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PH-20 but Not Acrosin Is Involved in Sperm Penetration of the Macaque Zona Pellucida

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ABSTRACT In this study, we investigated the functions of PH-20 and acrosin during the interaction of macaque sperm with the zona pellucida. Both of these sperm enzymes have been reported to be present on the inner acrosomal membrane of acrosome reacted sperm, and have been suggested to play a role during secondary sperm-zona binding in other species. Anti-macaque PH-20 IgG, anti-pig acrosin IgG and soybean trypsin inhibitor (SBTI) were used as probes for immunolocalization of the two proteins at the ultrastructural level, and as reagents for blocking sperm penetration of the macaque zona pellucida in vitro. As a control, we performed similar studies with antibodies to CD-46, which is also located on the inner acrosomal membrane, but has no known function in sperm-zona pellucida interaction. After labeling with anti-acrosin IgG, gold label was not present on the sperm surface before the acrosome reaction, but was detected over the entire head of sperm that were induced to acrosome react with calcium ionophore A23187. In contrast, when sperm were induced to acrosome react by binding to intact zona pellucida, acrosin was present in the acrosomal shroud but not on the inner acrosomal membrane. Similar results were obtained when SBTI was used as a probe for enzyme localization. PH-20 and CD-46 were demonstrated on the inner acrosomal membrane of sperm induced to acrosome react by ionophore treatment and by zona binding. Neither anti-acrosin IgG nor anti-CD-46 IgG affected sperm penetration of the zona at concentrations up to 300 μg/ml, but zona penetration was blocked completely when anti-PH-20 IgG (100 μg/ml) was present during sperm-oocyte interaction. Ultrastructural observations of oocytes incubated with anti-PH-20 IgG showed that acrosomal shrouds were present on the zona surface but no sperm had begun to penetrate into the zona substance. We conclude that anti-PH-20 IgG prevented sperm penetration of the macaque zona pellucida by interference with secondary sperm-zona binding, rather than primary sperm-zona binding or the zona-induced acrosome reaction. Acrosin was not detected on the inner acrosomal membrane of sperm that are induced to acrosome react after zona binding, and acrosin does not appear to be critical for sperm penetration of the macaque zona pellucida. *Mol. Reprod. Dev. 53:350–362, 1999.* © 1999 Wiley-Liss, Inc.

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Key Words: acrosin; PH-20; CD-46; sperm; zona pellucida

INTRODUCTION

When the first sperm enter the oviductal ampulla after ovulation, the oocytes in most species are surrounded by at least two investing layers, the zona pellucida and the cumulus oophorus, composed of cumulus cells and their extra cellular matrix. This extra cellular matrix, which surrounds and even extends into the outer region of the zona pellucida, has its origin from the cumulus cells, and hyaluronic acid is its major constituent (Talbot and DeCarlantonio, 1984). The zona pellucida is produced by the oocyte and is composed of at least three heavily glycosylated peptides, which in the mouse are termed ZP-1, ZP-2, and ZP-3 (Wassarman and Mortillo, 1991). Zona pellucida glycoproteins which are homologous to mouse ZP-1, ZP-2, and ZP-3 have now been identified in a variety of mammalian species, and in macaques they are termed ZPA (largest), ZPB (intermediate), and ZPC (smallest) (Martinez et al., 1996).

The most widely accepted model for sperm-zona interaction in mammals is based on studies of fertilization in mice. In this model, the specific recognition between gametes is mediated by carbohydrate binding proteins on the sperm surface and glyco-conjugates on the zona pellucida (Wassarman, 1992). The ZP-3 glycoprotein is involved in primary binding to the plasma membrane of the acrosome intact mouse sperm (Bleil and Wassarman, 1980), and ZP-3 also has the capability to induce the acrosome reaction of bound spermatozoa (Bleil and Wassarman, 1983). Acrosome reacted mouse sperm bind by the inner acrosomal membrane to the ZP-2 glycoprotein (Bleil and Wassarman, 1986; Bleil et al., 1988; Mortillo and Wassarman, 1991). This phase of sperm-zona pellucida interaction is termed

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secondary binding, and also may include interactions between components of the inner acrosomal membrane and zona pellucida that take place during sperm penetration.

The question of whether sperm employ enzymes as they traverse the oocyte investments has been debated by researchers for many years. Sperm penetration of the cumulus has long been thought to require the activity of a sperm hyaluronidase (Austin, 1960). The release of hyaluronidase activity from sperm had been associated with the acrosome reaction (Yanagimachi, 1981), but this information could not be reconciled with evidence that linked the acrosome reaction to the process of sperm binding to the zona pellucida (Saling et al., 1979; Bleil and Wasserman, 1983) or with the inability of acrosome reacted sperm to enter or pass through the cumulus (Suarez et al., 1984; Cherr et al., 1986; Cummins and Yanagimachi, 1986). The role of sperm hyaluronidase in cumulus penetration became clearer with the discovery by Gmachl and Kreil (1993) that PH-20, a known sperm plasma membrane protein, had significant DNA homology with bee venom hyaluronidase. Subsequently, the hyaluronidase activity of PH-20 has been shown to be involved in sperm penetration of the mouse and macaque cumulus (Lin et al., 1994; Meyers et al., 1997; Myles and Primakoff, 1997), and the plasma membrane-bound protein rather than a soluble enzyme appears to be responsible for this activity (Cherr et al., 1996, Li et al., 1997a).

There is substantial evidence linking two sperm enzymes with the process of zona penetration. These enzymes include the serine protease, acrosin, and the hyaluronidase, PH-20. Acrosin has been reported to be present on the exterior surface of the human sperm prior to the acrosome reaction and on the posterior head and inner acrosomal membrane after the reaction (Tesarik et al., 1988; Barros et al., 1992). Acrosin can cause structural modification of the zona pellucida (Urch, 1991). The location and physiological activity of acrosin have suggested that this enzyme may be required during zona penetration (Yanagimachi, 1994, for review). However, it has been reported recently that homozygous mice, in which the acrosin gene has been deleted, have no reduction in fertilization (Baba et al., 1994; Adham et al., 1997). PH-20 also has been localized to the inner acrosomal membrane; and PH-20 on the plasma membrane of the posterior head of the guinea pig sperm moves to and supplements the PH-20 already present on the inner acrosomal membrane (Myles and Primakoff, 1984). Monoclonal antibodies to PH-20 have been shown to block sperm-zona binding in guinea pigs, and immunizing guinea pigs with PH-20 blocked fertilization (Primakoff et al., 1988).

The objective of the present experiments was to evaluate the requirement for PH-20 and acrosin during sperm penetration of the macaque zona pellucida, by attempting to block zona penetration in vitro with specific antibodies. As a part of these studies, we also sought to confirm the location of both enzymes on the inner acrosomal membrane of sperm that were induced to acrosome react as a consequence of sperm binding to the zona. As a control in these experiments, we carried out similar evaluations of CD-46, another inner acrosomal membrane constituent, which is thought to aid in protection of the sperm after the acrosome reaction, but is not involved in zona penetration (Fénichel et al., 1990; Anderson et al., 1993).

