Modifications of Anionic-Lipid Domains Preceding Membrane Fusion in Guinea Pig Sperm

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ABSTRACT   The relationship between anionic-lipid concentration and the functional properties of plasma-membrane domains was explored using the guinea-pig sperm membrane as a model, with polymyxin B (PXB) as a probe. Areas of plasmalemma specialized for fusion during the acrosome reaction had a higher affinity for the probe than adjacent nonfusigenic regions. In addition, capacitation—a process preceding acrosome:plasma-membrane fusion—markedly enlarged the area susceptible to PXB binding over the acrosomal cap. Protease treatment mimicked capacitation by increasing the acrosome-reaction incidence as well as PXB binding, at enzyme concentrations not affecting the surface coat nor altering filipin/sterol localization. Both proteolytic digestion and capacitation failed to augment PXB- or filipin-affinity in nonfusigenic zones, such as the post-acrosomal segment, including its particle-free maculae. Incubation of sperm in capacitating medium supplemented with ^{32}P-labeled phosphate, followed by lipid extraction, thin-layer chromatography, and autoradiography, revealed a radioactive band comigrating with cardiolipin and phosphatidic acid. Vermiform protrusions elicited by PXB in the outer lamellae of cardiolipin-phosphatidylcholine liposomes resembled those seen in fusional regions of sperm membrane. We conclude that (a) differing concentrations of anionic lipids are found in adjacent domains of the sperm plasma membrane; (b) these domains mirror the functional regions of the membrane, with higher anionic-lipid concentrations localized over fusional zones; (c) the surface coat does not participate in the maintenance of such domains; (d) anionic-lipid synthesis may contribute to their formation; and (e) anionic-lipid concentrations increase as the membrane becomes fusionally competent, indicating that cellular modulation of lipid domains accompanies regulation of membrane function.

That phospholipids mediate fusion in diverse biological or model membrane-systems has been advocated by a number of investigators. Particularly, workers in reproductive biology have shown that fertilization is not prevented by the treatment of eggs with proteolytic or carbohydrate-hydrolyzing enzymes which leave phospholipids intact (26). Moreover, biophysicists have established that phospholipid vesicles can fuse, not only with each other (44) but with natural membranes (23, 55). Certain lipids have a greater potential for fusion than others. For example, liposomes composed solely of phosphatidylcholine (PC) resist merger for hours, yet vesicle membranes incorporating anionic lipids fuse readily upon the introduction of divalent cations (44).

The requirement for negatively charged lipids in the events of membrane:membrane fusion has been demonstrated in various systems—e.g., the entry of Semliki Forest virus into cells via the viral envelope-membrane union with the cell's lyso-

somal membrane (55), or the coalescence of lipids from two different model liposome populations (44).

Some acidic lipids are isothermically triggered by calcium (Ca^{2+}) to freeze from the liquid-crystalline to the gel state; whereas others partially melt, forming hexagonal arrays (14, 32). Accompanying the induction of a new phase, permeability, compressibility, and surface-potential become altered (43, 44). These phase transitions have been hypothesized to create disorganized lipid arrays necessary for the initiation of fusion (44). In cells as well as in liposomes, Ca^{2+} triggers membrane fusion, suggesting that an analogous mechanism is active in vesicle:vesicle coalescence and in cellular processes such as exocytosis and fertilization. Cholesterol also affects these phase transitions; thus its presence or absence as evidenced by filipin is a clue to the physical state of the membrane.

That a cell can concentrate anionic lipids in areas of the membrane bilayer where fusion occurs, and conversely, that

these lipids can be excluded from stable regions of membrane, is the subject of this paper. Other membrane components appear to have a differential topography in the plane of the membrane. Many integral membrane proteins (e.g., the acetyl-choline receptor and ouabain-sensitive ion pumps) reside in preferential foci within the plane of the bilayer (17, 35). In addition, labeling of β-OH sterols with the polyene filipin has demonstrated the heterogeneity of these components in the plasmalemmae of various cell types (15, 36). This differential topography correlates with specific functional domains of the membrane. Furthermore, we propose that fusigenic domains, high in anionic-lipid concentration, form in dynamic response to the fusional activity of the cell rather than permanently occupying exact sites in the plane of the membrane, awaiting fusion activation.

The findings reported here primarily stem from the trans-mission electronmicroscopy of freeze-fracture replicas, which permits large vistas of membrane to be examined. We used the antibiotics polymyxin B (PXB) as a probe for anionic lipids, and filipin as a probe for β-OH sterols such as cholesterol. It has already been established that PXB binds anionic lipids (52, 54), and through this binding, disrupts the normally smooth contours of eukaryotic-cell membranes (1, 4). Filipin binding to sperm has been described (15, 19); thus we could use it as a tool to monitor the availability of the plasma membranes to such probes, as well as their ability to deform in the presence of a lipid-binding agent. The model we have selected is the guinea-pig spermatozoon (Fig. 1). This highly polarized cell has the advantage of an extensive area of fusigenic plasma membrane: the acrosomal cap, easily recognized in freeze-fractures. Contiguous with the fusigenic region lies the stable post-acrosomal membrane. Newly removed from the tail of the epididymis or from the vas deferens, sperm must be capacitated before the addition of Ca^{2+} kindles fusion between the plas-malemma and the outer portion of the acrosomal membrane—the acrosome reaction (3). Capacitation, the process which readies these membranes for the reaction, can be achieved in vitro (58). The absence of calcium, however, can halt fusion, arresting the cells at the threshold of the acrosome reaction. In this paper, we have compared the binding of PXB to the head membranes of fresh spermatozoa with that of cells incubated in calcium-free capacitating media, to assess their relative distribution of anionic lipids. Thus, changes in distribution which accompany capacitation, but occur before the acrosome reaction, can be described.

