Regionalization of Transmembrane Glycoproteins in the Plasma Membrane of Boar Sperm Head Is Revealed by Fracture-Label

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ABSTRACT We used fracture-label and surface labeling techniques to characterize the distribution and topology of wheat germ agglutinin (WGA) receptors in the plasma membrane of boar sperm heads. We show that freeze-fracture results in preferential, but not exclusive, partition of WGA-binding sites with the outer (exoplasmic) half of the plasma membrane. Labeling of the inner (protoplasmic) half of the membrane is significant, and is denser over the areas that overlie the acrosome. Exoplasmic membrane halves are uniformly labeled. Analysis of freeze-fracture replicas revealed that the distribution of intramembrane particles over protoplasmic faces parallels that of WGA-binding sites as observed by fracture-label. Coating of intact spermatozoa with cationized ferritin results in drastic reduction of the labeling of both protoplasmic and exoplasmic membrane halves. Labeling of sperm cells lysed by short hypotonic shock fails to reveal the presence of WGA-binding sites at the inner surface of the plasma membrane. We conclude that: (*a*) all WGA-binding glycoconjugates are exposed at the outer surface of the membrane; (*b*) some of these glycoconjugates correspond to transmembrane glycoproteins that, on fracture, partition with the inner half of the membrane; (*c*) these transmembrane proteins are accumulated in the region of the plasma membrane that overlies the acrosome; and (*d*) parallel distribution of intramembrane particles and WGA-binding glycoproteins provides renewed support for the view of particles as the morphological counterpart of integral membrane proteins.

The mammalian sperm head is covered by a plasma membrane that is unique in its differentiation into two major topographic regions: a distal area that overlies the acrosome and a proximal zone which coats a segment of the nucleus. The two portions of the membrane show, at the outer surface, unequal complements of negative charges (1, 2), enzymes (3), glycoconjugates (4–9), antigens (10–21), and of a collagen-binding protein (22), as well as distinct amounts of sterols (23, 24) and anionic lipids (25–27). Different roles are committed to these two membrane areas during fertilization: the distal portion fuses with the acrosomal envelope, an event that leads to exocytosis of the enzymes contained in the acrosome (28, 29); the proximal portion, which remains intact during the acrosome reaction, participates in gamete fusion (29, 30).

The relative ability to bind lectins—wheat germ agglutinin (WGA)[1] in particular—is one of the parameters used to distinguish the two surface domains in the mammalian sperm head (4–9, 31). WGA binds to sialic acid, the terminal saccharide in fully glycosylated membrane proteins, although binding can also involve subterminal *N*-acetyl glucosamine residues (32, 33). Recently, we introduced a new system of techniques, fracture-label, that permits in situ cytochemical labeling of membrane halves obtained by freeze-fracture of cells or tissues (34–36). With fracture-label, questions related to the expression of specific components in each membrane

[1] *Abbreviations used in this paper:* IMP, intramembrane particles; SPBP, Sorensen's phosphate buffer with 4% polyvinylpyrrolidone; and WGA, wheat germ agglutinin.

half can be approached including, in favorable instances, the identification of transmembrane proteins (34, 37, 38). Here we used fracture-label and surface labeling techniques to investigate the distribution and partition of WGA receptors in intact, freeze-fractured, and physically disrupted plasma membranes of boar sperm head. Our results indicate that labeling of protoplasmic membrane halves by WGA corresponds to transmembrane WGA-binding glycoproteins intercalated across the plasma membrane of sperm cells. These transmembrane proteins appear preferentially accumulated over the region of the plasma membrane that overlies the acrosome, the same area in which intramembrane particles (IMP) are concentrated.

MATERIALS AND METHODS

Numerous samples of raw semen from mature boars, *Sus scrofa* (a generous gift of Dr. Larry Johnson, US Department of Agriculture, Beltsville, MD), were collected with the aid of an artificial vagina, and centrifuged (1,500 rpm) for 5 min, at room temperature. The spermatozoa-rich pellets were washed by successive pelleting and resuspension (three times) in Hanks' balanced salt solution or in PBS. Sperm cells were then fixed in 1.5% glutaraldehyde in PBS, pH 7.4, at ice bath temperature for 1 h, and washed twice in PBS. The samples were divided and treated differently, each method aimed to make distinct planes of the plasma membrane accessible to cytochemical labeling.

Thin-Section Fracture-Label: Fixed and washed sperm were thoroughly mixed in 30% (wt/vol) BSA in PBS, and centrifuged (3,000 rpm) for 10 min. After removal of the BSA supernatant, the mixture of BSA-cells was gelled by the addition of glutaraldehyde to a total concentration of 1%. The gels were processed for freeze-fracture cytochemistry, according to a method previously described (34, 36). The gels were sliced, impregnated in 30% glycerol in PBS (3 h), frozen in partially solidified Freon 22, transferred to liquid nitrogen, and finely crushed with a glass pestle. The fragments of freeze-fractured gels were thawed, deglycerinated, and incubated in a solution of WGA (0.25 mg/ml, Sigma Chemical Co., St. Louis, MO) in 0.1 M Sorensen's phosphate buffer with 4% polyvinylpyrrolidone (SPBP), pH 7.4, for 1 h at 37°C. Controls were preincubated in 0.4 M *N*-acetyl-D-glucosamine in SPBP (15 min), and in the same WGA solution in the presence of the sugar (0.4 M) during 1 h at 37°C. After washing in Sorensen's buffer, the fragments were incubated in ovomucoid-coated colloidal gold (39–41) in SPBP for 3 h at 4°C. Finally, the fragments were washed in SPBP and Sorensen's buffer and processed for thin-section electron microscopy.

Some samples of fixed and washed sperm cells were pre-coated with cationized ferritin in PBS, pH 7.2 (1 mg/ml, Miles Biochemicals, Elkhart, IN) before fracture label. Three successive incubations of 15 min were performed. Coated spermatozoa were washed in PBS, and mixed in 30% BSA in PBS. BSA-cells gels were obtained by glutaraldehyde cross-linking, and processed for freeze-fracture and WGA-ovomucoid-colloidal gold labeling as described above.