MATERIALS AND METHODS

Reagents and Antibodies

Most of the chemicals used in media preparation and for sperm treatment were purchased from Sigma Chemical Company (St. Louis, MO). All of the electron microscopy supplies were purchased from Ted Pella (Tustin, CA). Three antibodies were used for immunolocalization of sperm antigens and for experimental treatments. A rabbit polyclonal antibody to purified pig sperm acrosin was a gift from Dr. Jerry Hedrick and the late Dr. Umbert Urch. Antibodies to recombinant cynomolgus macaque PH-20 were raised in rabbits as previously described (Lin et al., 1994) and characterized (Lin et al., 1994; Overstreet et al., 1995). Mouse monoclonal antibodies to CD-46 were purchased from Immunotech Inc. (Westbrook, ME). IgG was purified from sera using protein A columns from Pierce Scientific (Rockford, IL). The sources for all other reagents are given in the descriptions of specific methods.

Sperm Collection and Treatment

Adult male cynomolgus macaques were caged individually at the California Regional Primate Research Center in compliance with the Federal Animal Welfare Act and the NIH Guidelines for Care and Use of Laboratory Animals. The animals were maintained with a 6:00 a.m. to 6:00 p.m. light schedule at 25°C to 27°C and were fed a diet of Purina monkey chow and water ad libitum. The males were trained to chair restraint and were electroejaculated with direct penile stimulation (Sarason et al., 1991). Ejaculates were collected in 15 ml centrifuge tubes and allowed to stand at room temperature for 30 min prior to washing. All steps prior to incubation were done at room temperature. Coagulum was removed and the semen was suspended in 10 ml of a modified Biggers, Whitten and Whittingham (BWW) medium (Overstreet et al., 1995). After centrifugation at 300 × g for 10 min, the resulting sperm pellet was resuspended, centrifuged, and adjusted to a concentration of 10×10^6 sperm/ml prior to incubation for 1.5 to 3 hr at 37°C in 5% CO_2 in air (pH 7.35 to 7.40). The sperm suspension was divided into 1 ml aliquots, and following the addition of 1 mM caffeine and 1 mM dbcAMP (Sigma Chemical Co.), the aliquots were incubated an additional 30 min to initiate activation (VandeVoort et al., 1992). Activated sperm samples were evaluated for the quality of motility and the percentage of motile sperm. Only samples which had hyperactivated motility by subjective assessment (VandeVoort et al., 1992) were used in any of the experiments. In some experiments, activated sperm

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suspensions (10×10^6/ml) were induced to undergo the acrosome reaction by addition of 15 μM (final concentration) of calcium ionophore A23187 in dimethyl sulfoxide (DMSO).

Oocyte Collection

Ovaries were obtained at necropsy from adult female cynomolgus macaques; and oocytes were collected from ovarian follicles and stored at −80°C, as described previously (VandeVoort et al., 1992). Prior to experiments, oocytes were thawed at room temperature, washed in phosphate buffered saline (PBS), and equilibrated into the appropriate buffer for 1 hr at 37°C. The oocytes were evaluated at 200× magnification using an inverted Olympus CK2 microscope equipped with Hoffman optics (Scientific Instruments Company). Only those oocytes that had an intact cytoplasm and zona pellucida were used in the experiments.

Electrophoresis and Western Blotting

In preparation for analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), activated sperm were thoroughly washed in PBS (pH 7.3) containing 1 mM para-aminobenzamidine. Solubilizing buffer (0.3 M Tris, 5% SDS and 50% glycerol, pH adjusted to 6.8 with HCl) was brought to a boil and immediately added to the washed, pelleted sperm, and the solubilized sperm were boiled for an additional 3 to 5 min. Polyacrylamide gels (7.5% SDS) were purchased from BioRad Corp. (Hercules, CA). After gel electrophoresis proteins were transferred to nitrocellulose membranes and blocked with 9% nonfat dry milk, 0.1% Tween 20 in Tris-buffered saline for approximately 2 hr. After blocking, the samples were probed with a 1:1000 dilution of either anti-PH-20, anti-acrosin, or anti-CD-46 IgG. After washing overnight, membrane blots were labeled with either anti-rabbit or anti-mouse IgG conjugated to alkaline phosphatase (1:3000) as the secondary antibody (Pierce). Molecular weights were determined by prestained markers (BioRad). Sperm samples were stained for total sperm proteins with Gel Code Blue (Pierce).

Ultrastructural Localization of PH-20, Acrosin, and CD-46

Acrosome intact sperm were fixed for ultrastructural observation before and after activation (incubation for 30 min with caffeine and dbcAMP), and acrosome reacted sperm were fixed at 10, 20, and 30 min after addition of calcium ionophore. Each sample was fixed in 2% paraformaldehyde in PBS (pH 7.4) for 30 min and then washed twice with a blocking buffer of 1% bovine serum albumin (BSA) in PBS. After blocking, the sperm samples were resuspended in a 1:100 dilution with either anti-PH-20, anti-acrosin, or anti-CD-46 IgG in blocking solution. For some observations of acrosin localization, anti-acrosin IgG was added to the sperm suspension and was present during induction of the acrosome reaction. When SBTI was used as a probe, the inhibitor was added to the medium (100 μg/ml) immediately prior to addition of the ionophore. SBTI-treated sperm were fixed, blocked, and then incubated with anti-SBTI IgG (100 μg/ml; Biogenesis Inc., Sandown, NH). After 2 hr exposure to the specific IgG, the sperm were washed extensively in blocking solution prior to incubation with either goat anti-rabbit or goat anti-mouse IgG tagged with 15 nM gold particles (E-Y Laboratories, San Mateo, CA). After 2 hr of labeling, the sperm were fixed overnight at 4°C in 2% glutaraldehyde buffered with 0.1 M cacodylate, pH 7.4.

To induce acrosome reactions by sperm-zona interaction, washed oocytes were coincubated with activated sperm (3×10^6/ml) for 30 sec and then transferred to a fresh drop of BWW medium for 10 min before fixation in 2% paraformaldehyde in DPBS. In each replicate experiment, three fixed oocytes were probed with each of the three IgGs and with SBTI. After primary fixation, the sperm-oocyte samples were treated as previously described for labeled sperm. The experiment was repeated three times. In some experiments anti-acrosin IgG and SBTI were added to the sperm suspension and therefore, were present during gamete interaction.

After overnight fixation, samples were washed in 0.1 M cacodylate buffer for 1 hr, placed into 1% OsO_4 for 2 hr, and then extensively washed in 0.1 M cacodylate for 1 hr. Samples were dehydrated through a graded alcohol series and embedded in a Spurrs epoxy resin. Sections were cut with a diamond knife, stained with lead citrate, and viewed at 80 KV on a Phillips 410 transmission electron microscope.

Sperm Penetration of the Zona Pellucida

Two different experiments were carried out to compare the effects of the antibodies on sperm penetration of the zona pellucida. Each experiment was replicated four times and each replicate utilized sperm from a different male. In the first experiment, the effects of anti-PH-20 IgG were compared with those of anti-CD-46 IgG. In the second experiment, the effects of anti-acrosin IgG were compared with those of soybean trypsin inhibitor (SBTI). Activated sperm were diluted to a concentration of 3×10^6/ml in BWW medium containing both dbcAMP and caffeine (1 mM each). Anti-PH-20 IgG or anti-CD-46 IgG was added to sperm suspensions at concentrations of 100 μg/ml. Anti-acrosin IgG and SBTI were also tested at concentrations of 300 μg/ml. In each experiment, the IgG or inhibitor was added to the sperm suspension 5 min prior to the addition of oocytes, and one aliquot of sperm suspension was left untreated as a control. Sperm suspensions (200 μl) were transferred to small petri dishes, 5 to 7 oocytes were added per dish, and the sperm-oocyte suspensions were covered with mineral oil and coincubated for 18 hr at 37°C in 5% CO_2 in air. Aliquots of all sperm suspensions were assessed prior to coincubation for the percentage of motile sperm and for the percentage of viable, acrosome reacted sperm using fluorescein isothionate-conjugated Pisum sativum agglutinin (FITC-PSA, Vector Laboratories), as described by VandeVoort et al. (1997). All sperm suspensions used in these experiments had greater than 85%

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motility and less than 5% viable acrosome reacted sperm.

