding, such oocytes did not become activated nor did they engulf the oolemma-bound spermatozoa, thus substantiating the role of sperm plasma membrane receptor(s) in gamete fusion. At present, it is not known how and when the plasma membrane is removed from the fertilizing spermatozoon, although we can speculate that at least some parts of it fuse with the oolemma into a continuous zygotic plasma membrane, which may therefore become directly anchored to the sperm PT.

FUNCTION OF THE PT-BORNE SPERM PROTEINS IN FERTILIZATION, OOCYTE ACTIVATION AND ANTI-POLYSERMY DEFENSE

Oocyte activation in mammals encompasses the completion of second meiotic division and the activation of anti-polyspermy defense, paralleled by calcium oscillations that periodically cross oocyte cytoplasm (reviewed by Schultz and Kopf, 1995; Williams, 2002). The fertilization-induced oocyte activation is followed by the assembly of nuclear pore complexes (NPC) into the cytoplasmic annulate lamellae (AL), and by the insertion of NPCs into a de novo-formed nuclear envelope (NE) of the female and male pronuclei (Sutovsky et al., 1998, and references therein). Three hypotheses were offered to explain the sperm-induced oocyte activation in mammals: The conduit, or calcium bomb hypothesis (Jaffe, 1980), implicates the direct, sperm-generated "injection" of Ca^{2+} ions into oocyte cytoplasm at fertilization. The receptor hypothesis (Jones and Whittingham, 1996; Swann et al., 1989) maintains that the specific receptors on the sperm and oocyte plasma membranes activate the signaling cascade leading to the release of Ca^{2+} from internal stores in oocyte endoplasmic reticulum. Finally, the oscillogen/SOAF hypothesis favors a soluble oscillogenic factor, presumably a polypeptide that is released from the sperm head into the oocyte cytoplasm at the time of gamete fusion (Kimura et al., 1998; Parrington et al., 1996; Perry et al., 1999b; Swann, 1990). Recent studies seem to support the validity of SOAF hypothesis in mammals. The injection of sperm heads, but not sperm tails alone into oocyte