Critical-point Drying Fracture-Label: Fixed and washed sperm were impregnated in 30% glycerol in PBS, and mixed in a solution of 30% BSA in 30% glycerol (in PBS). Small droplets of the mixture were dispersed on precleaned glass coverslips that were superimposed on other coverslips paved with an equal number of droplets (total volume ~4 μl) of 3% glutaraldehyde in 30% glycerol/PBS. The pairs of coverslips strongly held together due to the cross-linking of BSA by glutaraldehyde. The preparations were frozen in the liquid phase of partially solidified Freon 22, and stored in liquid nitrogen. Freeze-fracture of the BSA-cells gels was performed by mechanical separation of complementary coverslips immersed in liquid nitrogen. The coverslips, paved by the fractured gel, were thawed, deglycerinated, and incubated in WGA and ovomucoid-colloidal gold as described above. After cytochemical labeling, we fixed the preparations in 1% osmium tetroxide in veronal acetate buffer for 30 min, dehydrated in ethanol, and critical-point dried in ethanol/carbon dioxide (35). The fractured gels were shadowed with platinum/carbon. We obtained replicas of the fracture-faces by digestion of the gel in sodium hypochlorite, and mounted on Formvar-coated grids for electron microscopic observation.

Conventional Freeze-Fracture: In these experiments, we used droplets of fresh boar semen directly dropped into the fixative in addition to fixed and washed sperm. The cells were washed in PBS, glycerol-permeated, and squeezed between two copper disks of a double-replica device (42). The samples were frozen in the liquid phase of partially solidified Freon 22 cooled by liquid nitrogen, and stored in liquid nitrogen. Freeze-fracture was carried out at −140°C in a Balzers 301 apparatus (Balzers, Hudson, NH). Replicas were obtained by evaporation from a platinum/carbon electron source, and cleaned in sodium hypochlorite and distilled water. Nomenclature of fracture faces is according to Branton et al. (43).

Surface Labeling: Fixed and washed spermatozoa were incubated with WGA and ovomucoid-colloidal gold (as described above), washed in SPBP and PBS, and processed for thin-section electron microscopy (see below).

Labeling of the Inner Surface of the Plasma Membrane: Osmotic shock was induced on unfixed spermatozoa by incubation of the cells in distilled water for 5 min at room temperature. The sperm were washed twice in PBS, fixed in 1.5% glutaraldehyde in PBS (1 h), washed in the buffer, and incubated in WGA followed by ovomucoid-colloidal gold, as described above. The samples were washed in SPBP and PBS, and processed for thin-section electron microscopy.

Processing for Thin-Section Electron Microscopy: Isolated cells and fractured gels were postfixed in 2% osmium tetroxide in veronal acetate buffer, reduced by the addition of 1% potassium ferricyanide (44), stained en bloc with uranyl acetate (5 mg/ml) in the same buffer, pH 5.8, dehydrated in acetone, and embedded in Epon 812. We used light microscopic examination of thick sections to select fracture areas for thin sectioning. Thin sections were observed by electron microscopy after counterstaining with lead citrate only.