MATERIALS AND METHODS

Fresh Sperm

Spermatozoa were removed from the vasa deferentes and epididymal tails of mature (500 g), ether-anesthetized guinea pigs. The pooled sperm at a concentra-tion of 10^7 cells/cm^3 were then mixed in Ca^{2+}-free Tyrode's solution (TS), pH 7.4, and treated as follows: (a) Control sperm were incubated in TS containing comparable (molar) sucrose concentrations to replace PXB. (b) For polymyxin-B (Sigma Chemical Co., St. Louis, MO) labeling, sperm were treated as previously reported (1), except that TS was substituted for HEPES-Tris. (c) Filipin labeling was accomplished according to the method used in earlier experiments (15), but with the omission of dimethylsulfoxide (DMSO). (d) Hemocyanin-PXB conju-gation was achieved by using a modification of the method of Jan and Revel (28) wherein we substituted keyhole limpet hemocyanin (KLH) (Sigma Chemical Co.) for that of Busycon canaliculatum, and 10 mg of PXB for IgG. After dialysis for 24 h at 4°C, a Bio-Gel A 1.5-m column (2.5 × 20 cm; Bio-Rad Laboratories, Richmond, CA) was used to separate nonreacted PXB from the conjugate. Hemocyanin-PXB was recovered by monitoring absorption spectra of the frac-tions at 280 nm, and subsequent ultrafiltration of the absorbing fractions. A final volume of 10 ml contained protein concentrations of 1.5–2.5 mg/ml, as measured
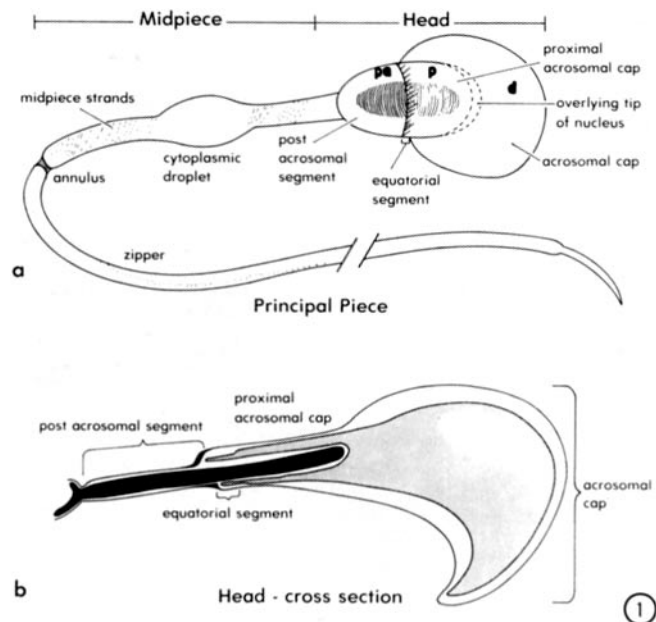

FIGURE 1 (a) Diagram of the surface of a guinea-pig sperm; (b) cross-section of the head only, as it appears in thin section.

by the Bio-Rad protein-determination method (4). (e) Next, the sperm were incubated for 30 min at 37°C in a phosphate-buffered saline (PBS)/hemocyanin-PXB solution (pH 7.4).

Enzyme-treated Sperm

Sperm were incubated in TS supplemented with 1 mg/ml proteinase (crude trypsin extract, Sigma Chemical Co., Type II, pancreatic; trypsin activity 1,050 U/mg; chymotrypsin activity 1,040 U/mg) for 30 min at 37°C (pH 7.2–7.4), followed by the addition of 1 mg/10 ml soybean trypsin inhibitor (Sigma Chemical Co.; 0.9 mg inhibits 10,000 U trypsin). Subsequently the cells were washed twice and labeled with PXB or filipin, or incubated with sucrose as described above. Comparable aliquots of sperm were incubated for 30 min without proteinase.

Capacitated Sperm

Sperm were incubated in Ca^{2+}-free TS, with 2% bovine serum albumin (BSA) and 1% glucose in 1-ml aliquots at 37°C under sterile mineral oil for 24–26 h according to the method of Yanagimachi and Usui (58). To some of these aliquots of sperm, we added 0.4 cm^3 of a 10-mM $CaCl_2$ solution. Droplets of these sperm were taken at 10-min intervals, examined to establish motility and quantitate acrosome reactions, and then allowed to air-dry for subsequent staining (see below). The rest were washed once and treated with the lipid-binding agents as previously described here. Only dishes containing 90% motile sperm were re-trieved for further processing.

Quantification of Acrosome Reactions

Ten aliquots from preparations of unfixed, fresh, protease-treated, TS-incu-bated, and capacitated sperm (with or without calcium) were first monitored at the light-microscopic level to determine motility and acrosome reactions, then permitted to air-dry on glass microscope-slides. They were subsequently stained with erythrosin B and naphthol yellow S (9).

Preferentially staining acrosomal contents bright red with these dyes greatly facilitates counting reacted vs. nonreacted sperm by light microscopy (Figs. 2 and 3). To eliminate the effect of air-drying and staining on acrosomal patency, we subtracted the fraction of acrosome-reacted cells in non-calcium-treated samples from those with calcium. The fraction of sperm which lost their acrosomal contents after incubation and subsequent addition of calcium was calculated according to the following formula:

$$\left[\frac{\text{Number of acrosome-reacted sperm 20 min post-}Ca^{2+}}{\text{Total sperm 20 min post-}Ca^{2+}} \right] \text{ minus } \left[\frac{\text{Number of acrosome-reacted sperm pre-}Ca^{2+}}{\text{Total sperm pre-}Ca^{2+}} \right]$$

We counted sperm at random by "blind" selection of a high-power field,

followed by enumeration of all the sperm within that field. Ten fields were counted for each droplet.

Preparation of Sperm for Electronmicroscopy

All preparations were fixed in 1.25% glutaraldehyde buffered with 0.1 M sodium cacodylate, 1% sucrose (pH 7.4). Freeze-fractures were obtained as described (21); and surface replicas were processed by critical-point-drying (45), mounting in a Balzers apparatus, and shadowing with carbon-platinum at −2 × 10^{-6} torr. For surface replicas, samples were kept at room temperature, although the cutting arm was cooled to liquid nitrogen temperature. Thin sections of standard material were prepared with tannic acid according to the technique of Simionescu and Simionescu (50). Sections were post-stained with uranyl acetate (20 min) and lead citrate (2 min).

Radioactive Labeling

Sperm were incubated under sterile oil at 37°C in Ca^{2+}-free TS containing 1 mg/ml glucose and 2 mg/ml BSA and supplemented with 1 mCi/ml ^{32}P-labeled inorganic phosphate. Motile sperm were collected after 30 min and after 24 h, and lipid extraction was conducted as detailed below. Additionally, protease incubation was performed as above, but also in the presence of 1 mCi/ml ^{32}PO$_4$ with subsequent lipid extraction.

Liposomes

Liposomes were prepared and treated as we have previously reported (1). These liposomes consisted of equimolar ratios of egg phosphatidylcholine and bovine heart cardiolipin (Avanti Biochemicals, Inc., Birmingham, AL).

Lipid Extraction and Separation

Lipids were extracted from sonicated sperm in TS with chloroform: methanol. After filtration of the lower phase through glass wool, the solvent was evaporated under nitrogen at 35°C. The yellowish deposit was resuspended in dichloromethane and applied to silica gel G-250 (Merck AG, Darmstadt, FRG), along with lipid standards supplied by Dr. Papahadjopoulos (Cancer Research Institute, University of California School of Medicine, San Francisco, CA). The plate was developed in chloroform:acetone:methanol:acetic acid:water (6:8:2:2:1 vol/vol). After determination of the presence of ninhydrin-stainable and sulfuric-acid-burnable material, plates of radioactively labeled sperm lipids were autoradiographed (12 h, 4°C).

RESULTS

Viewed under the light microscope, freshly removed epididymal or vasal sperm were freely motile; >90% retained the acrosome upon air-drying and staining for light microscopy. After 24–26 h of incubation at 37°C in calcium-free Tyrode's solution, the acrosome reaction was triggered in 60–80% of the cells (Figs. 2 and 3) by the addition of calcium to motile sperm cultures.