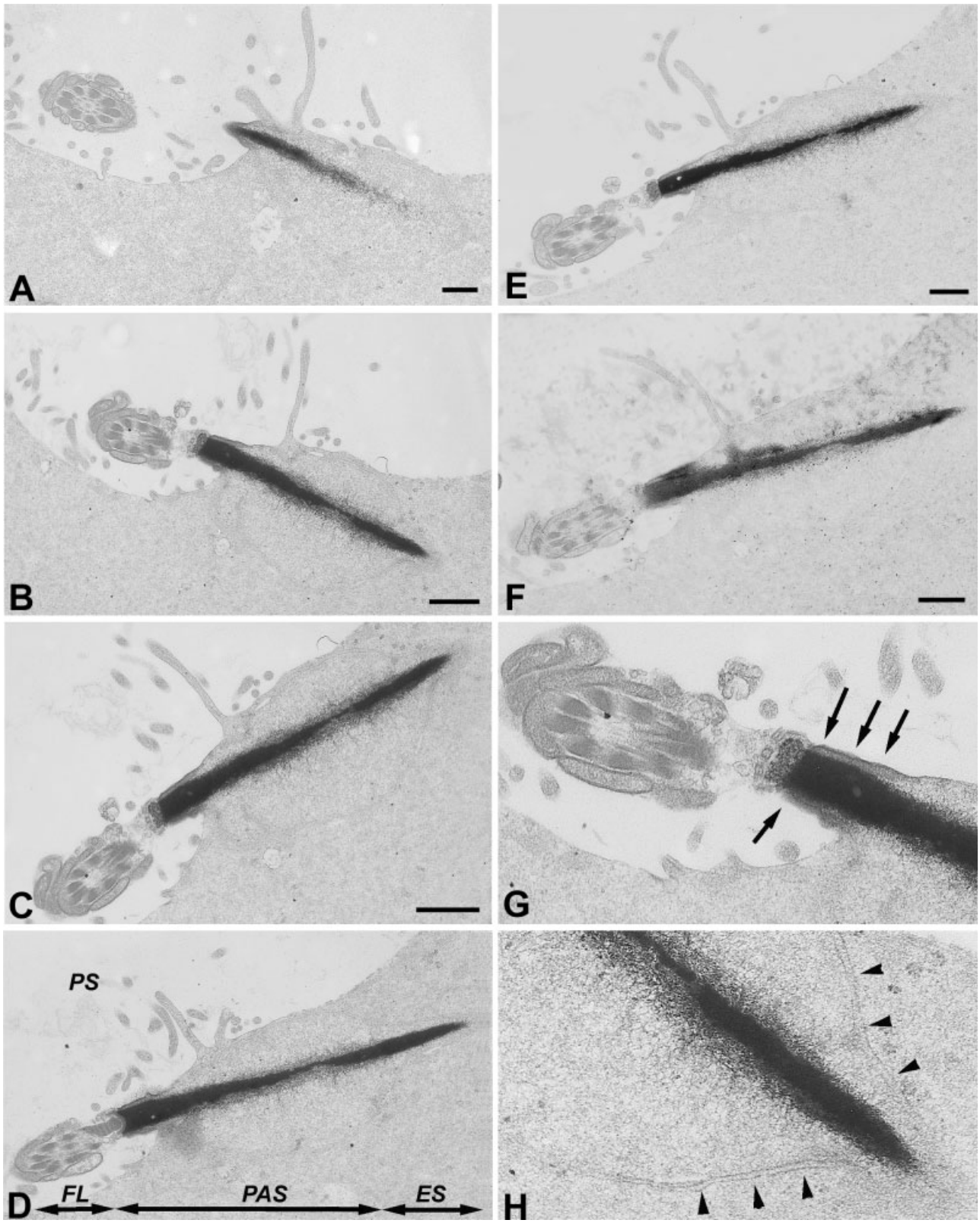

Fig. 5. Solubilization of the sperm PT during sperm incorporation into oocyte cytoplasm, as shown by serial sectioning. A complete entry of this spermatozoon in the oocyte cytoplasm was probably prevented by anti-polyspermy defense. The obligue sections span the post-acrosomal sheath (*PAS*; **A–F**) and a part of the equatorial segment (*ES*). A major part of the sperm head is engulfed by oocyte cytoplasm, whereas the distal portion of the post-acrosomal sheath and the entire flagellum (*FL*) emanate into the perivitelline space (*PS*). Note the intact PT (arrows in **G**) on the non-incorporated part of the sperm nucleus, which contrasts with the absence of PT, decondensation of the sperm chromatin, and formation of the new nuclear envelope (arrowheads in **H**) around the incorporated part of the sperm nucleus. Scale bars = 500 nm.