Following coincubation, oocytes were removed from the sperm suspension, washed extensively, and pipetted vigorously to remove sperm from the zona surface. Oocytes then were placed individually on a glass slide with 25 µl of medium and were covered with a glass cover slip supported at its corners with silicone grease containing 75–150 µm glass beads. The cover slip was carefully depressed until the zonae could be rolled between the slide and cover slip. The zonae were observed at 200× magnification using an inverted microscope equipped with Hoffman optics. Oocytes were gently rolled under the cover slip and observed for sperm penetration of the zona pellucida. Oocytes were scored as penetrated if one or more sperm had fully traversed the zona pellucida and were present in the perivitelline space.

For ultrastructural observations of the effects of IgGs and inhibitors on sperm-zona pellucida interaction, anti-PH-20 IgG, anti-CD-46 IgG, anti-acrosin IgG, or SBTI were added to activated sperm suspensions (3×10^6/ml) at concentrations of 100 µg/ml, 5 min prior to the addition of oocytes. Sperm and oocytes were coincubated for 30 sec and then oocytes were transferred to a fresh drop of BWW medium for 3 hrs before fixation in 2% paraformaldehyde in DPBS. In each of three replicate experiments, three oocytes were coincubated with sperm suspensions in the presence of each of the IgGs and with SBTI. After fixation, the oocytes were prepared for electron microscopy as described previously.

RESULTS

Electrophoresis and Western Blotting

When activated acrosome intact sperm were solubilized and analyzed by SDS-PAGE, all three IgGs (anti-PH-20, anti-acrosin, and anti-CD-46) revealed a single band on the Western blot (Fig. 1). Acrosin and PH-20 are both known to undergo molecular modifications after the acrosome reaction that give rise to multiple isoforms; but prior to the acrosome reaction the antibodies recognized a single protein. Anti-PH-20 IgG recognized a protein with an apparent molecular weight of 64 kDa and anti-acrosin IgG recognized proacrosin with a molecular weight of approximately 55 kDa (Fig. 1). Anti-CD-46 recognized a protein with a molecular weight of approximately 48 kDa (Fig. 1).

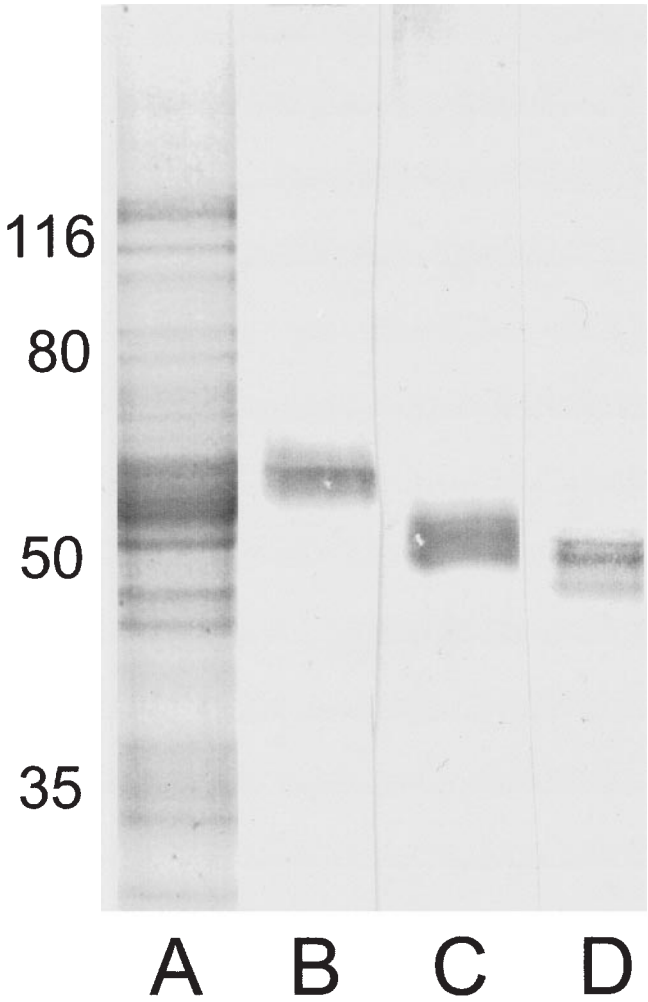

Fig. 1. Western blot analysis of activated, acrosome intact macaque sperm probed with the three different IgGs used for immunolocalization and for sperm-zona pellucida penetration experiments. Lane A shows the sperm preparation stained with Gel Code Blue for total sperm proteins. Lane B is probed with anti-PH-20 IgG. Lane C is probed with anti-acrosin IgG. Lane D is probed with anti-CD-46 IgG. Positions of molecular weight standards (kDa) are shown on left.

Ultrastructural Localization of PH-20, Acrosin, and CD-46

We have shown previously that PH-20 is located on the plasma membrane of the macaque sperm head including the acrosomal cap, equatorial region and posterior head (Overstreet et al., 1995). The present study confirmed this finding for sperm evaluated before and after activation (not shown). There was no labeling of the sperm plasma membrane of non-activated or activated acrosome intact sperm by either anti-CD-46 or anti-acrosin antibodies (not shown). Sperm that were exposed to the released or soluble acrosin showed no evidence of acrosin adsorption on their surface prior to membrane fusion, even during the early stages of the acrosome reaction, such as acrosomal swelling and membrane ruffling (Fig. 2). Both non-activated sperm and activated sperm were incubated with SBTI and subsequently probed with anti-SBTI, and no labeling of the sperm plasma membrane was detected (not shown). The lack of SBTI binding is consistent with the results obtained with anti-acrosin IgG and further suggests the absence of any SBTI-sensitive serine proteases on the sperm surface.