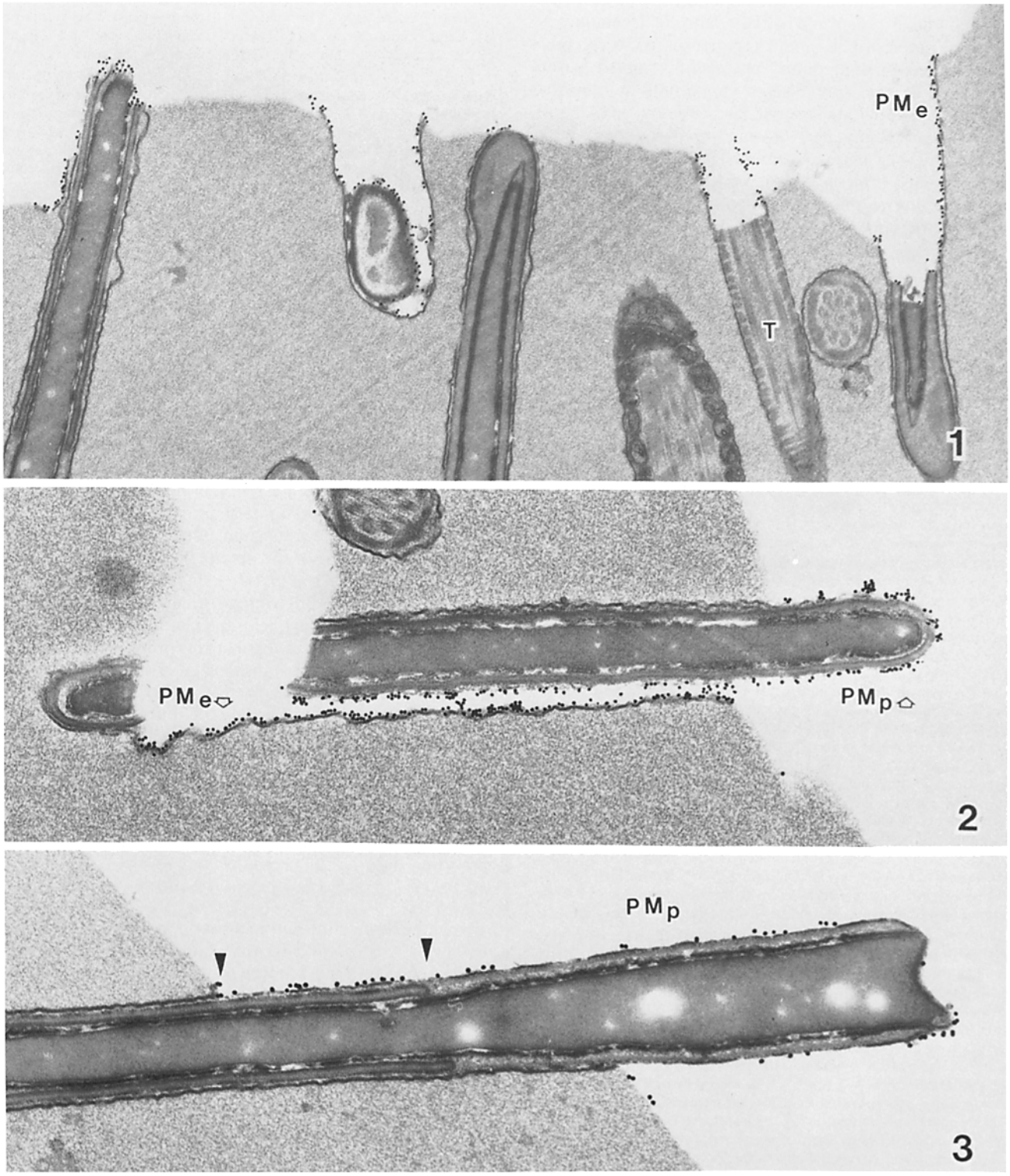
RESULTS

Ultrastructure

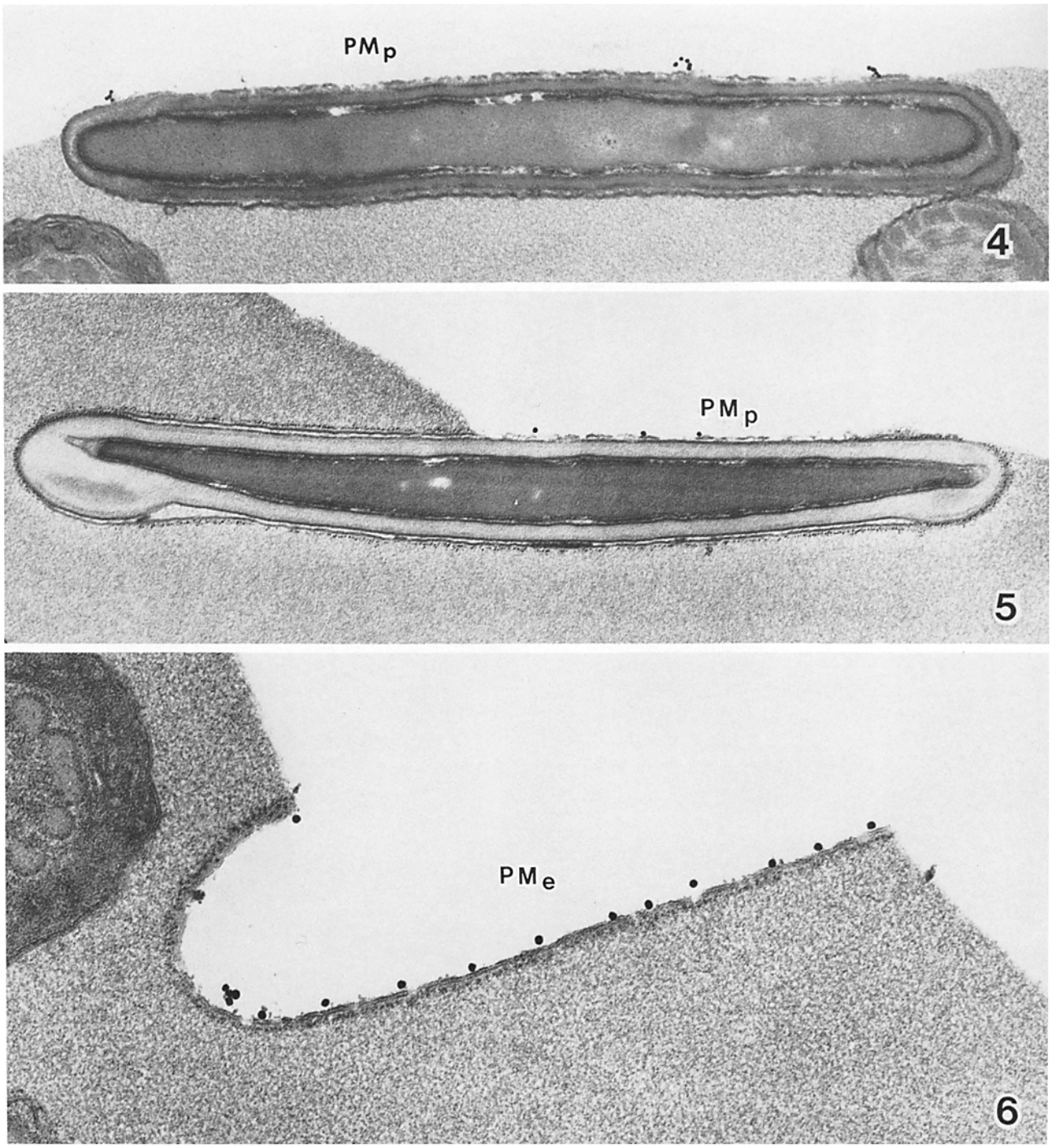
The quality of preservation of sperm cells prepared for thin-section fracture-label was comparable to that obtained after conventional processing for electron microscopy (Figs. 1–6). This shows that the additional steps (glycerination, freezing, fracture, thawing, and deglycerination) necessary to achieve cytochemical labeling of membrane halves do not affect the fine morphology of spermatozoa. In addition, sperm cells embedded (after fixation) in glutaraldehyde-cross-linked BSA show a decrease in wrinkling of the plasma membrane (Figs. 1–6) generally observed in spermatozoa processed without the support of a matrix. The morphology of membrane halves, as seen in thin sections, was identical to that observed in other fracture-labeled cells and tissues; both inner and outer halves of the membrane were seen as interrupted trilaminar profiles (Figs. 2–6). (The appearance of membrane halves as interrupted unit-membrane segments results from reorganization of membrane apolar groups exposed, upon fracture, to a polar (aqueous) environment, as evidenced and discussed in detail in previous papers from this laboratory [34, 36, 37].)