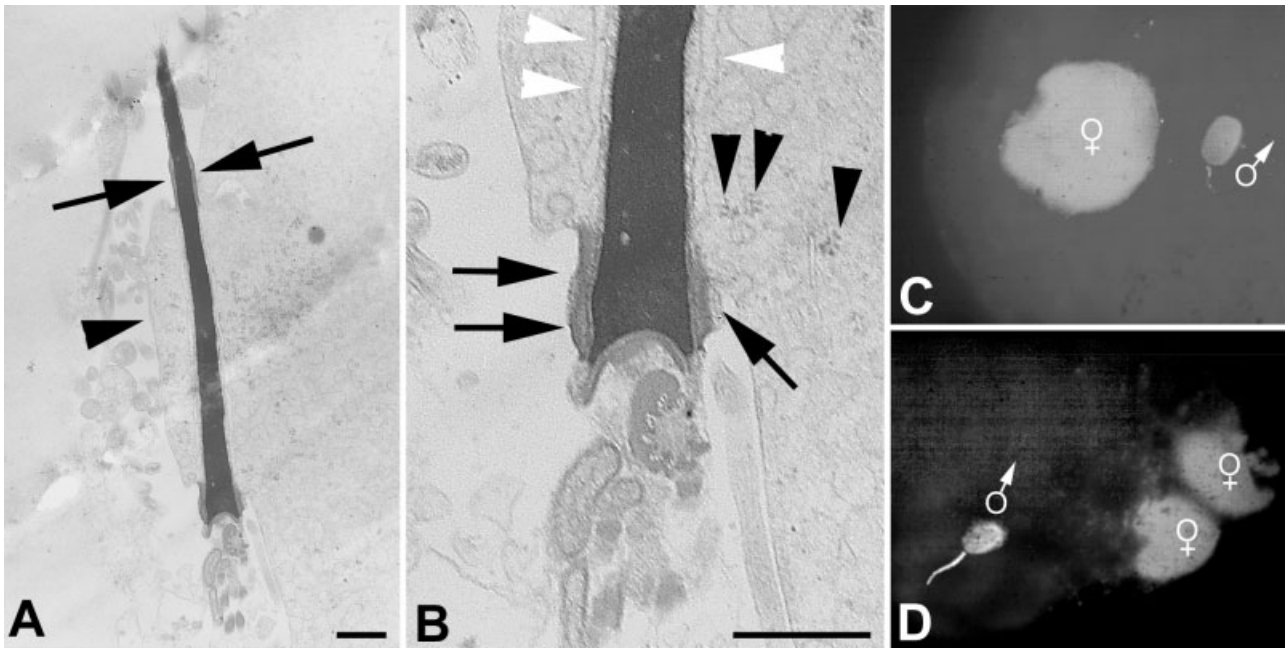

Fig. 6. The effect of microfilament disruptor cytochalasin B on sperm head incorporation into bovine oocyte cytoplasm. **A:** Bull spermatozoon at 20 hours post-insemination fused with the oollemma in a position parallel to the oocyte surface. Arrowhead points to oocyte cytoplasm partially engulfing the PAS of the spermatozoon just below the ES (arrows), which is not completely incorporated, supporting the view that the sperm-borne, oocyte-activating factors are released mainly from the PAS. Note the conspicuous absence of long oocyte microvilli, caused by cytochalasin treatment. **B:** Detail of the spermatozoon in A highlighting the intact PAS-PT (arrows) in the distal PAS, not exposed to oocyte cytoplasm. PT is replaced by a new nuclear envelope (white arrowheads) in the segment of PAS fused with the oocyte. Black arrowheads point to small particles, perhaps proteasomes, present in the vicinity of the sperm nucleus inside the oocyte cytoplasm. **C,D:** Oocyte activation during in vitro fertilization in the presence of CB. One (C) or two (D) female pronuclei (♀) are seen in the presence of an intact spermatozoon (♂), presumably fused with the oolemma. Scale bars = 500 nm.

cytoplasm induces activation. Factors released from the mammalian sperm head by freeze-thawing or by chemical extraction contain SOAF activity, and work across mammalian species and even in invertebrates (reviewed by Fissore et al., 2002). Since the sperm acrosome does not enter the oocyte cytoplasm during natural fertilization (Yanagimachi, 1994), SOAF activity should not be derived from the acrosomal content known to contain a complete calcium signaling system, including IP3-receptors (Minelli et al., 2000) and several PLC species (Choi et al., 2001; Fukami et al., 2001), which are also present in the oocyte cytoplasm and were implicated in the process of oocyte activation. Similar to the identity of SOAF, the actual mechanism, by which the spermatozoon introduces the oscillogenic molecules into the oocyte cytoplasm, is not known.

By strict definition, the SOAF should be a testis-specific, evolutionarily conserved polypeptide or a set of polypeptides present in the acrosome-reacted sperm head, most likely in the PAS and or in the ES, expressed during the final stages of spermatid elongation but not prior to the acquisition of spermatids' ability to activate an oocyte. Injection of one sperm equivalent or less of such hypothetic SOAF should trigger calcium oscillations, anti-polyspermy defense, and second polar body extrusion, and induce embryonic development in the absence of a spermatozoon. The timing of the PAS-PT dissolution in the oocyte cytoplasm (Sutovsky et al., 1997a) and the ability of isolated sperm PT fractions to induce oocyte activation (Perry et al., 2000) indicate that the PT of mammalian spermatozoa contains factors capable of triggering oocyte activation, including the activation of mechanisms responsible for anti-polyspermy defense. Supportive of such a role for the PT are the studies in the globozoospermic infertility patients, whose spermatozoa lack a PT and fail to induce oocyte activation after ICSI (Battaglia et al., 1997; Rybouchkin et al., 1996, 1997). Furthermore, crude whole sperm/whole sperm head extracts (Wu et al., 1998; Swann, 1990) and those prepared from the isolated sperm heads (Kimura et al., 1998; Perry et al., 1999b) by freeze/thaw or by DTT-extraction induced oocyte activation after extract microinjection in rodents.