Fresh guinea-pig sperm revealed smooth expanses of plasma membrane when examined in freeze-fracture replicas (Fig. 4). After treatment with the anionic-lipid-binding agent PXB, however, the distal acrosomal cap became crenulated; while the proximal cap membrane retained the flat aspect of untreated sperm (Figs. 5 and 7).

Keyhole limpet hemocyanin conjugated to PXB rendered the antibiotic visible in surface replicas (Fig. 6), disclosing PXB binding to the same regions of membrane which appeared crenulated or bubbled in fractured specimens.

Incubation of the sperm in capacitating media increased the extent of PXB disturbance (Fig. 8). Whereas the distal acrosomal cap was perturbed in both fresh and capacitated sperm, the proximal membrane, free of PXB damage before incubation, became susceptible to the antibiotic only after incubation in capacitating medium, thus making it competent to undergo fusion (cf. Figs. 7 and 8). Yet this enhancement in sensitivity did not encroach upon a band of membrane fronting the tip of the nucleus, even when the bilayer on either side of the tract crenulated. In both fresh and capacitated sperm, round maculae barren of intramembranous particles (IMP) frequently punctuated this undisturbed band (19). These circles, too, remained unaffected by PXB (Fig. 8) or the sterol-binding agent filipin. In tannic-acid-stained thin sections, a density lay between this area of the outer leaflet of the granule membrane and the PXB-resistant plasma membrane (Fig. 9). Neither the equatorial segment nor the postacrosomal region of the plasmalemma—areas of nonfusigenic membrane uninvolved in acrosomal granule exocytosis—had any PXB affinity, either before or after capacitation (Figs. 10 and 11). Again these PXB-resistant areas displayed a thick density beneath the postacrosomal membrane in thin sections (Fig. 12). Intramembranous-particle-free maculae in front of the tip of the nucleus (Fig. 13), as well as those just subjacent to the equatorial zone, were undisturbed by filipin or PXB (Figs. 14 and 15).

Tannic-acid staining of sperm from the cauda of the epididymis or vas deferens revealed a dense extracellular material with a periodicity of 200 Å lying over the acrosomal caps of fresh spermatozoa (Fig. 9) and diminishing after incubation in
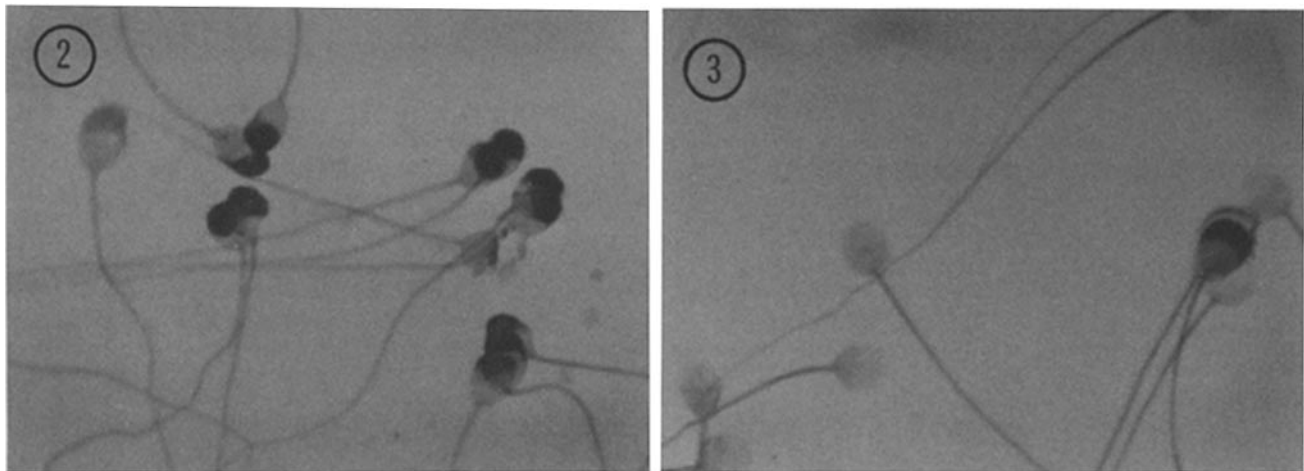
FIGURES 2 and 3   Light micrographs of fresh and capacitated sperm. Fig. 2 shows the acrosomal granules in these fresh sperm were deeply stained. After washing and treatment with Ca^{2+}, they were allowed to dry on the slide and stained with naphthol yellow S and erythrosin B. × 1,000. Fig. 3 shows acrosome-reacted sperm. Capacitated sperm, in contrast, lose the brightly stained granule upon the addition of Ca^{2+}. × 1,000.

capacitating media (21). This "coat" was also visible in our surface replicas of unwashed sperm freshly removed from the cauda or vas (Fig. 16). Occupying large plaques on the membrane, the coat manifested the same 200-Å periodicity as that of the quilt (22), embossed on the inner face of the bilayer in freeze-fracture preparations (Fig. 21 shows the quilt accentuated by filipin treatment). Neither corpus epididymal sperm nor any of the incubated sperm, exhibited this structure (Figs.
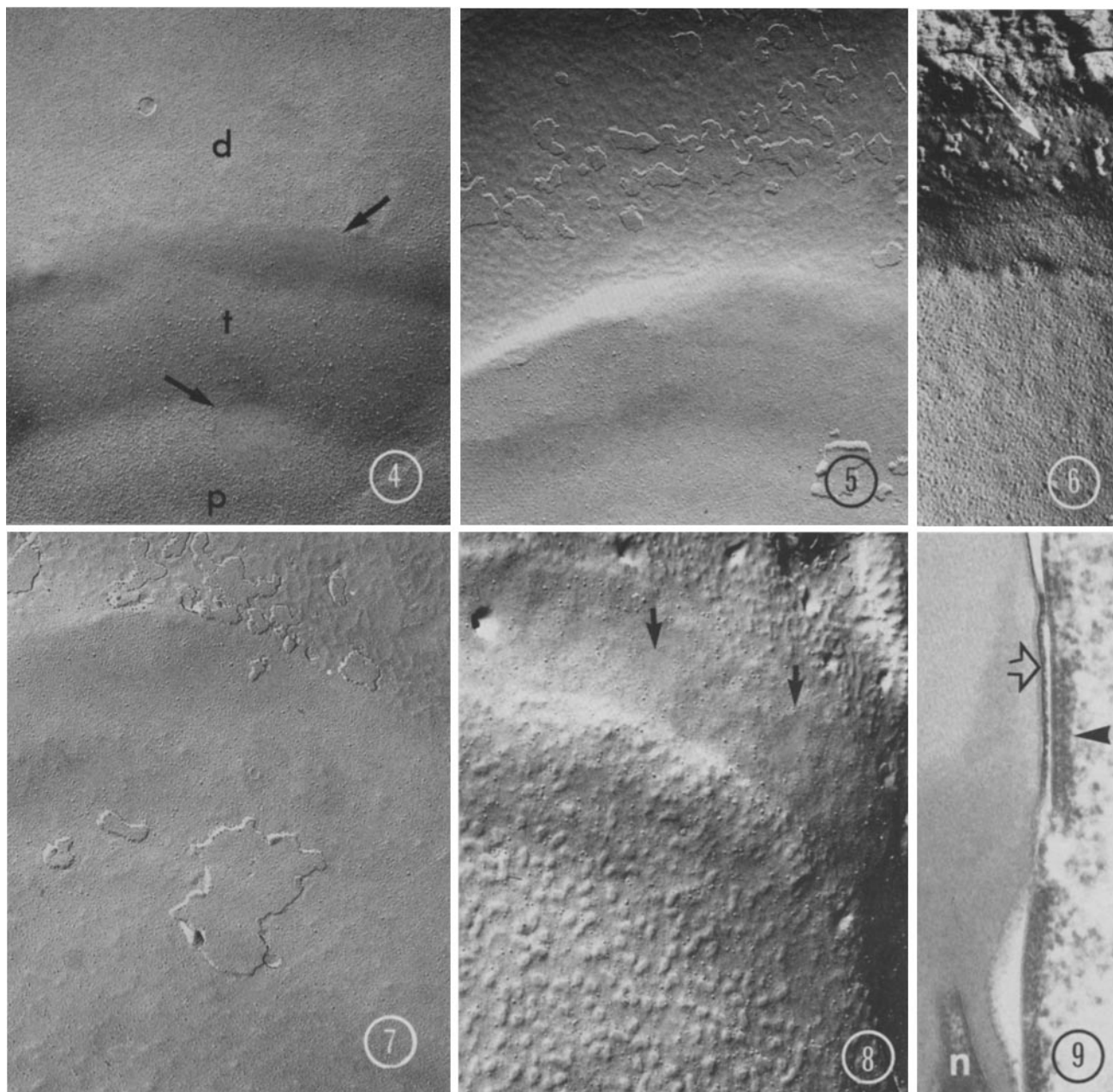