Observations were made of ionophore-treated sperm at three time points in order to observe the early (10 min), intermediate (20 min), and later (30 min) ultrastructural alterations associated with the acrosome reaction. We have previously documented the association of PH-20 with the acrosomal shroud and vesicles of sperm induced to acrosome react by treatment with

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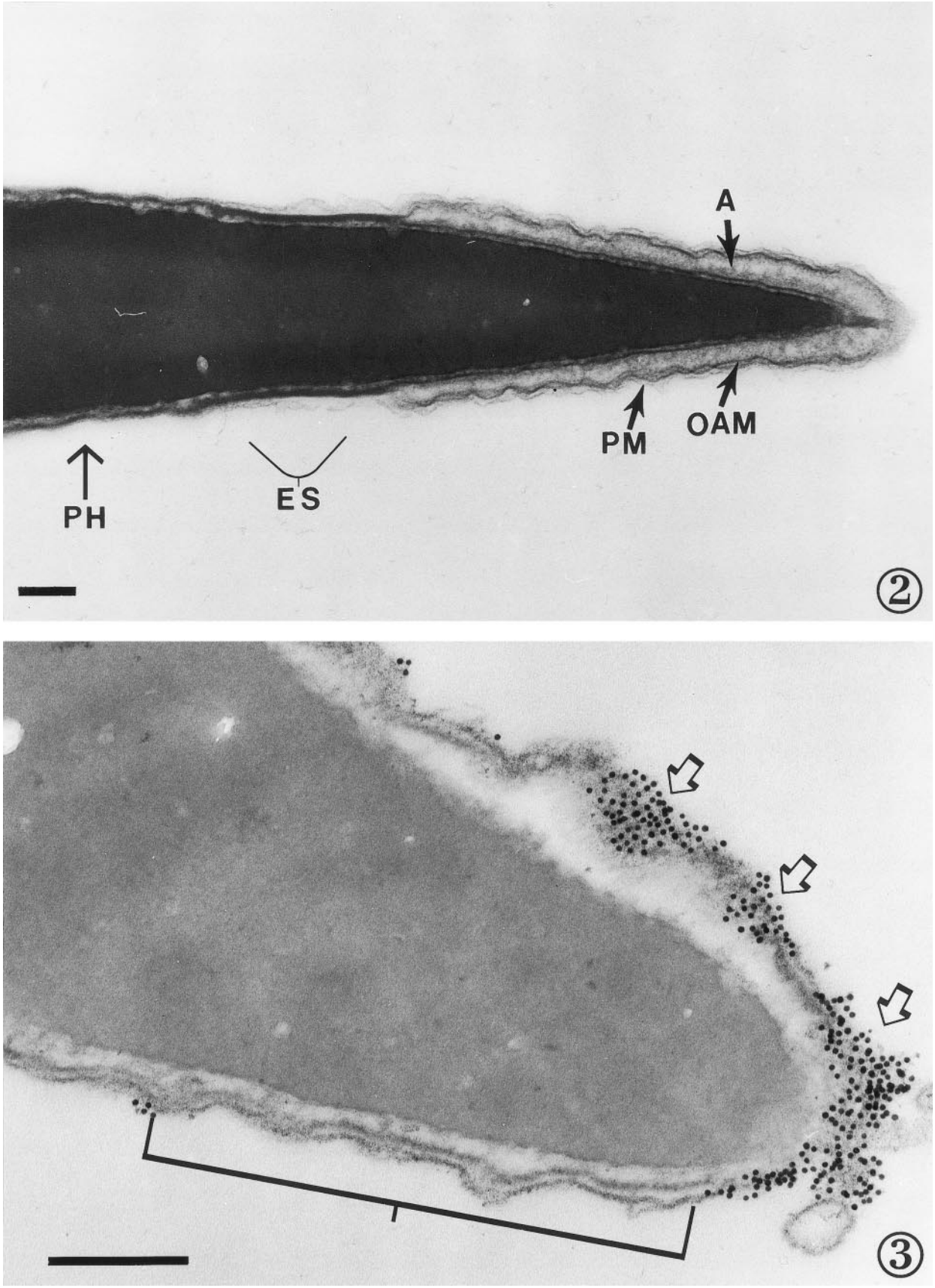

Figures 2 and 3.

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calcium ionophore, as well as the location of PH-20 on the inner acrosomal membrane of these acrosome reacted sperm (Overstreet et al., 1995; Cherr et al., 1996; Yudin et al., 1998). These previous observations were confirmed in the present study (not shown). Labeling of the acrosomal contents with anti-acrosin IgG was apparent at the first sign of vesiculation of the plasma membrane and the outer acrosomal membrane (Fig. 3). Similar observations were made when SBTI was used as the probe (not shown).

In cynomolgus macaque sperm, the acrosomal cap persists following the acrosome reaction, and is composed of the vesiculated plasma membrane and outer acrosomal membrane along with an insoluble matrix that maintains a shroud-like structure (VandeVoort et al., 1997). When anti-acrosin IgG or SBTI was present in the medium prior to initiation of the acrosome reaction, labeling was observed at the earliest stage of membrane fusion leading to a build up of acrosin labeling along the periphery of the acrosomal shroud (Fig. 4). A similar pattern of labeling was observed throughout the shroud and along the inner acrosomal membrane when SBTI was used as the probe (not shown). In cases where the shroud was dislodged, acrosin labeling could be demonstrated on the entire surface of the sperm head, including the inner acrosomal membrane, equatorial segment, and posterior head (Fig. 5). Similar labeling patterns were observed with the SBTI probe (not shown). However, labeling with both probes was confined to the sperm head and no label was observed on the sperm middle piece or flagellum (not shown).

When anti-CD-46 IgG was used as a probe, some labeling could be found within the acrosomal shroud (not shown), and when the shroud was missing, there was strong labeling of CD-46 along the inner acrosomal membrane and little or no labeling on the posterior head and equatorial segment (Fig. 6).

Observations were made of sperm-zona pellucida interaction after 10 min of gamete coincubation in order to observe the induction of acrosome reactions following sperm-zona binding and to observe the initial stages of sperm penetration into the zona. Following the zona pellucida-induced acrosome reaction, cynomolgus macaque sperm are bound to the zona by the acrosomal shroud, through which the sperm penetrates to enter the zona substance (VandeVoort et al., 1997). When anti-acrosin IgG was present during gamete interaction most of the label could be found sandwiched between the zona and the acrosomal shroud (Fig. 7). When oocytes were fixed after gamete interaction and probed with anti-acrosin IgG, the label was distributed throughout the shroud (Fig. 8). The lack of gold observed between the shroud and zona when the sample was fixed prior to labeling was probably due to the inability of the probes to gain access into this region (Fig. 8). Whether oocytes were fixed before or after labeling, there was no evidence of gold particles on the inner acrosomal membrane (Figs. 7 and 8). Similar labeling patterns were observed with the SBTI probe (not shown). Zona-bound sperm that were probed with anti-CD-46 IgG had a labeling pattern of the acrosomal shroud and inner acrosomal membrane that resembled those patterns observed after ionophore treatment (not shown). When oocytes were fixed after gamete interaction and probed with anti-PH-20 IgG, there was evidence of gold label on the inner acrosomal membrane as well as on the acrosomal shroud (Fig. 9).

Sperm Penetration of the Zona Pellucida

Anti-acrosin IgG, SBTI, and anti-CD-46 had no effect on sperm penetration of the macaque zona pellucida in vitro (Table 1). In contrast, anti-PH-20 IgG completely blocked sperm entry into the perivitelline space of the oocytes (Table 1). Ultrastructural observation of oocytes treated with anti-acrosin IgG, SBTI, and anti-CD-46 revealed sperm in the thickness of the zona pellucida after 3 hr of gamete interaction (Fig. 10), supporting the observations by light microscopy of sperm in the perivitelline space. In oocytes treated with anti-PH-20 IgG, electron microscopy revealed no sperm at any stage of zona penetration, suggesting that the immunological block was present at the initial stages of sperm-zona interaction. However, acrosomal shrouds were present on the zona surface of oocytes treated with anti-PH-20 IgG (not shown), indicating that the treatment did not block the zona-induced acrosome reaction.