Further efforts in this research will be focused on the identification of individual PT proteins with SOAF activity. One such candidate is PT32, the 32-kDa polypeptide that has been cloned (Gong et al., 1997; Wu et al., 2001) and found in the sperm PT of several mammalian species including bovine, mouse, and human. In addition to having numerous attributes of SOAF (Sutovsky et al., 2002), this protein shares a high N-terminal sequence identity and proline rich motifs with the WW domain-binding protein 2 (WBP2), which binds to the WW-domain of Yes-associated protein in the Src-family tyrosine kinase cascade (Chen and Sudol, 1995; Sudol et al., 1995; Sudol, 1998). The significance of this resemblance could reside in the recent findings that various tyrosine kinases, including *c-Src, c-Fyn*, and *c-Yes* gene products participate in the

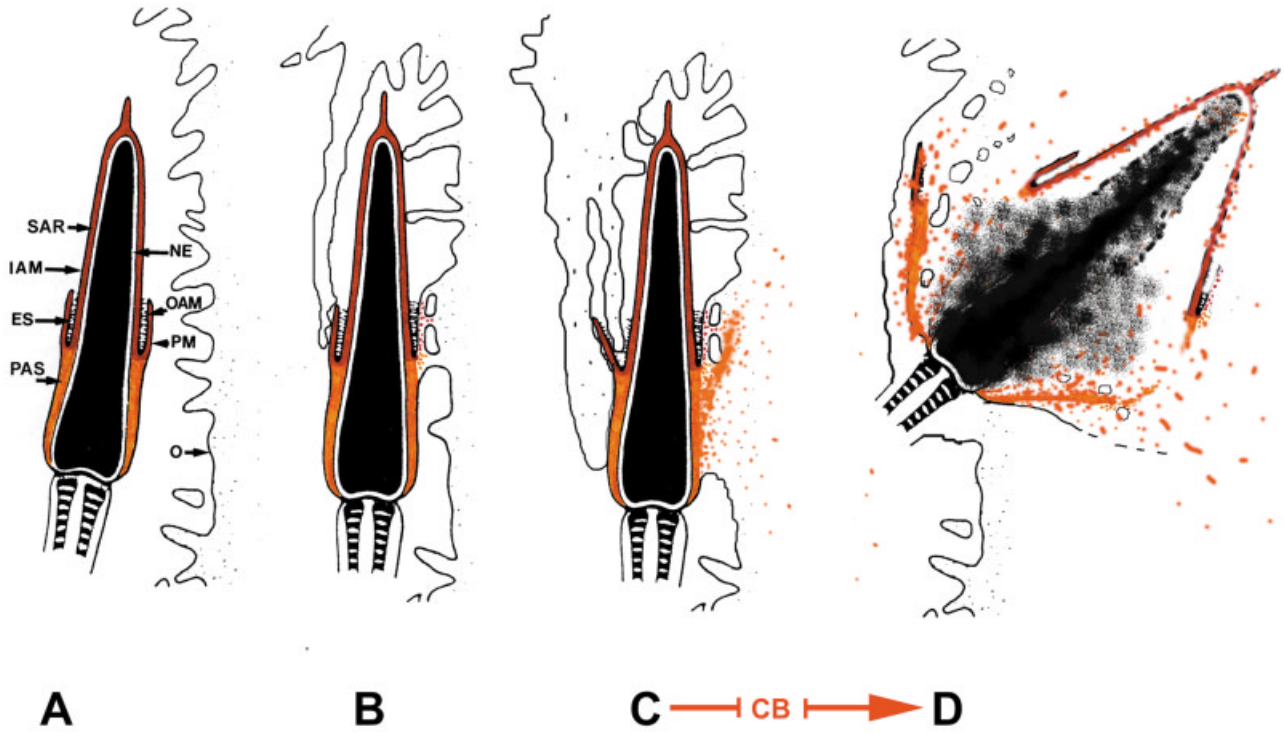

Fig. 7. Schematic interpretation of the release of sperm PT-borne, oocyte-activating factors (SOAF) into oocyte cytoplasm at fertilization. **A:** Acrosome reaction exposes the inner acrosomal membrane (IAM) underlaid by the sub-acrosomal PT layer (SAR); equatorial segment (ES) and post-acrosomal sheath (PAS) regions of the sperm head are accessible to the oocyte microvilli once the spermatozoon reaches the perivitelline space. **B:** Oocyte microvilli fuse with the sperm plasma membrane over the equatorial segment, effectively exposing the perinuclear theca to the oocyte cytoplasm. **C:** PT (PAS) in the regions already fused with the oolema becomes solubilized very rapidly, releasing the SOAF and possibly other factors into oocyte cytoplasm. Disulfide-bonds-reducing factors such as glutathione, present in the ooplasm, may facilitate the dissolution and dispersion of sperm PT at this stage. **D:** The oocyte microvilli pull the PT and sperm nucleus into the cytoplasm, and the PT starts to solubilize in the oocyte cytoplasm. The arrest of sperm-incorporation (at step C), induced by microfilament disruption with cytochalasin B (CB), does not prevent oocyte activation, as the oocyte microvilli retain their ability to fuse with the sperm plasma membrane, effectively exposing the sperm PT to oocyte cytoplasm. PT from those parts of the sperm head engulfed by the oocyte is then released into the oocyte cytoplasm, thus explaining the ability of bull spermatozoa to activate the oocytes in the absence of complete sperm incorporation after CB-treatment. ES = equatorial segment, IAM = inner acrosomal membrane, NE = nuclear envelope, OAM = outer acrosomal membrane, PM = plasma membrane, PAS = post-acrosomal sheath, SAR = sub-acrosomal region. Adapted from Yanagimachi (1994) after Bedford and Cooper (1978).