FIGURE 4   The smooth contours of a fresh, untreated sperm as observed in conventional freeze-fracture replicas. Note the circular clearing of IMP (arrows) at the tip of the nucleus (t) which delineates the proximal (p) and distal (d) cap. X 36,000.

FIGURE 5   PXB treatment of fresh sperm creates crenulations in the distal cap. X 24,000.

FIGURE 6   Hemocyanin-conjugated PXB, as seen in critical-point-dried surface replicas, deposits large globules (arrow) on the distal cap; while the tip of the nucleus and proximal cap are left clean. X 16,000.

FIGURE 7   A fresh sperm treated with PXB exhibits only a slight dimpling of the proximal cap. The tip of the nucleus is undisturbed. X 24,000.

FIGURE 8   Drastic perturbations of the membrane cover the acrosomal cap of this capacitated sperm, except for the persistently smooth membrane arching the tip of the nucleus. Unaffected maculae are visible in this smooth band of membrane (arrows). X 45,000.

FIGURE 9   In a thin section of a fresh sperm, a density (arrow) lines the granule's outer leaflet at the tip of the nucleus (n). Notice the surface coat (arrowhead). X 70,000.

17–20). Instead, incubation for 30 min in TS alone or in TS-protease produced a filigree design in the surface components which apparently lie beneath the quilt (Figs. 18 and 19). In contrast, fresh corpus epididymal sperm had rough yet regular surfaces but no quilt (Fig. 17). And capacitated sperm exposed surface designs ranging from rough, through filigreed, to smooth (Fig. 20). Sperm which remained in stacks throughout the 24-h incubation even retained vestiges of the quilt.

To determine the effect of these coats on PXB binding, we attempted to remove them with proteinase. However, the
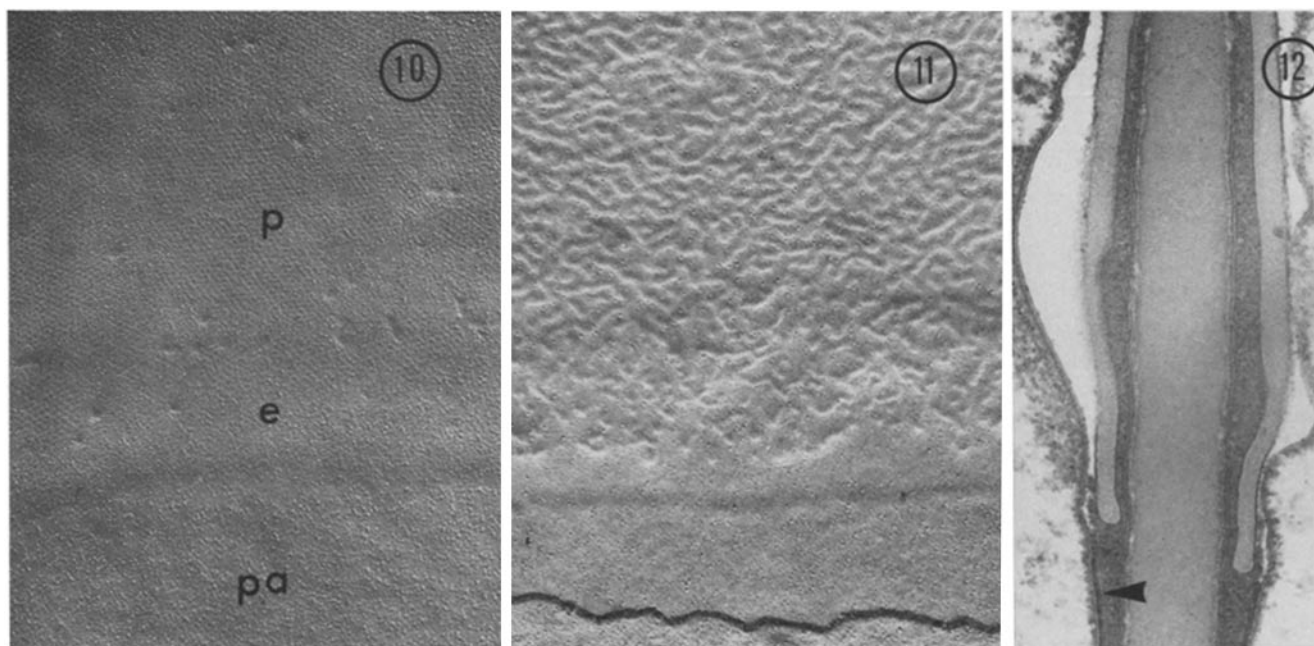
FIGURES 10, 11, and 12   The equatorial region as a boundary between acrosomal cap and post-acrosomal membrane. Fig. 10 shows a few protrusions poking through the quilt over the proximal acrosomal-cap (p) membrane of a fresh sperm, but without penetrating beyond the equatorial segment. × 50,000. Proliferation of disturbance (Fig. 11) in this same region of capacitated sperm is also arrested at the end of the equatorial segment. The post-acrosomal (pa) membrane is undisturbed by PXB in both specimens. × 38,000 (shadowed from above). Fig. 12 shows a density (arrowhead) underlining the cytoplasmic surface of the post-acrosomal plasma membrane of a non-capacitated sperm. × 60,000.
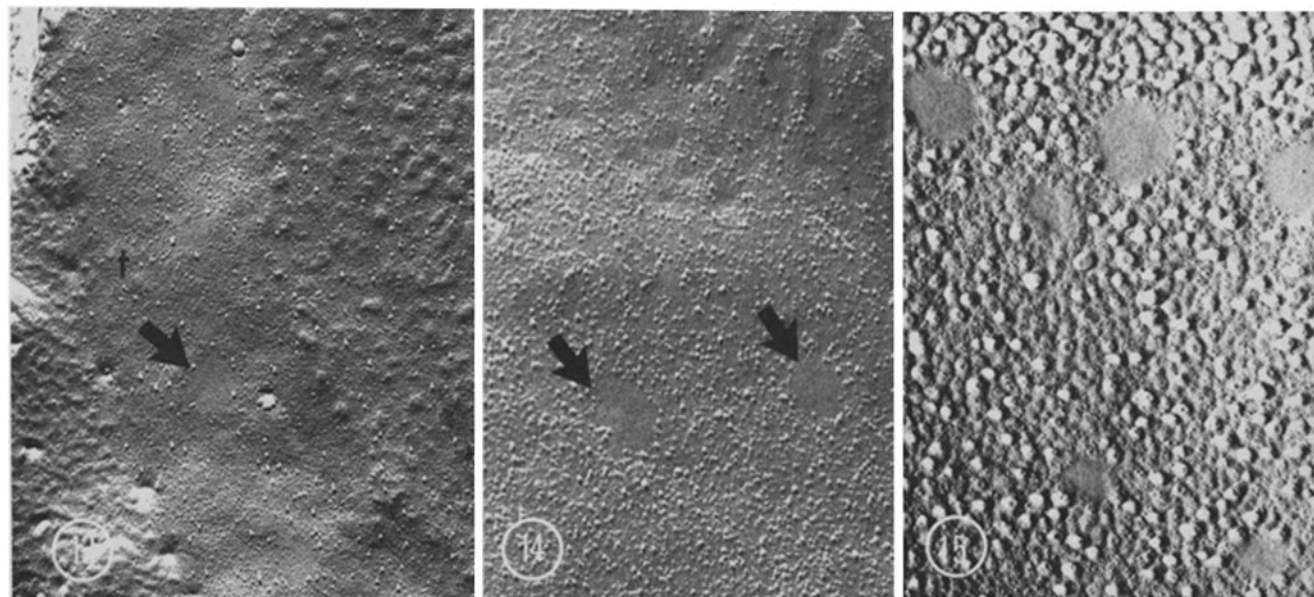