Thin-section Fracture-Label

Upon freeze-fracture and incubation in WGA followed by ovomucoid-coated colloidal gold, both protoplasmic (inner) and exoplasmic (outer) halves of the plasma membrane of boar sperm head were well labeled, whereas the surface of fractured BSA was virtually clean of colloidal gold spheres (Figs. 1–3). Comparison of the density of colloidal gold label between the two membrane halves showed preferential association of WGA with exoplasmic halves. This was best seen in gel "cracks" because they provide the visualization of complementary halves of fractured membranes (Fig. 2). WGA receptors were uniformly distributed along exoplasmic membrane halves (Figs. 2 and 3). In contrast, the inner half of the membrane showed reduced density of colloidal gold spheres over the postacrosomal region (Fig. 3). The specificity of WGA labeling was demonstrated by its drastic reduction (≥90%) when fractured cells were incubated in WGA in the presence of *N*-acetyl-D-glucosamine (Fig. 4). Coating of sperm cell surface with cationized ferritin prior to freeze-fracture also led to marked decrease (≥70%) in the density of label over both protoplasmic and exoplasmic membrane halves (Figs. 5 and 6).



FIGURES 1–3 Fig. 1: Thin section of freeze-fractured gel of sperm-BSA incubated in WGA followed by labeling with ovomucoid-coated colloidal gold. Exoplasmic halves of the plasma membrane (PM_e) from sperm head and sperm tail (*T*) are strongly labeled. The surface of BSA is devoid of colloidal gold. × 29,000. Fig. 2: WGA labeling of complementary halves of the plasma membrane of sperm head at the acrosomal-cap region. Both exoplasmic (PM_e) and protoplasmic (PM_p) halves of the membrane are well labeled. The density of WGA-ovomucoid-colloidal gold complexes is higher on the PMe than on the PMp. × 39,000. Fig. 3: Thin section fracture-label showing unequal density of WGA receptors along the protoplasmic membrane half (PM_p) of the plasma membrane of boar sperm head: the postacrosomal zone of the membrane is less intensely labeled by colloidal gold than the distal (acrosomal cap) area (between arrowheads). × 42,900.



FIGURES 4–6 Fig. 4: Control of the specificity of WGA labeling. Thin-section fracture-label shows only a few WGA-ovomucoid-colloidal gold complexes associated with the protoplasmic membrane half (PM_p) of boar sperm head plasmalemma. The fractured gel was incubated in WGA in the presence of the competitive sugar (*N*-acetyl-D-glucosamine), followed by ovomucoid-coated colloidal gold. × 45,000. Figs. 5 and 6: Thin section fracture-label of boar sperm head coated with cationized ferritin prior to freeze-fracture and incubation in WGA. Ovomucoid-colloidal gold label is scarce on both protoplasmic (PM_p, Fig. 5) and exoplasmic (PM_e, Fig. 6) halves of the plasma membrane. × 40,000 and × 83,000.

Critical-Point Drying Fracture-Label

A variant of the basic method to obtain fracture faces of BSA-cells gels for critical-point drying fracture-label (40) was introduced in this study. The sperm head has a disk-like shape that on fracture with a scalpel, as used before (35, 37, 38, 45), gives limited views of the membrane halves. Here, we were able to obtain extended views of the plasma membrane of sperm heads by fracturing spermatozoa "sandwiched" between two glass coverslips, followed by thawing and cytochemical labeling (Figs. 7–9). Upon critical-point drying, these specimens show clear differences in the appearance of protoplasmic membrane halves in postacrosomal and acrosomal-cap areas (Fig. 7). While postacrosomal regions have a mosaic-