sperm-induced oocyte activation by regulating various signal transduction pathways including phospholipase C activity in the oocyte cytoplasm (Carrol et al., 1999; Glahn et al., 1999; Kinsey, 1997; Mehlmann et al., 1998; Runft et al., 2002; Schultz and Kopf, 1995; Sette et al., 1998; Talmor et al., 1998; Tsai et al., 1998). Our findings do not rule out that a parallel, or a backup, mechanism to PT-induced oocyte activation exists in mammals. One possible candidate is the truncated *c-kit* tyrosine kinase (Sette et al., 1997, 1998, 2002), which is, atypically for an SOAF-candidate, present in both the sperm head and flagellum (Sette et al., 1997). Oscillin/glucosamine-6-phosphate isomerase, predominantly found in the sperm head (Parrington et al., 1996) and possibly bound to the PT, was proposed to be a sperm-born oscillogen in mammals, yet the recombinant protein lacks the ability to activate mammalian oocytes after microinjection (Wolny et al., 1999; Wolosker et al., 1998). Kimura et al. (1998) discussed the possibility that calicin, a cytoskeletal component of mammalian PT (Longo et al., 1987; Olson and Winfrey, 1988; Paranko et al., 1988), may be involved in oocyte activation. A combination of two or more sperm-contributed components may be responsible for triggering the signaling pathway of oocyte activation (Perry et al., 1999b). Herrada and Wolgemuth (1997) reported the presence of a DNA-binding transcription factor Stat4 in murine sperm PT. This and other PT proteins, namely histones found in the PT (Aul and Oko, 2002; Tovich and Oko, 2001), may also be responsible for the binding of extrinsic DNA to the partially demembranated spermatozoa during ICSI-mediated transgenesis (Perry et al., 1999a). Finally, new evidence shows that the over-expression of testicular phospholipase C-variant, PLC-ζ, in the mouse oocyte triggers calcium oscillation similar to those induced by SOAF injection (Cox et al., 2002; Saunders et al., 2002). While this is solid evidence, it is cautioned that several other PLC species with similar activity are not restricted to sperm PT or even to the sperm head or testis (reviewed by Runft et al., 2002; Williams, 2002). It remains to be demonstrated if the PLC-ζactivity is confined to the sperm head PT of the acrosome-reacted spermatozoa, with particular emphasis on the "acrosome-reacted."

The major problem of studies involving injection of the sperm extract is that in most of them, the acrosomes are not completely removed from spermatozoa extracted by repeated freezing-thawing, thus likely contaminating the presumed SOAF extract with acrosomal fractions containing a complete calcium signaling system including several phospholipase species. Rapid, short calcium oscillations, probably mediated by an active sperm PLC-species, are seen in the sperm head during sperm hyperactivation (Suarez et al., 1993).