FIGURE 13   Circular clearings of IMP (arrow), unaffected by PXB, punctuate the also-undisturbed band at the tip of the nucleus (t) in this capacitated PXB-treated sperm. × 54,000.

FIGURE 14   A capacitated sperm, with macular clearings (arrows) in the post-acrosomal segment, remains unaffected by PXB, which induced perturbations of the adjacent proximal cap. × 36,000.

FIGURE 15   Filipin attacks the proximal cap of a capacitated sperm heavily, and more lightly affects the post-acrosomal segment; but, like PXB, the polyene leaves the particle-free clearings barren. × 45,000.

plaques of the quilt disappeared in 30 min without the enzyme, and the "subcoat" diminished in both treated and untreated sperm equally. But protease did accelerate functional capacitation, so that after a 30-min incubation with enzyme, >60% of the acrosomes were lost within 20 min subsequent to the addition of calcium. In comparison, <20% of the acrosomes reacted when calcium was added to sperm incubated without enzyme for 30 min.

Preincubation in the crude protease extract also influenced the binding of PXB, but not that of filipin. The binding of the latter antibiotic over the acrosomal caps of protease-treated sperm was identical to that of TS-incubated control preparations, but differed radically from the polyene/sterol distribution in unwashed sperm (Figs. 21 and 22). After 30 min, regardless of the presence of enzymes, spermatozoa resembled fully capacitated cells in respect to the loss of the quilt pattern and the increased number of sterol/filipin complexes in comparison to those of fresh sperm: complexes now covered the entire acrosomal cap. That capacitation enhances filipin binding has already been reported (20).

Whereas filipin binding demonstrated no disparity between fusionally competent and fusionally incompetent cells, PXB was dramatically augmented in the fusionally competent cell, whether protease-treated or capacitated by standard incubation (Figs. 23 and 24). In contrast, 30-min incubation without enzymes produced no elevation in PXB binding.

Protease failed to enhance either PXB or filipin perturbations in areas of membrane normally unaffected by these agents (Fig. 24). The equatorial segment, the postacrosomal membrane, and the band at the tip of the nucleus remained smooth after treatment. Circular clearings of IMP and filipin/sterol complexes were more prevalent in protease-treated sperm, but also appeared in the nontreated cells. Like the postacrosomal segment, these barren maculae remained free from polyene or antibiotic assault, even after proteolysis. A summary comparing the effects of filipin and PXB on fresh, capacitated, protease-treated, and briefly TS-incubated sperm is given in Table I.

To identify the anionic phospholipid responsible for the increased binding of PXB during capacitation, we incubated sperm in TS containing ^{32}P-labeled phosphate. Thin-layer chromatographic (TLC) separation of the chloroform:methanol lipid extract and subsequent autoradiography of the TLC plate revealed a dark band comigrating with phosphatidic acid (PA) and with cardiolipin, as well as some radioactivity over other lipid fractions (Fig. 25 B). Sperm incubated for 30 min in ^{32}PO$_4$-supplemented TS, with or without protease, showed only one, slightly radioactive band again comigrating with PA and cardiolipin (Fig. 25 A). When liposomes composed of equimolar ratios of PC and cardiolipin were treated with PXB, they exhibited vermiform protrusions and rivulets (Figs. 26 and 27). Such protrusions were reminiscent of those mediated by PXB in the capacitated spermatozoan proximal acrosomal-cap membrane.

DISCUSSION

We interpret this diversity in PXB binding as indicative that anionic lipids in the external half of the plasma-membrane bilayer are restricted to the fusigenic acrosomal-cap region, since they are undetectible in the adjacent but stable postacrosomal segment. Furthermore, the enhanced PXB binding over the fusigenic area after proteolysis or capacitation suggests that these procedures make more anionic lipid accessible to the probe. Internal organelle membranes do not crenulate when

PXB is in the bathing media, supporting the view that the antibiotic does not readily penetrate lipid bilayers. Anionic lipid, therefore, must be exposed on the outer leaflet to be attacked by PXB. "Flip-flop" of lipid from the cytoplasmic to the external leaflet could thus explain the enhanced labeling after capacitation or proteolysis. Very rarely is the principal piece perturbed by PXB (1). Again, the inability of PXB to pass through the bilayer may account for this, because only those tails with broken membranes would be affected if the anionic lipid resided primarily in the cytoplasmic leaflet, as has been advocated for other cells (8).