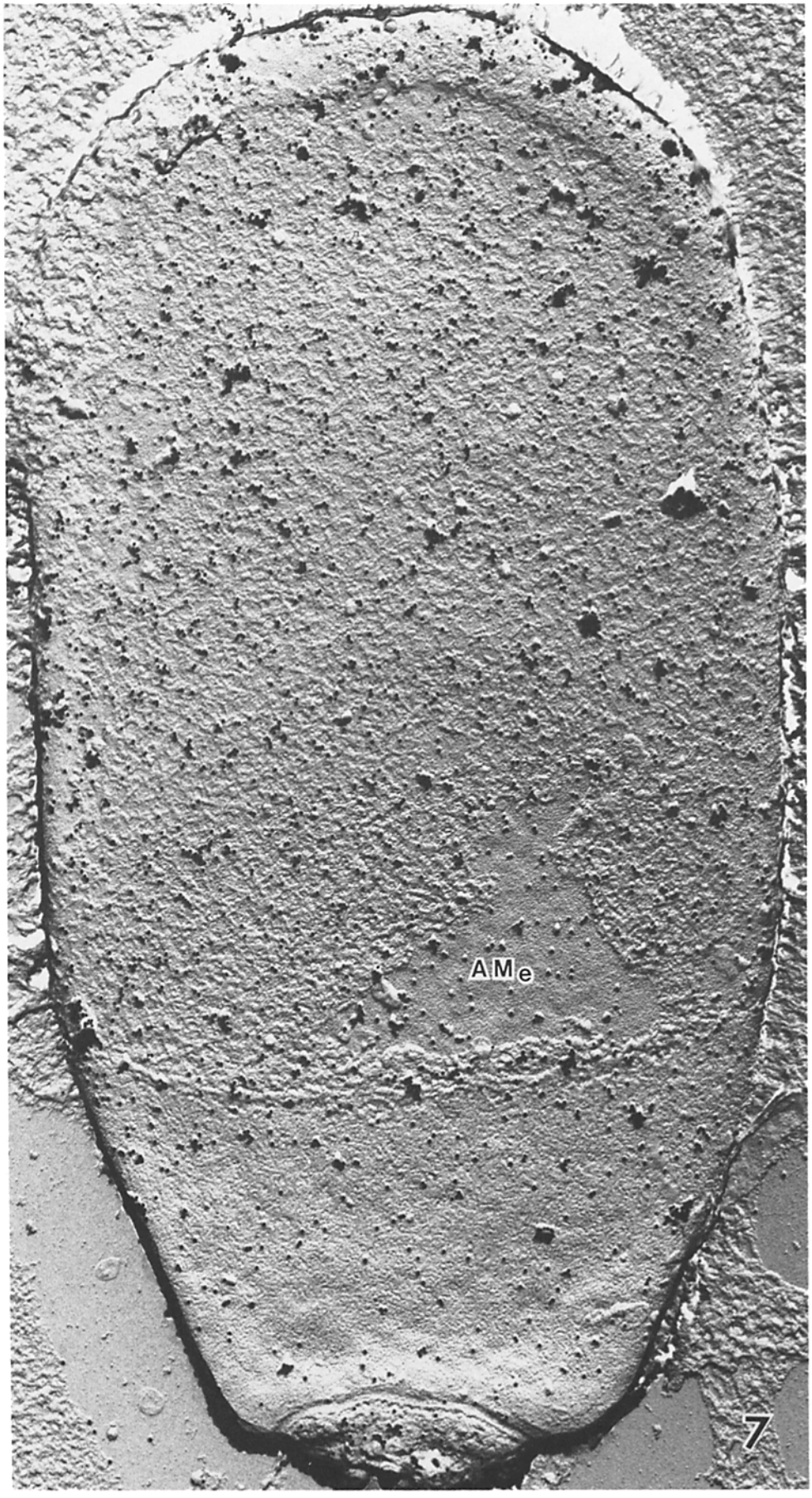


FIGURE 7 Critical-point drying fracture-label of boar sperm head shows the distribution of WGA-ovomucoid-colloidal gold along the protoplasmic half of the plasma membrane. The density of colloidal gold spheres is higher over the distal area (acrosomal cap) than in the proximal (postacrosomal) region of the membrane half (for tracing of colloidal gold, see Fig. 8). The two domains of the membrane half show different morphology: the mosaic-like structure of the postacrosomal region (lower portion in the figure) contrasts with the rough texture of the acrosomal-cap area. A small area of the exoplasmic membrane half of the acrosomal membrane (AM_e) is also shown. × 25,700.

like structure similar to that observed in protoplasmic halves of other cell membranes identically processed (35, 37, 38, 45), the distal region displays a rough texture which is akin to that generally seen in exoplasmic membrane halves. As previously described, membrane-intercalated particles are not identifiable in critical-point-dried preparations (35).

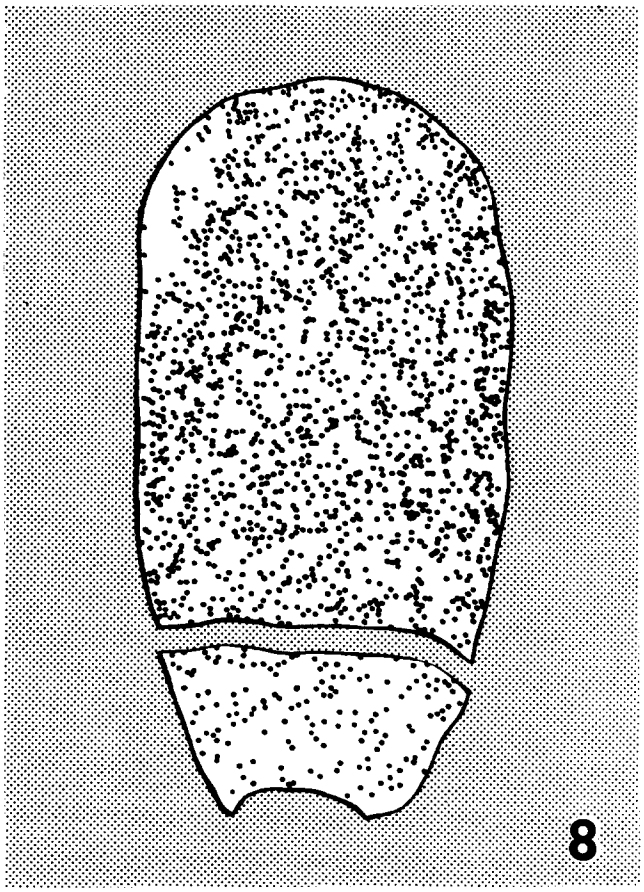


FIGURE 8 Tracing of colloidal gold spheres seen in Fig. 7. The density of the marker is approximately 2.3 times higher on the acrosomal-cap than on the postacrosomal region of the plasma membrane half.

Observation of the partition and planar distribution of WGA-colloidal gold complexes in preparations of critical-point-dried fracture-label confirmed and extended thin-section observations. Exoplasmic membrane halves were densely and uniformly labeled (Fig. 9), whereas label over protoplasmic membrane halves was less intense (Figs. 7 and 8). Significantly, along the inner halves of the plasma membrane the label was denser over the area overlying the acrosome (Figs. 7 and 8). Quantitative evaluation, in both thin sections and replicas, of the number of colloidal gold spheres associated with protoplasmic membrane halves showed that the density of WGA receptors in the postacrosomal area was 2 to 2.5 times lower than that observed in the region of the acrosomal cap (distal region).