The early removal of the PT from the sperm nucleus and its rapid solubilization, especially in the case of PAS-PT, corroborates the proposed role of sperm PT as a carrier of SOAF responsible for the fertilization-induced calcium oscillations and oocyte activation (Kimura et al., 1998). The present study clearly demonstrates that after being detached from the sperm nucleus, the PT is incorporated into oocyte cytoplasm and completely dissolved in it before the pronuclei reach their full size and become apposed. Furthermore, the segment of PAS-PT exposed to the oocyte cytoplasm dissolves in it even before the incorporation of the sperm head is completed (this study), or even if its completion is prevented by the disruption of oocyte and/or sperm SAR microfilaments with cytochalasin (McAvey et al., 2002; Sanchez-Gutierez et al., 2002; Sutovsky et al., 1996b). These observations indicate why sperm-oolemma fusion, but not the complete sperm incorporation into the oocyte cytoplasm, is necessary for oocyte activation in mammals (Sutovsky et al., 1996b). The dispersion of the perinuclear material, or PT, in the oocyte cytoplasm after fertilization was previously shown in mouse (Usui, 1996; Usui and Yanagimachi, 1976; Usui et al., 1997). The PT-mediated introduction of sperm head-anchored SOAF into the oocyte cytoplasm (see Fig. 7) may explain the sperm-induced oocyte activation in mammals without the need for the hypothetic large, activation-permissive pores in the oolemma, proposed by Jones et al. (1998).

PATHOLOGY OF SPERM PT DURING SPERMATOGENESIS AND AFTER ASSISTED REPRODUCTION

Globozoospermia

Recently, infertility treatment trials were performed with spermatozoa donated by infertile men suffering of globozoospermia (Battaglia et al., 1997; Edirishinge et al., 1998; Rybouchkin et al., 1996), a rare spermatogenic disorder in which the absence of the PT causes the fertilization prohibiting, round shape of the sperm nucleus (Escalier, 1990). With few exceptions, perhaps cases of incomplete globozoospermia, such spermatozoa failed to induce oocyte activation after intracytoplasmic sperm injection (ICSI). Human (Palermo et al., 1992; Van Steiterghem et al., 1993) and non-human primate (Sutovsky et al., 1996b) oocytes are activated by ICSI with normal spermatozoa, and the intracytoplasmic injection of crude (Swann, 1990), or partially purified (Kimura et al., 1998; Perry et al., 1999), sperm extracts activates rodent oocytes. In light of these observations, our previous studies on the removal of the PT at the egg cortex during fertilization (Sutovsky et al., 1997a) seem to be compatible with the proposed role of the sperm PT in oocyte activation (Kimura et al., 1998).

Intracytoplasmic Sperm Injection

ICSI is a successful assisted reproductive technology, producing mostly chromosomally normal embryos. It is still debated whether a slight increase in sex chromosomal abnormalities and a few cases of high incidence thereof (e.g., Int'Veld et al., 1995) are due to the deleterious effect of the technique, or to inferior quality of gametes obtained from infertility patients. However, cell biological studies of ICSI in primates and in the failed, ICSI-fertilized human zygotes do support a concern that this technique could produce defective embryos that could contribute to the pool of failed in vitro developed embryos, if not to the pool of abnormal, implanted embryos, fetuses, and newborns with chromosomal abnormalities (Schultz and Williams, 2002). The phenomenon of the SAR-PT persistence after ICSI was originally described in primates (Sutovsky et al., 1996a) and later in failed human ICSI zygotes (Bourgain et al., 1998; Küpker et al., 1998; Sathananthan et al., 1997). Further studies demonstrated that the persistence of the SAR-PT on the apex of the male pronucleus disrupts the S-phase not only in the male, but also in the female pronuclei, probably through a cell cycle check point assuring synchronous S-phase in these respective DNA-containing entities (Hewitson et al., 1999; Ramalho-Santos et al., 2001). The latter study provided evidence that in such cases, the ES-PT persisted along with SAR-PT after ICSI, while the oocyte activation seemed to occur normally and was paralleled by complete solubilization of the PAS-PT, already shown in the initial primate study (Sutovsky et al., 1996a). The somewhat preferential localization of sex chromosomes in the sperm head apex (Luetjens et al., 1999), known to contain a SAR-sheltered heterochromatin region after aberrant ICSI, could then account for uneven partition of sex chromosomes inside the male pronucleus. Residual SAR-PT co-localized with the sperm-derived sex chromosomes, as identified in the ICSI zygotes by anti-PT antibodies and sex chromosome-specific fluorescent in situ hybridization (FISH), respectively, in human sperm injected into hamster oocyte (Terada et al., 2000). Most recently, the delayed breakdown of the acrosome (and presumably SAR-PT) was described in porcine ICSI zygotes, showing an aberrant PN development paralleled by normal oocyte activation (Katayama et al., 2002).