In fresh or capacitated sperm, three foci within the plasmalemma of the head fail to crenulate in the presence of the probe: the band at the tip of the nucleus, the equatorial segment, and the nearby postacrosomal segment. The distribution of hemocyanin-conjugated PXB in surface replicas parallels that of the protuberances in freeze-fractured preparations, supporting the conclusion that alterations in membrane contour correlate with binding. Hence this lack of crenulation indicates that decreased binding probably reflects a relative paucity of anionic lipids (although other explanations may also apply). The capacity of the probe to detect anionic lipids may be influenced by such factors as: (a) membrane lipids in the gel state may have a lower affinity for lipid-binding agents; (b) peripheral proteins, which can induce the gel state (3), may also restrict the insertion of probes into the bilayer or prevent the probe from reaching the lipid; (c) positive surface charges may repulse cationic molecules such as PXB; and (d) membrane-bound calcium, which competitively inhibits PXB binding (25, 33), may occupy binding sites.

Of the three foci that fail to bind PXB, the postacrosomal segment is presumably less fluid than the acrosomal-cap region. When human spermatozoa are exposed to anilinonaphthalene sulfate (34), or guinea-pig sperm to merocyanin S 540 (2)—agents known to partition into liquid-crystalline but not gel lipid bilayers (48)—fluorescence is observed in the acrosomal cap, yet is absent in the postacrosomal membrane. However, a less fluid state of the lipid bilayer is not likely to affect PXB binding, because protrusions have been reported in liposomes incubated with PXB below their transitional temperatures (1, 51). Moreover, decreased fluidity may contribute to the maintenance of these lipid domains by depressing the lateral diffusion rate of anionic lipids from adjacent areas. Conversely, the gel state could be a result of peripheral-protein-mediated inhibition of lateral mobility. Structures suggestive of peripheral proteins or glycoconjugates appear on both sides of these membrane sites in thin sections: cytoplasmic densities underlie the plasmalemma; and a thick extracellular coating can be discerned as well. That external peripheral proteins do not prohibit PXB from approaching the postacrosomal membrane is demonstrated by the inability of protease to increase PXB binding in this segment. In view of the function of the postacrosomal membrane at this stage of the spermatozoan lifespan (i.e., to remain stable during acrosomal exocytosis), it would be expected to be less fluid and to contain little if any anionic lipid, as either condition would render it labile. It is not until after the acrosome reaction that this membrane acquires its ability to fuse with the egg (3, 4, 11).

Calcium may be culpable in the inhibition of binding at the tip of the nucleus. Pyroantimonate, which precipitates where divalent cationic metals are present in high concentrations, is deposited in this band (20), and calmodulin is localized there, as demonstrated by antibody-specificity and Stelazine binding
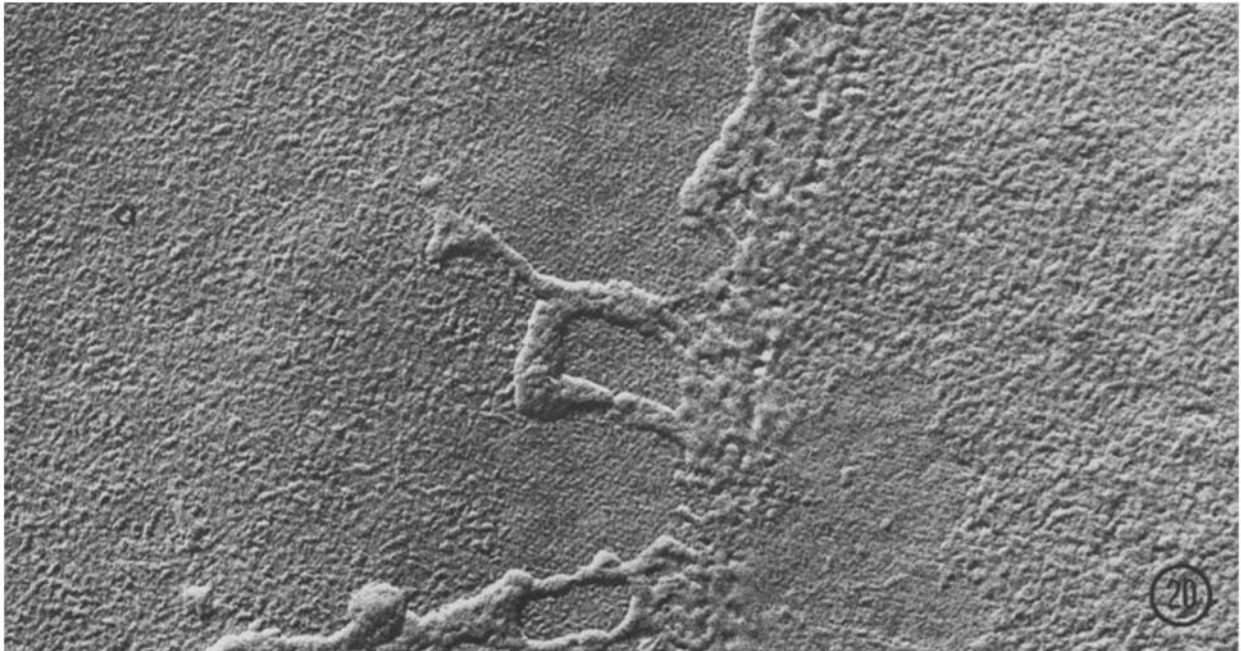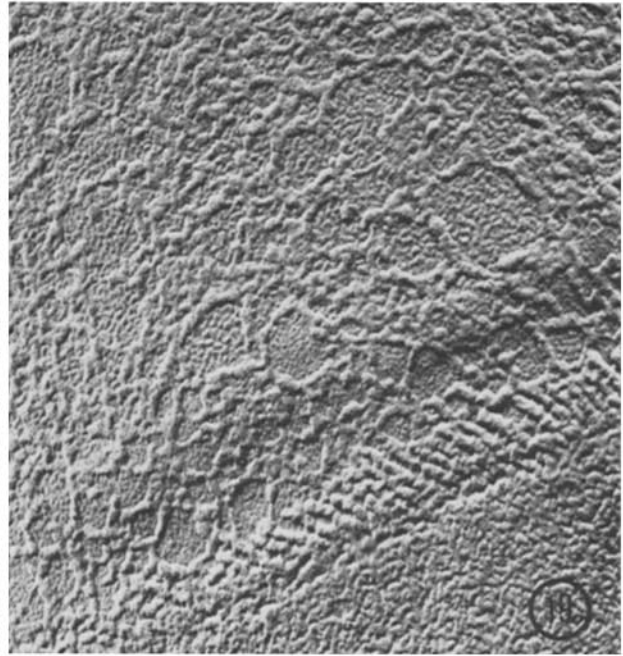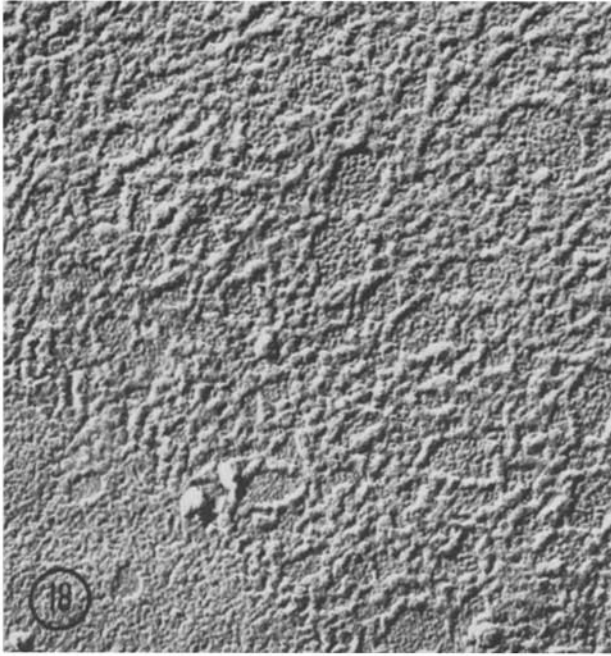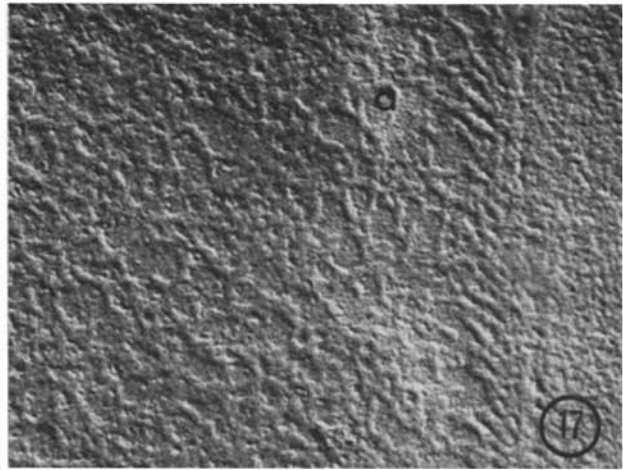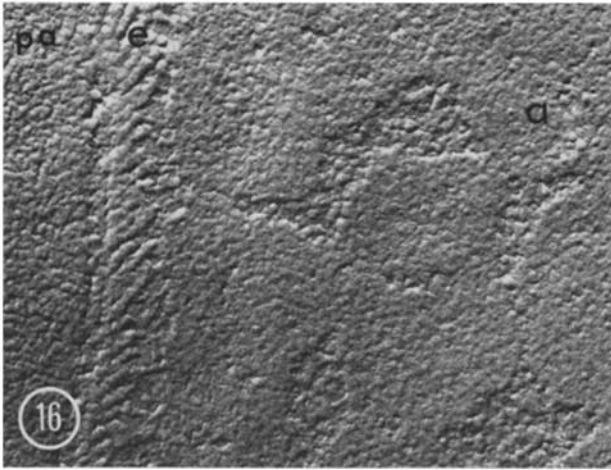
(30, 39). Because calcium competitively inhibits PXB binding (25, 33), its presence could readily explain the dearth of protuberances here. Finally, it is doubtful that charge-charge repulsion plays a role in obstructing PXB, because it does not prevent colloidal iron hydroxide (18, 57) from attaching to these membrane regions.