DISCUSSION

The results of these experiments demonstrate that antibodies directed against PH-20 are capable of blocking sperm penetration of the macaque zona pellucida in vitro, but antibodies to acrosin have no effect on zona penetration. These findings support the model, developed from work with guinea pigs, that PH-20 is involved in secondary sperm-zona binding (Primakoff and Myles, 1983; Primakoff et al., 1988). Acrosin and proacrosin also have been suggested to act as sperm ligands for zona pellucida during secondary binding (Jones et al., 1988; Hedrick et al., 1989; Urch and Patel, 1991; Crosby et al., 1998), but our observations demonstrate that acrosin is not located on the inner acrosomal membrane of the zona pellucida-induced, acrosome reacted macaque sperm, but is found in the acrosomal shroud.

Fig. 2. Immunolocalization of acrosin on acrosome intact macaque sperm 10 min after treatment with Ca^{++} ionophore A23187. This sperm has undergone swelling of the acrosomal cap (A), but there is no sign of fusion between the plasma membrane (PM) and the outer acrosomal membrane (OAM). Prior to membrane fusion there is no evidence of labeling in the acrosomal region, the posterior head (PH) or equatorial segment (ES). Bar = 0.2 μm.

Fig. 3. Immunolocalization of acrosin on acrosome reacting macaque sperm 10 min after treatment with Ca^{++} ionophore A23187. This glancing section of a sperm head captures the initial stages of the membrane fusion events of the acrosome reaction. The three large arrows denote the first signs of fusion and the labeling of acrosin as it is released from the vesiculating acrosome. Regions of the sperm head in which there are no signs of fusion have little or no labeling. Bar = 0.2 μm.

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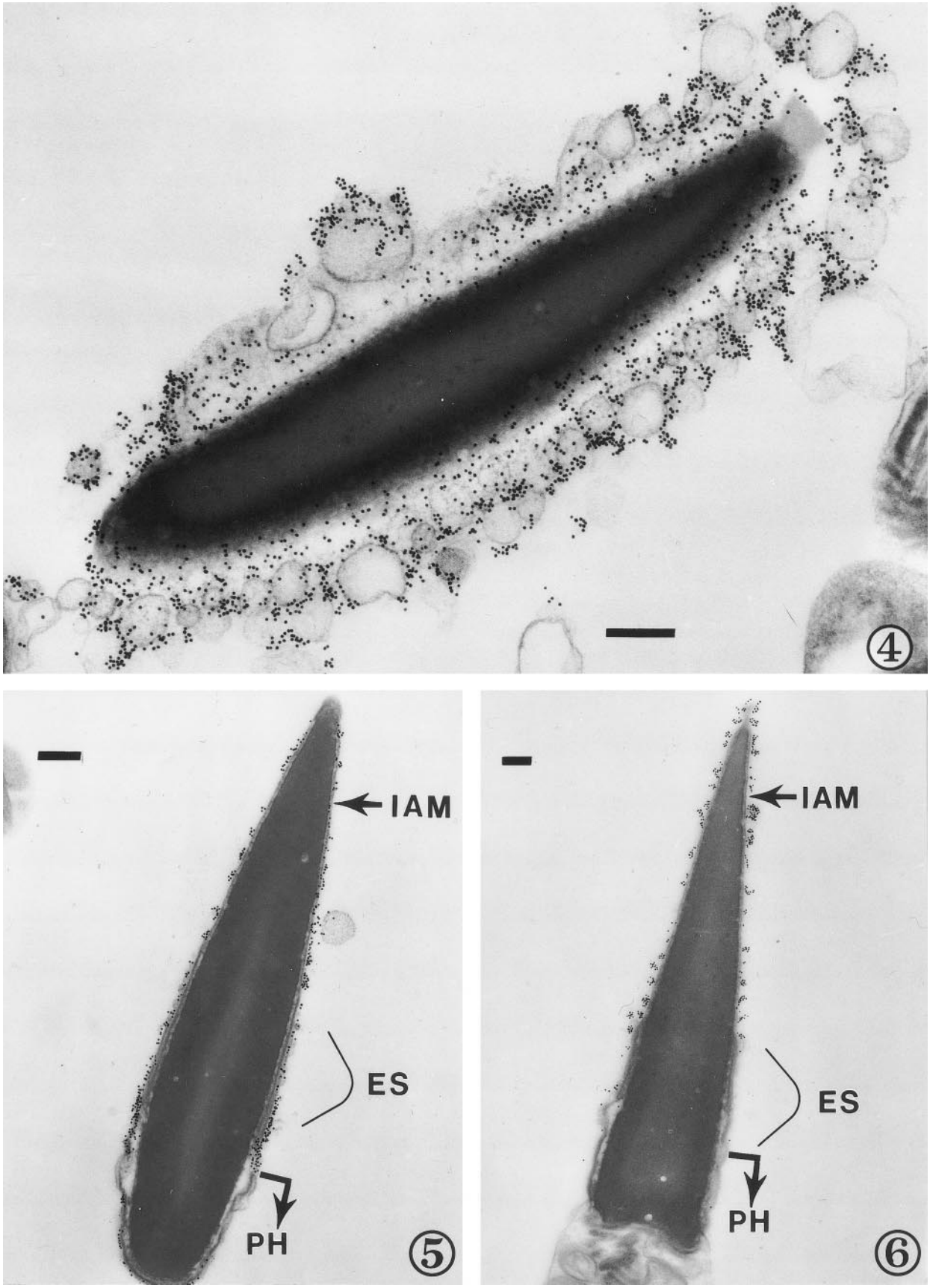

Figures 4–6.

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The mechanisms by which mammalian sperm penetrate the zona pellucida have been a subject of speculation for a number of years (Green, 1988). There is no consensus on whether sperm enzymes are required for zona penetration, whether they enhance penetration or whether flagellar activity alone is sufficient for sperm passage through the zona (Drobnis et al., 1988; Bedford, 1991, 1998, for review). It has been reported that the sperm head has a radically sharpened blade angle following the acrosome reaction (Bedford and Hoskins, 1990), that hydrolytic enzymes appear on the inner acrosomal membrane (Lopez and Shur, 1987; Phelps and Myles, 1987; Barros et al., 1992), and that there is a change in the flagellar beat pattern (Yanagimachi, 1988; for review). The morphological changes and increase in flagellar thrust that are associated with the acrosome reaction have been well documented and accepted, but the appearance and function of sperm-bound enzymes following the acrosome reaction is still controversial (Saling, 1981; Tesarik et al., 1988; Liu and Baker, 1993).

Both acrosin and PH-20 have been located on the inner acrosomal membrane (Phelps and Myles, 1987; Barros et al., 1992) and both enzymes have a component of the zona that could serve as substrate (Urch et al., 1985; Kan, 1990). Acrosin and PH-20 are also reported to have adhesive functions, as well as lytic activities (Myles et al., 1987; Jones, 1991). Specific anti-acrosin antibodies and protease inhibitors (SBTI) do not block primary sperm-zona binding, but low molecular weight protease inhibitors (i.e., benzamidine) block induction of the acrosome reaction (Tesarik et al., 1990; Pillai and Meizel, 1991; Llanos et al., 1993). In the present experiments, sperm incubated with SBTI or anti-acrosin IgG and a secondary gold label showed no sign of acrosin or SBTI-sensitive protease activity on the sperm surface until the first sign of fusion between the plasma membrane and outer acrosomal membrane. Even when sperm were incubated in the presence of a high percentage of acrosome reacted sperm (>60%) there was no evidence of acrosin on the surface of acrosome intact sperm. These results agree with Jones and Williams (1990) that acrosin is present on the external sperm surface only after the acrosome reaction, but they disagree with the findings of Tesarik et al. (1988).