Conventional Freeze-Fracture

Fracture faces of the plasma membrane of boar sperm head were obtained with the use of a double-replica device. Because of the flattened shape of sperm heads, this method provided higher numbers of membrane fracture faces than the commonly used technique of cleaving frozen cells with a blade. Protoplasmic fracture faces of the membrane exhibited a distinctly larger density of membrane-intercalated particles than exoplasmic fracture faces. Both membrane fracture faces show a remarkable regionalization of particles: very low density in the postacrosomal area contrasted with high density in the region of the membrane that overlies the acrosome (Figs. 10 and 11). Small rugosities densely cover protoplasmic faces. The boar sperm head plasmalemma did not present any crystalline arrangements or palisade of ridges over its equatorial segment, previously described in sperm of other mammals (46, 47).

Surface Labeling

Incubation of fixed spermatozoa in WGA followed by ovomucoid-coated colloidal gold showed elective binding of the lectin to the distal (acrosomal cap) area of boar sperm head surface (Figs. 12 and 13). The non-uniform distribution of labeled WGA receptors depended, as it has been described for lectins (48) and other cytochemical probes (14), on the

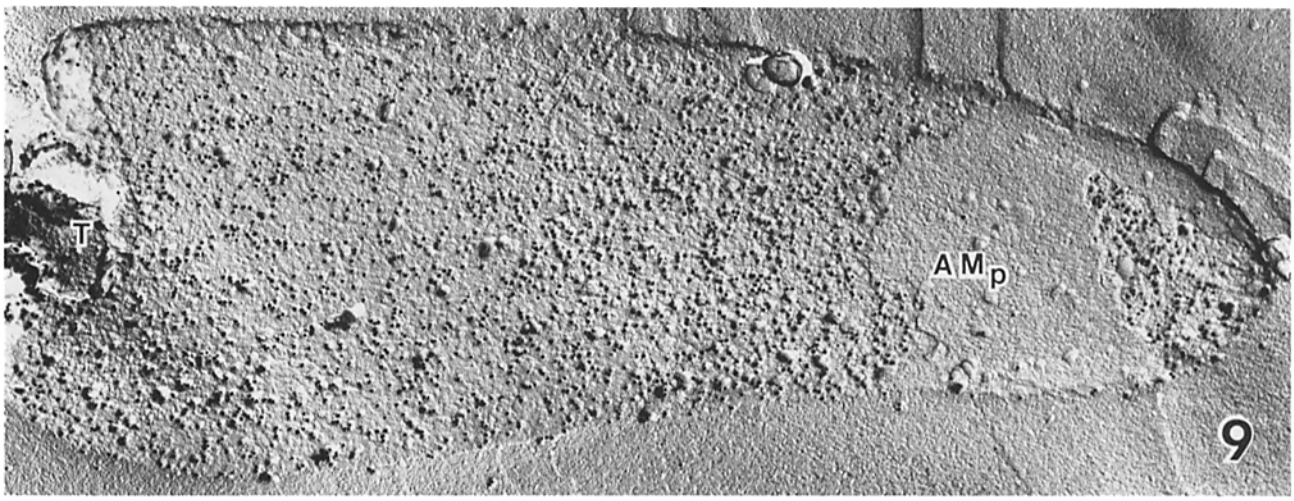
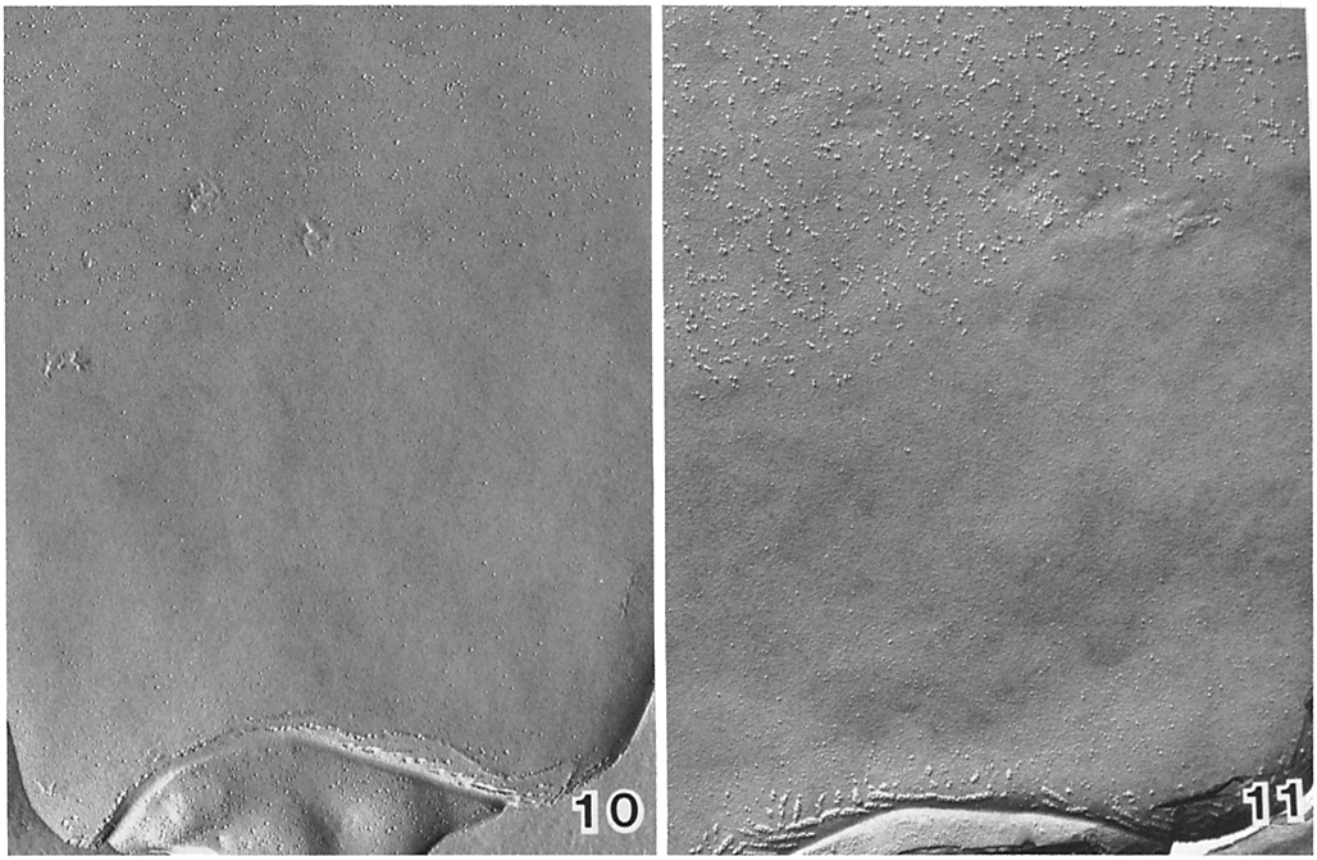