In contrast to high fertilization rates after ICSI in primates, rodents, and rabbits (reviewed by Sutovsky and Schatten, 2000), bovine appears to be a difficult species for this form of assisted reproduction, yielding low-to-moderate fertilization and cleavage rates even in the protocols employing artificial activation (Goto, 1990; Rho et al., 1998). This is likely due to a higher rigidity of the perinuclear theca in bull, as compared to other mammals (Perreault et al., 1988; Sutovsky et al., 1997b). The best results so far, with an unassisted 40% activation rate, were achieved using a protocol that combines the pretreatment of bull spermatozoa with the disulfide bond-reducing agent dithiothreitol (DTT), and the chemical activation of the sperm-injected oocytes with the combination of ionomycin and 6-dimethylamino-purine (Rho et al., 1998). Such a technique, however, may not induce optimal oocyte activation and the DTT treatment may affect the biological activity of the

SOAF (Perry et al., 2000) as well as the integrity of zygotic chromatin (Szczygiel and Ward, 2002). The use of purified or recombinant PT proteins combined with piezo-injection of spermatozoa (Horiuchi et al., 2002) may be a viable alternative for achieving higher success rates in mammalian species with poor ICSI fertilization. Similarly, the use of such PT extracts, or of the recombinant PT proteins, may be helpful in the treatment of infertility by ICSI in globozoospermic patients. Equally effective, yet objectionable due to environmental and ethical concerns, may be the activation by the microinjection of mRNAs encoding for SOAF components (when identified) or oocyte-derived, constitutively active kinases involved in the triggering of calcium oscillations during fertilization.

MATERIALS AND METHODS

Gamete Preparation and In Vitro Fertilization of Bovine Oocytes With MitoTracker-Tagged Spermatozoa

Straws of frozen bull sperm (ABS, DeForest, WI) were thawed and centrifuged for 10 min at 700*g* through a two-layer (45 and 90%) Percoll gradient, then resuspended and incubated for 10 min at 37°C in a modified Tyrode's medium (Sperm-TL; Parrish et al., 1986), supplemented with 400 nM MitoTracker Green FM (Molecular Probes Inc., Eugene, OR), a vital, fixable mitochondrial dye with high affinity to sperm mitochondrial membranes (Sutovsky et al., 1996b).

Oocytes were isolated by aspiration from ovaries obtained from a local abattoir and matured in vitro for 24 hours (metaphase II) in TC 199 medium (Gibco) as described previously (Sutovsky et al., 1996b). MitoTracker-tagged sperm were resuspended in fertilization medium (TL; Parrish et al., 1986) supplemented with 0.25–5 μg/ml heparin, and pipetted into 50-μl drops of fertilization medium, to give a final concentration of 1×10^6 sperm/ml. Zygotes were incubated at 39°C in a humid atmosphere of 5% CO_2. In some experiments, 10 μg/ml of cytochalasin B (Sigma, St. Louis, MO) were added to fertilization medium prior to transfer of the gametes.

Perinuclear Theca-Oolemma Binding Assay

Bull spermatozoa were processed as described above and deprived of their plasma membranes by a 20-minute incubation at 37°C in 0.05% lysophosphatidylcholine (Lysolecithin; Sigma) diluted in the KMT medium (100 mM KCl, 2 mM $MgCl_2$, 10 mM Tris-HCl; pH 7.0). Mature, metaphase-II-arrested oocytes were deprived of zonae pellucidae as described for immunofluorescence and transferred into drops of fertilization medium. One million demembranated spermatozoa per milliliter were mixed with zona free oocytes, cultured for 4 hours, and fixed for immunofluorescence (pAb 427/DAPI, or DAPI only) or electron microscopy as described below.