Following the acrosome reaction and prior to zona penetration, macaque sperm adhere to the zona pellucida by a matrix of fused acrosomal vesicles, which is termed the acrosomal shroud (VandeVoort et al., 1997). In the present study, we demonstrated that this shroud is heavily labeled by anti-acrosin antibodies. This observation is consistent with a function of proacrosin and acrosin in binding sperm to the zona pellucida after the acrosome reaction (Jones et al., 1988; Hedrick et al., 1989), and it is possible that acrosin is responsible for adhesion of the acrosomal shroud to the zona surface. Bliel et al. (1988) have shown that the ZP-2 component of mouse zona pellucida can bind to acrosome reacted mouse sperm, although it is not clear whether this binding was to an acrosomal shroud or to the surface of the inner acrosomal membrane. Nevertheless, it is believed that ZP-2 binds to the inner acrosomal membrane of mouse sperm (Bliel et al., 1988) and that acrosin is the ligand to which ZP-2 is bound (Huang and Yanagumachi, 1985; Mortillo and Wasserman, 1991).

In our experiments, acrosin was present on the inner acrosomal membrane as well as the equatorial segment and posterior head of macaque sperm that were induced to acrosome react by treatment with calcium ionophore. However, when sperm acrosome reacted as a consequence of binding to the zona, acrosin was present in the acrosomal shroud but no acrosin could be demonstrated on the inner acrosomal membrane. The appearance of acrosin on the surface of ionophore-treated sperm may be a result of adhesion of soluble acrosin to the sperm surface. Although the adhesion of acrosin to sperm membranes appears to be an experimental artefact, there is some specificity in the binding, because acrosin did not bind to any surface of acrosome intact sperm and it was never observed on the sperm midpiece or flagellum. It is likely that a similar adhesion of soluble acrosin to acrosome reacted sperm was not observed on the zona pellucida because of the relatively low local concentration of the soluble enzyme in these preparations. Our demonstration that macaque sperm do not express acrosin on the inner acrosomal membrane raises the question of what sperm component may bind ZP-2, if this aspect of secondary sperm-zona binding is important during zona penetration. Recently, the demonstration of fertility in acrosin null mice led to speculation that a sperm-bound protease other than acrosin may facilitate sperm penetration of the zona (Baba et al., 1994). Our observations suggest that there are no SBTI-sensitive serine proteases on the inner acrosomal membrane of macaque sperm that are induced to acrosome react by contact with the zona pellucida (not shown).

On the other hand, there is strong evidence that PH-20 is present on the surface of the plasma membrane overlying the acrosome in sperm of mice, rats, monkeys, and humans and on the inner acrosomal membrane in all of these species, as well as the guinea pig (Phelps and Myles, 1987; Lin et al., 1993; Overstreet et al., 1995; Jones et al., 1996). The present

Fig. 4. Immunolocalization of acrosin on acrosome reacting macaque sperm 20 min after treatment with Ca^{++} ionophore A23187. Gold labeling of acrosin is observed throughout the vesiculated acrosomal shroud. Bar = 0.2 μm.

Fig. 5. Immunolocalization of acrosin on acrosome reacting macaque sperm 30 min after treatment with Ca^{++} ionophore A23187. The acrosomal shroud has been lost and the inner acrosomal membrane (IAM) has been exposed. Gold labeling of acrosin is observed along the entire sperm head, including the IAM, equatorial segment (ES) and posterior head region (PH). Bar = 0.2 μm.

Fig. 6. Immunolocalization of CD-46 on acrosome reacting macaque sperm 30 min after treatment with Ca^{++} ionophore A23187. The acrosomal shroud has been lost and the inner acrosomal membrane (IAM) has been exposed. After probing with the CD-46 antibody, the majority of the label could be found along the IAM with little or no labeling of the equatorial segment (ES) and posterior head (PH). Bar = 0.2 μm.

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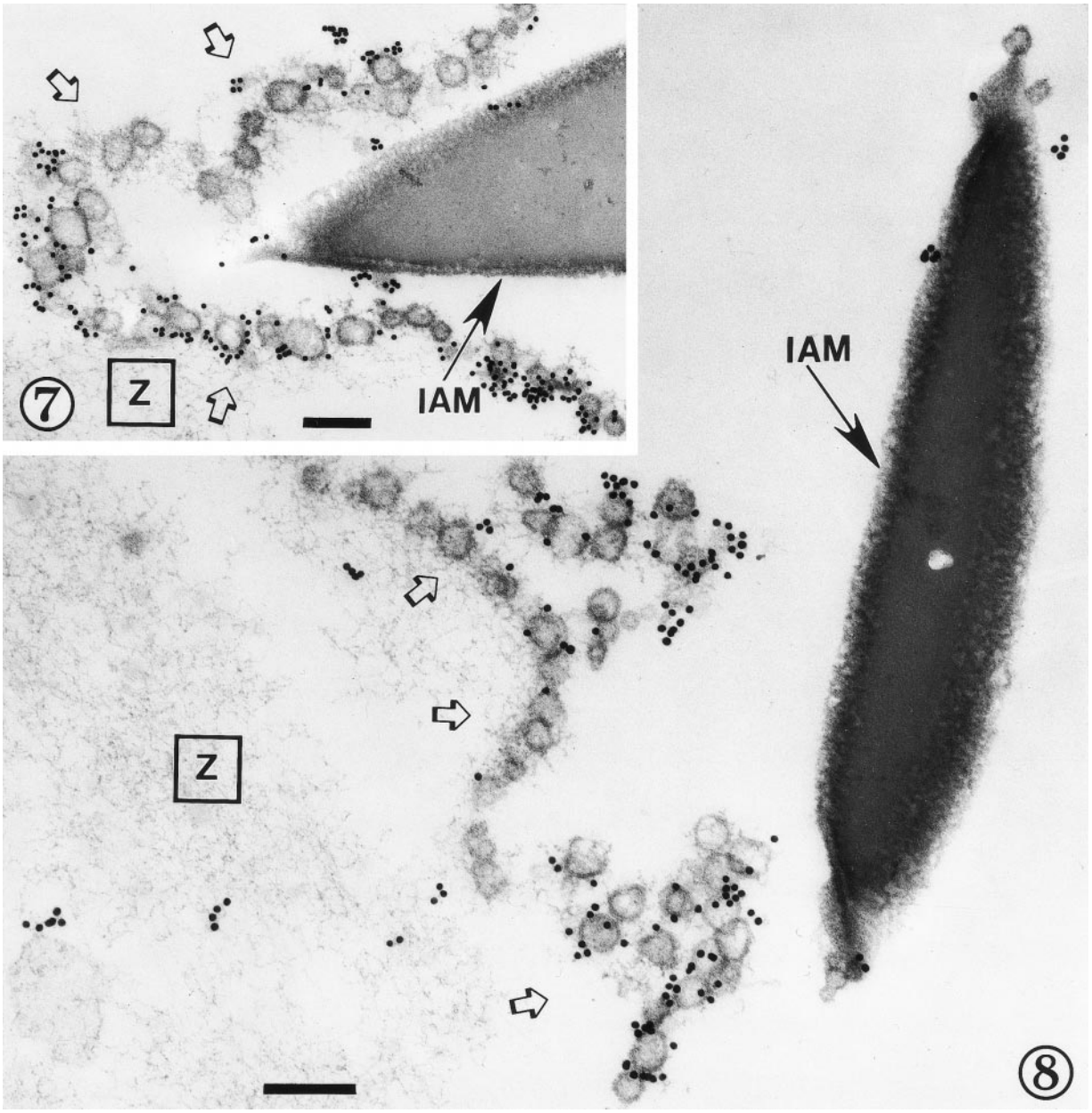

Fig. 7. Immunolocalization of acrosin on acrosome reacted sperm bound to the macaque zona pellucida following 10 min of gamete coincubation. Acrosin labeling was primarily found sandwiched between the vesiculated shroud (arrows) and the zona pellucida (Z). The inner acrosomal membrane (IAM) had minimal labeling. Bar = 0.2 µm.