IMP Clearings

Circular clearings of intramembranous particles have been described in various other fusional systems, such as frog neuromuscular junctions, pancreatic islet cells, and *Phytophthora* zoospores (10, 42, 46). It has been argued that these maculae are artifactually produced by either glutaraldehyde or glycerol, for they were not seen in freeze-fracture images of quick-frozen mast cells (12). Nevertheless, we have observed these clearings in the cytoplasmic droplet of quick-frozen sperm, neither fixed nor cryoprotected (22). In the present study, we report IMP clearings in traditionally fixed and cryoprotected sperm membranes. They appeared at two sites on the head: in the band fronting the tip of the nucleus, where (we presume) the acrosomal granule first begins to fuse with the plasma membrane; and in the postacrosomal segment near the equatorial region, where sperm:egg fusion ultimately occurs (4, 11). While the latter clearings were present regardless of the capacitation status of the membrane, those near the tip of the nucleus became evident only after the quilt pattern melted, that is, within half an hour of incubation in capacitating medium. Both areas of membrane manifested densities on the cytoplasmic surface, and neither group of maculae was susceptible to PXB or filipin attack. In addition, the clearings were still extant after protease digestion, indicating that polypeptide moieties exposed to the outer milieu were not involved in their maintenance. These clearings appeared in regions of membrane where fusion takes place. However, because they were present in membrane before it attained fusional competence, they were therefore unable to induce such fusion by themselves.

Effects of Crude Trypsin Extract on the Incidence of the Acrosome Reaction, Surface Coat, and Lipid Topography

Protease incubation mimics capacitation by increasing both the incidence of the acrosome reaction and the anionic-lipid concentration in the fusional membrane, leaving surface appearance and sterol distribution unchanged. Neither trypsin nor chymotrypsin used separately can accelerate capacitation, although pronase can (56). A common component of the crude bovine pancreatic extract we used was β-amylase (13), which

has been purported to facilitate capacitation (29, 31).

Although it could expedite functional capacitation, the crude extract was incapable of altering the normal morphological maturation of the spermatozoan surface. That the surface coat is modified both during transit through the epididymis and after in vitro or in vivo capacitation is demonstrable by differences in lectin agglutination (53) and binding (24, 49); in antigenic determinants (37); and in surface charge as measured by the electrophoretic mobility of whole sperm (38) or by surface affinity for charged colloidal iron (18, 57). In the course of passage through the cauda of the epididymis in the rat, a highly glycosylated secretory product adheres to the sperm and masks previously accessible surface components from radioiodination (41); while the heads of both rat and rabbit sperm increase in affinity for concanavalin A (24, 40). The coat probably corresponds to the quilted plaques described here, because they, too, cannot be detected in earlier maturational stages and disappear rapidly upon incubation.

Antibody agglutination (53) and elegant fluorescence microscopy (49) have revealed that some lectin receptors increase in abundance after capacitation of guinea pig sperm, perhaps because they are unveiled by the loss of the coat (24); whereas others decrease. It is likely that the rough mat draping the acrosomal caps of all sperm shown here, although overlain by the quilted plaques in caudal specimens, represents an architectural counterpart to the lectin receptors and antigenic sites that are masked by the epididymal secretory product. The mat peels more slowly than the quilt and remains extant in a large proportion of sperm that have been incubated for 24 h in capacitating media, and thus are capable of granule exocytosis as well as PXB binding throughout the region covered by the mat. This attests to the mat's inability to prevent the acrosome reaction or to maintain lipid boundaries.