FIGURE 9 Critical-point drying fracture-label of boar sperm head. Labeling of the exoplasmic membrane half by WGA-ovomucoid-colloidal gold is intense and uniformly distributed. The protoplasmic half of the acrosomal membrane (AM_p) is virtually unlabeled. *T*, cross fracture of sperm tail. × 27,000.



FIGURES 10 and 11 Conventional freeze-fracture of the plasma membrane of boar sperm head. In both exoplasmic (Fig. 10) and protoplasmic (Fig. 11) fracture faces, intramembrane particles are preferentially accumulated on the distal (acrosomal cap) portion of the membrane. Numerous small rugosities cover both distal and proximal areas of the protoplasmic fracture face (Fig. 11). (Fig. 10) × 30,000. (Fig. 11) × 31,000.

concentration of the lectin and/or ovomucoid-colloidal gold complexes. If the concentration of label molecules was increased, a fraction of boar sperm heads appeared evenly marked by colloidal gold spheres (Fig. 14).

Labeling of Disrupted Plasma Membranes

Rupture and detachment of the cell membrane from the underlying acrosome occurs in boar sperm heads submitted to osmotic shock in distilled water for 5 min. The procedure was used to search for eventual binding of WGA to the inner (cytoplasmic) surface of the plasma membrane. In these preparations, only rare colloidal gold spheres were found attached to the inner surface of the plasma membrane (interpreted as corresponding to inspecific label), whereas the outer surface of the membrane remained well labeled (Fig. 15). Labeling of the cytoplasmic surface of the acrosomal membrane was also observed (Fig. 15), a finding that must be interpreted with caution (Aguas and Pinto da Silva, manuscript in preparation).

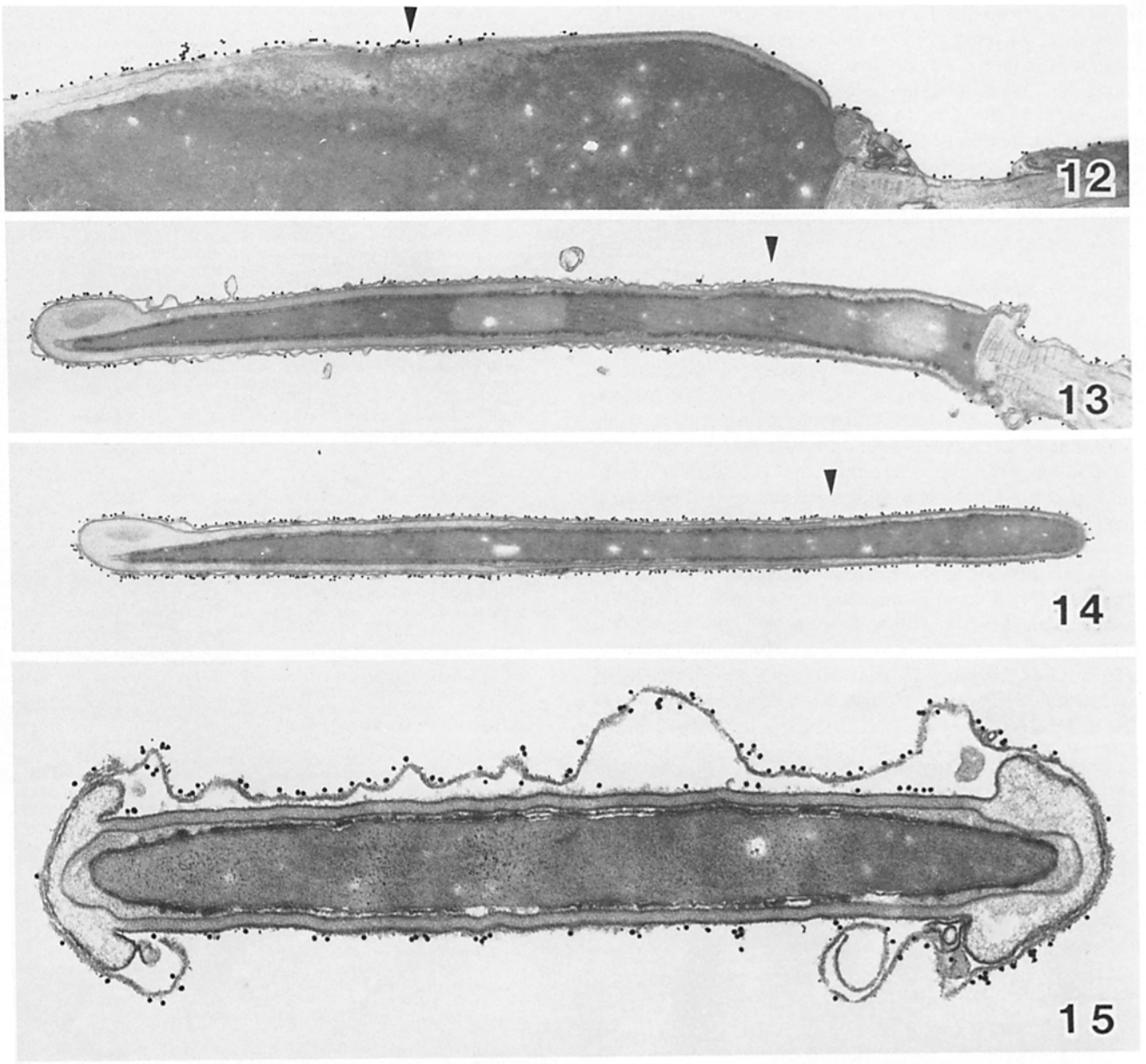
DISCUSSION

We show here that on freeze-fracture WGA receptors partition with both protoplasmic and exoplasmic halves of the plasma membrane of boar sperm head. While WGA preferentially labels exoplasmic membrane halves, labeling of protoplasmic halves of the membrane is significant and displays regional variation.