Fig. 8. Immunolocalization of acrosin on acrosome reacted sperm bound to the macaque zona pellucida following 10 min of gamete coincubation. The oocytes were fixed prior to addition of anti-acrosin IgG. The acrosomal shroud (arrows) is closely apposed to the zona pellucida (Z). Acrosin labeling was distributed throughout the shroud, but very little label was associated with the inner acrosomal membrane (IAM). Bar = 0.2 µm.

experiments demonstrate that PH-20 is present on the inner acrosomal membrane of macaque sperm following the zona pellucida-induced acrosome reaction. There is evidence that guinea pig sperm PH-20 which is present in the posterior head region migrates to the inner acrosomal membrane after the acrosome reaction (Primakoff et al., 1988), and that the PH-20 along the inner acrosomal membrane contributes to sperm-zona adhesion (Myles et al., 1987). The PH-20 on the inner acrosomal membrane has been endoproteolytically cleaved, which may be necessary for its zona binding capability (Primakoff et al., 1988; Cherr et al., 1996). While there is ample evidence that the PH-20 on the inner acrosomal membrane of guinea pig sperm functions as a zona binding molecule, there has been no evidence for a similar function of PH-20 in other mammalian sperm (Hunnicutt et al., 1996).

Acrosome intact and acrosome reacted sperm can bind to and fertilize the oocytes of guinea pigs and rabbits, but whether sperm that are acrosome reacted

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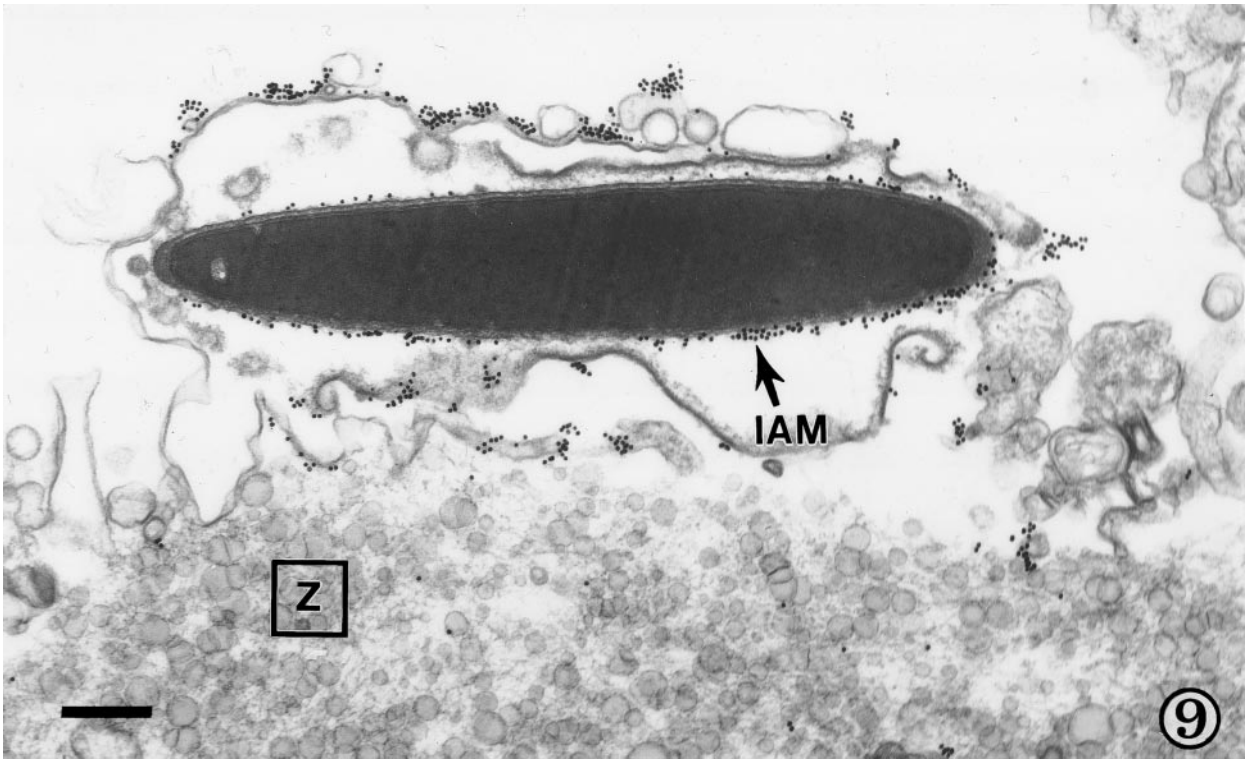

Fig. 9. Immunolocalization of PH-20 on acrosome reacted sperm bound to the macaque zona pellucida following 10 min of gamete coincubation. The oocytes were fixed prior to addition of anti-PH-20 IgG. PH-20 labeling was found associated with the acrosomal shroud and the inner acrosomal membrane (IAM). The sperm is bound to the zona pellucida (Z). Bar = 0.2 μm.

prior to zona binding can fertilize the oocytes of humans, monkeys, or mice is either not known or doubtful (Kuzan et al., 1984; Myles et al., 1987; Morales et al., 1989; VandeVoort et al., 1997). It is not known whether sperm of all mammalian species bind to the zona by an acrosomal shroud following the zona-induced acrosome reaction, and it may be necessary to distinguish the mechanisms for sperm-zona binding via the shroud versus those that mediate binding between the zona and components of the inner acrosomal membrane. Although macaque sperm are bound to the zona by their shrouds after the acrosome reaction is induced on the zona surface, when sperm are induced to undergo the acrosome reaction and are then exposed to zona, shrouded sperm are less capable of binding to the zona in comparison with sperm having nude inner acrosomal membranes (VandeVoort et al., 1997). If acrosin is important in binding of the acrosomal shroud to the zona surface, then loss of acrosin from the acrosome reacted sperm could have reduced their zona binding capability, even though their acrosomal shrouds were retained. It is likely that true secondary sperm-zona binding is initiated in macaques when the sperm penetrates through the shroud and the inner acrosomal membrane comes in contact with the zona substance (VandeVoort et al., 1997). The previous experiments of VandeVoort et al. (1997) demonstrate that the inner acrosomal membrane of macaque sperm has an affinity for the zona pellucida.