Immunofluorescence

Oocytes were removed from the fertilization drops at various time points ranging from 8 hours (sperm incorporation) to 24 hours (first mitosis) after insemination and processed for immunofluorescence as described previously (Sutovsky et al., 1996b). A mixture of the perinuclear theca-specific rabbit polyclonal antibody pAb 427 (Oko and Maravei, 1994; dil. 1/200) and a nuclear pore-specific mouse monoclonal antibody mAb 414 (BabCo, Berkeley, CA; Davis and Blobel, 1987; Sutovsky et al., 1998; dil. 1/200) was followed by a 40-minute incubation with the red fluorescent, TRITC-conjugated goat anti-rabbit IgG and a far-red emitting, Cy5-conjugated goat anti-mouse IgG (both from Zymed Labs, South San Francisco, CA; both dil. 1/40). DNA was stained by DAPI (Molecular Probes, Eugene, OR). The coverslips were mounted on microscopy slides in a VectaShield mounting medium (Vector Labs, Burlingame, CA) and examined on a Zeiss Axiophot epifluorescence microscope equipped with an RTE/CCD 1217 camera (Princeton Instruments, Inc., Trenton, NJ), operated by MetaMorph software. Images were archived on recordable CDs. The fluorescence of the MitoTracker-labeled sperm tail mitochondria was retained after such processing and allowed us to discriminate between the male sperm-tail tagged pronuclei, and the female tail-free pronuclei. Final images were prepared by pseudo-coloring and superimposing the parfocal single channel images using Adobe Photoshop 4.0 software (Adobe Systems Inc., Mountain View, CA). Images were printed on Epson Stylus 1280 photo printer. A total of 250 zygotes and 50 oocytes were processed with the above antibodies and 50 zygotes were processed with preimmune rabbit serum as a negative control.

Transmission Electron Microscopy

Zygotes were fixed in formaldehyde-glutaraldehyde fixative of Ito and Karnofsky (1968), containing 5% of 2-4-6 trinitrophenol (picric acid; Sigma), post-fixed in 1% osmium tetroxide, dehydrated by an ascending ethanol series (30–100%), perfused with the solution of acetone and Epon 812, and embedded in Epon 812 resin. Tissue sections were cut using a Sorvall MT 5000 ultramicrotome, transferred onto 100 MESH Cu-grids, stained with uranyl acetate and lead citrate, and examined and photographed on a Philips 300 electron microscope. Negatives were scanned by an Umax Power Look 3000 scanner and printed using Adobe Photoshop 6.0 software.

Preparation of the Perinuclear Theca Extracts

Isolated bull sperm heads (Oko and Maravei, 1994) were exposed to three successive extractions, consisting of incubations in 0.2% Triton X-100 for 1 hour at 4°C with agitation, 1M NaCl with agitation for 1 hour at 4°C, and 100 mM NaOH overnight at 4°C. The first and second extractions solubilize the acrosome, sperm head membranes, and hydrophobic and ionically bound proteins, leaving essentially a shell of insoluble perinuclear theca surrounding the condensed nucleus (Oko and Maravei, 1994). Subsequent extraction with 100 mM NaOH solubilizes the PT but leaves the nucleus in its condensed form. The supernatant recovered in this last extraction step (PT extract) was neutralized, dialyzed, and lyophilized for use in SDS- PAGE analysis and Western blotting as described previously by Oko and Maravei (1994) and for microinjection into the oocytes. It is important to note that all the antibodies raised against this PT extract (see above reference), whether raised against the whole extract (pAb 427) or against each of its major proteins, exclusively immuno-

localize to the PT of the sperm head providing the confidence for the specificity of this extraction technique.

SDS-PAGE and Western Blotting

Lyophilized PT extracts were solubilized in 2% SDS, 5% -mercaptoethanol by boiling for 5 minutes and then run on linear gradient (8–18%) polyacrylamide gels according to the SDS- discontinuous system originally described by Laemmli (1970). Preparative gels were eletrophoretically transferred to nitrocellulose (Schleicher and Schuell Inc., Keene, NH) utilizing a Hoefer Wet Transphor apparatus according to the technique of Towbin et al. (1979). The immuno-reactivity of Western blotted proteins to pAb 427 was detected by developing the phosphatase color reaction on the secondary antibody phosphatase conjugate [alkaline phosphatase conjugated $F(ab)^2$ goat anti-rabbit IgG; Cappel-Cooper Biomedical Inc., Malvern, PA] according to McGadey (1970).

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