Labeling of Protoplasmic Membrane Halves Corresponds to Transmembrane Glycoproteins

The labeling that we observed over protoplasmic halves of fractured plasma membranes can have two conceivable origins: (*a*) putative WGA-binding sites originally exposed at the inner surface of the membrane; (*b*) WGA-binding sites exposed at the outer surface and associated with transmembrane glycoproteins that, on fracture, are dragged across the outer membrane half and partition with the inner half of the membrane. Two independent lines of evidence demonstrate that WGA-binding glycoconjugates are restricted to the outer surface of the membrane: in disrupted cells, access of the label to the cell interior does not result in significant labeling of the inner surface of the plasma membrane; in intact cells, coating of the outer surface of sperm cells with cationized ferritin (prior to fracture-label) results in drastic reduction in the labeling of both exoplasmic and protoplasmatic membrane halves. We conclude that the WGA-binding sites that are labeled on protoplasmic membrane halves must originate from the outer surface of the membrane; we propose that they are dragged, on fracture, across the exoplasmic half of the membrane. Therefore, labeling of protoplasmic membrane halves by WGA must reflect the presence of *transmembrane* WGA-binding glycoproteins vectorially oriented with their oligosaccharide exposed at the outer surface of the plasmalemma.

Previous fracture-label studies on human erythrocytes (34,



FIGURES 12–15 Figs. 12–14: Surface labeling of boar sperm head with WGA-ovomucoid-colloidal gold. WGA receptors are denser over the distal area of the plasma membrane profile (Figs. 12 and 13). The difference in label along the sperm head surface is not recognizable if high concentrations of WGA and ovomucoid-coated colloidal gold are used (Fig. 14). Arrowheads point to the limit between the acrosomal and postacrosomal areas of sperm head. × 25,000, × 20,000, and × 18,500, respectively. Fig. 15: Labeling of boar sperm head by WGA-ovomucoid-colloidal gold after disruption of the plasma membrane by osmotic shock. The inner (cytoplasmic) surface of the plasmalemma is virtually devoid of colloidal gold whereas its outer surface is well labeled. Some colloidal gold spheres are seen in association with the cytoplasmic surface of the acrosomal membrane, thus showing that the inner surface of the plasmalemma was accessible to the marker. × 45,000.

37) and T lymphocytes (38) have shown that during freeze-fracture transmembrane proteins are unequally partitioned with each membrane half: the process appears to be stochastic in nature and to reflect the relative expression of a given transmembrane protein in each membrane half. In boar sperm, peripheral proteins, glycolipids, as well as integral membrane proteins partitioned with the outer membrane half may all contribute, in proportions that our experiments cannot define, to the labeling of exoplasmic membrane halves. In other fracture-label studies, we showed (34, 36–38, 45) that, on thawing of freeze-fracture preparations, components of the split membrane reorganize into interrupted bilayer structures. It was recently demonstrated that on exoplasmic membrane halves this process appears to include repositioning of glycoconjugates, particularly glycolipids (49).

Regionalization of Transmembrane Glycoproteins Parallels That of Membrane Intercalated Particles

We document here that on the protoplasmic membrane halves of the plasma membrane labeling by WGA-colloidal gold is denser in the area that overlies the acrosome than over the postacrosomal region of sperm head. This demonstrates the existence of regional accumulation of transmembrane glycoproteins along sperm head plasmalemma. Studies of surface labeling with WGA-fluorescein isothyocyanide showed differences in the density of lectin receptors between

the proximal and distal areas of the mammalian sperm head (for review, see reference 9), a finding that has contributed to the current concept that the two regions form distinct membrane domains (for review, see reference 50). The use of fracture-label allows us to propose that the expression of domain distribution of WGA receptors at the cell surface of mammalian sperm head includes that of transmembrane glycoproteins. As exoplasmic membrane halves are always uniformly labeled, glycolipids and peripheral glycoproteins do not appear to be involved in the formation of the domain pattern.

The distribution of WGA-colloidal gold label on protoplasmic membrane halves of fracture-labeled preparations of sperm head plasmalemma parallels the unequal density of large IMP seen along protoplasmic faces of conventional freeze-fracture preparations. As discussed above, WGA-labeling of the protoplasmic membrane half corresponds to transmembrane glycoproteins. Therefore, the herein topological correlation between WGA receptors and IMP provides additional evidence for the prevailing view that IMP of biological membranes represent integral proteins (51–54), in this case transmembrane WGA-binding glycoproteins. Consequently, our results do not support alternative interpretations that envisage the IMP as lipid molecules in an inverted micellar configuration (55–57). The correlation does not imply that all the membrane-intercalated particles seen on protoplasmic faces are WGA-binding glycoproteins, but certainly suggests that integral membrane proteins are a decisive contributor for their expression.

We are grateful to Dr. Daniel Friend for kindly answering questions raised during the course of this study. We thank Dr. Larry Johnson (US Department of Agriculture, Beltsville, MD) for the gift of numerous samples of boar sperm, Dr. Jacques Chevalier for suggestions and help with conventional freeze-fracture, and Drs. M. L. Barbosa, G. Tadvalkar, and M. R. Torrisi for discussion.

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