In the present experiments, sperm were treated with anti-PH-20 antibodies during gamete interaction, and although vigorous sperm motility was maintained, penetration of the zona pellucida was blocked. Treatment of oocytes with an antibody to CD-46, another inner acrosomal membrane protein, had no effect on sperm penetration of the zona. Fine structural evaluation of oocytes treated with anti-PH-20 IgG revealed no evidence of sperm entry into the zona, but a number of empty acrosomal shrouds were present on the zona surface. This observation suggests that neither primary sperm-zona binding nor the zona-induced acrosome reaction was blocked by anti-PH-20 IgG. These observations are consistent with the results of our previously reported experiments in which Fab fragments of anti-PH-20 IgG did not block sperm binding to the macaque zona pellucida or the zona-induced acrosome reaction (Yudin et al., 1998). However, macaque sperm PH-20 became aggregated following sperm-zona binding, and when PH-20 aggregation was induced by treatment with anti-PH-20 IgG, there was an increase in internal sperm calcium, acrosomal swelling, and morphological acrosome reactions in approximately 20% of treated sperm (Yudin et al., 1998). We interpret these results to support the hypothesis that the PH-20 protein is involved in sperm-zona pellucida signaling and induction of the acrosome reaction, but possibly not as the primary recognition ligand for the zona.

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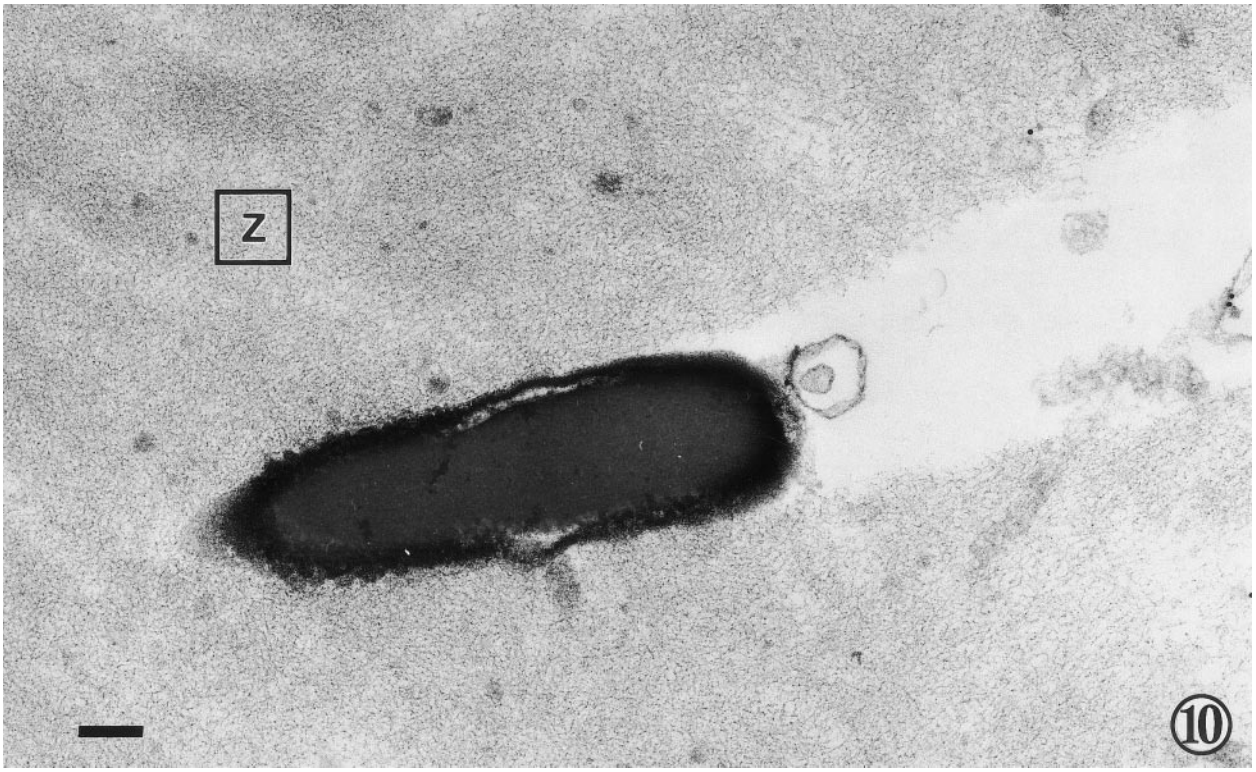

Fig. 10. Fine structural view of macaque sperm penetrating the macaque zona pellucida (Z). This micrograph was taken from an experiment in which sperm were treated with anti-acrosin IgG, but similar observations were made following treatment with SBTI and anti-CD-46 IgG. As the sperm penetrates the zona there is a well defined slit in its wake and no obvious sign of compaction along the leading edge. Bar = 0.2 µm.

TABLE 1. Penetration of Macaque Zonae Pellucidae by Sperm Treated With Specific Inhibitors or Antibodies*

Treatment	% Oocytes penetrated	No. penetrations/oocyte
Experiment 1		
Control	93.3 ± 11.5[a]	2.5 ± 0.4[a]
Anti-PH-20 IgG	0 ± 0[b]	0 ± 0[b]
Anti-CD-46 IgG	97.0 ± 8.0[a]	2.0 ± 0.8[a]
Experiment 2		
Control	100 ± 0	4.7 ± 0.6[a]
Anti-acrosin IgG	100 ± 0	3.1 ± 0.9[a]
SBTI	100 ± 0	4.1 ± 0.4[a]

*Data are presented as means ± SEM, N = 4.
[a,b]Different superscripts within a column indicate significant difference ($P < 0.05$).

The absence of sperm in any stage of zona penetration supports the conclusion that secondary sperm binding to the zona was blocked by treatment with anti-PH-20 IgG. PH-20 has been shown to be an adhesion molecule for both guinea pig and rat zonae (Hunnicutt et al., 1996; Jones et al., 1996), and it is likely that the mechanism of anti-PH-20 antibody-induced blockage of macaque zona penetration may involve interference with sperm- zona adhesion. Gacesa et al. (1994) have raised the question of whether PH-20 could bind to hyaluronic acid which is known to be a component of the zona (Kan, 1990). Hunnicutt et al. (1996) carried out exhaustive hyaluronidase treatments of guinea pig zonae and reported no decrease in sperm binding following such treatments. Because our experiments were performed with ovarian oocytes, which were recovered from follicles prior to cumulus formation, it is likely that they had little or no hyaluronic acid in the zona. Therefore, the present results also cast doubt that hyaluronic acid is the recognition molecule for PH-20 within the macaque zona pellucida.

The study of fertilization biology in non-human primates is complicated by the limited availability of oocytes, but the capability to study large numbers of morphologically normal sperm in synchronized capacitation systems (VandeVoort et al., 1992, 1997) is highly advantageous. The cell biology and biochemistry of the PH-20 protein have been thoroughly investigated in macaques (Cherr et al., 1996; Li et al., 1997a; Yudin et al., 1998), and the biochemical characteristics and functions of this non-human primate sperm are similar in most respects to those of human sperm (Sabeur et al., 1997, 1998). The results of the present study provide morphological and experimental evidence that implicates the PH-20 protein in the process of secondary sperm binding to the macaque zona pellucida. These findings, together with our previous observations on the enzymatic actions of PH-20 during cumulus penetration (Li et al., 1997b; Meyers et al., 1997) and its

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functions in cell signaling during interaction with the cumulus (Cherr et al., 1998) and zona pellucida (Yudin et al., 1998), underscore the importance of this sperm protein in macaque fertilization.

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