Like the quilt, the mat does not participate in restricting the access of either probe to the membrane. The quilt, however, may presumably inhibit filipin binding and contribute to patchy PXB binding over the distal acrosomal cap. But it has been lost long before PXB-induced crenulations invade the proximal acrosomal cap, strengthening the theory that the quilt is also incapable of maintaining lipid domains.

Enzyme treatment had no effect on the low binding-level of either probe to the postacrosomal segment. This further validates the conclusion that hydrolyzable surface components do not limit the entry of probes to the membrane or preserve lipid boundaries. Enzymatic extension of PXB binding could be due to the digestion of integral membrane components that control either lipid flow from the distal to the proximal acrosomal cap or the rate of flip-flop from the inner to the outer plasma-membrane leaflets.

FIGURE 16 Plaques of extracellular material coat the acrosomal cap (a) of unwashed epididymal or vasal sperm, while fingerlike projections demarcate the equatorial segment (e). The post-acrosomal surface exhibits no plaques. × 32,000.

FIGURE 17 Unwashed sperm from the body of the epididymis lack a quilt, but a rough mat drapes the surface of the acrosomal cap (a). × 30,000.

FIGURE 18 After 30-min TS-incubation, the quilt vanishes and a filigree design appears in the underlying coarse mat covering the cap (a). × 36,000.

FIGURE 19 Sperm incubated in crude trypsin extract display the same filigreed surfaces as their nontreated counterparts. × 36,000.

FIGURE 20 After incubation in capacitating media, the spermatozoan surface is polymorphic. Some cells, however, lose the rough mat as well as the quilt, exposing the membrane surface. × 36,000.
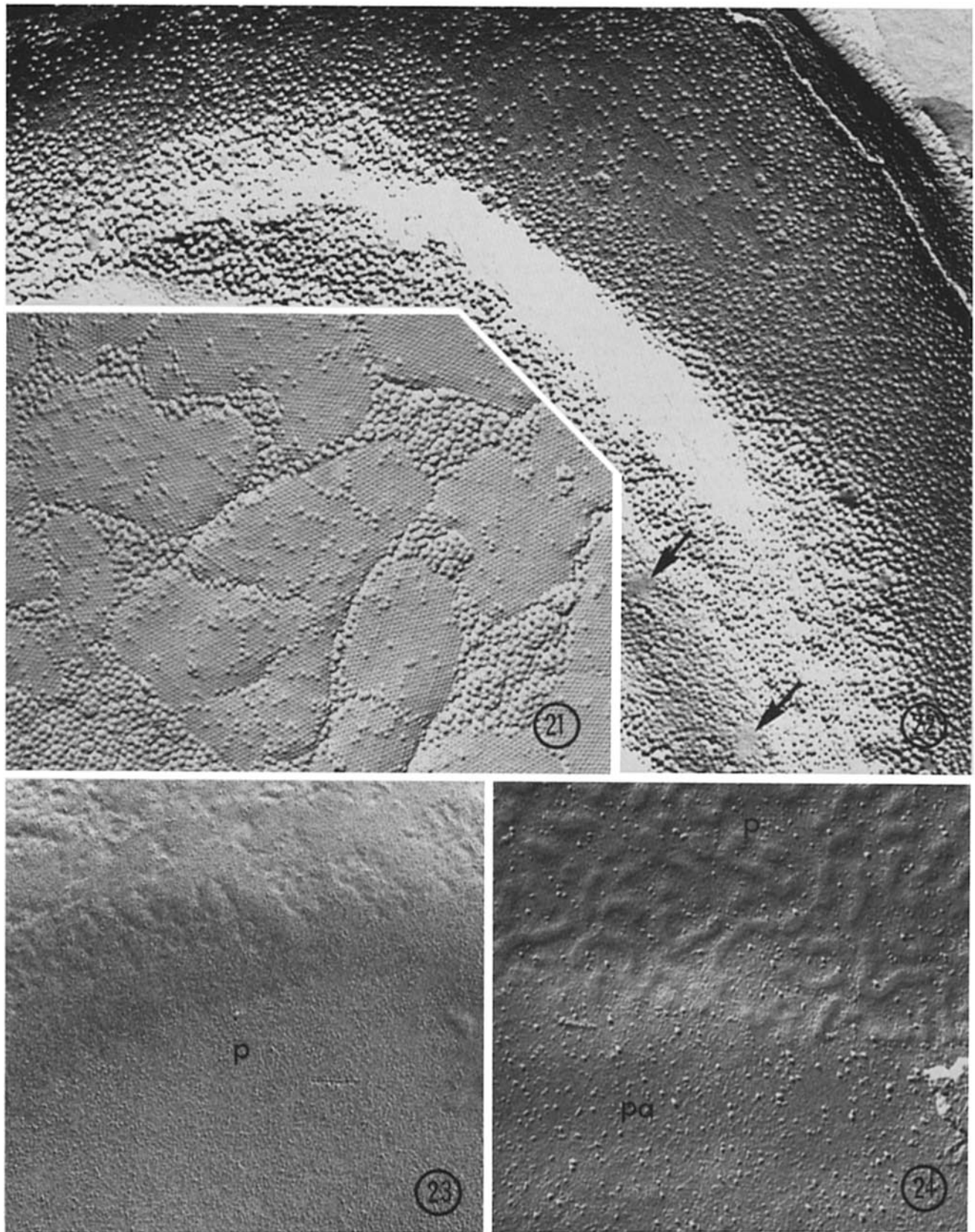
FIGURES 21–24 The effects of trypsinization on filipin and PXB distribution. In Fig. 21, filipin intensifies the quilt design in fresh sperm. × 24,000. Fig. 22 shows, after 30-min trypsinization, filipin/sterol complexes are diffusely dispersed over the whole cap, although the IMP clearings above the tip of the nucleus remain bare (arrows). × 25,000. In Fig. 23, sperm incubated for 30 min in TS have lost the quilt, but the proximal cap membrane (*p*) is still unaffected by PXB. × 34,000. Fig. 24 shows that trypsinization produces enhanced susceptibility to PXB over the entire acrosomal cap, particularly in the proximal portion (*p*), but does not influence the susceptibility of the postacrosomal membrane (*pa*). × 50,000.

Table I

Effects of Various Incubations and Enzyme Treatments on PXB- and Filipin-binding Distributions in Sperm

	Acro* reaction post Ca²⁺ ‡	Distal acro cap	Tip of nucleus§	Prox acro cap	Pa including Es	Acro cap
	%		PXB distribution/filipin distribution			sc/q‖
Fresh	10	+/+¶	−/+	−/+¶	−/+	+/+
24-h Incub ("capac")	60–80	++/++	−/++	++/++	−/+	−/−
30-min Incub	20	+/++	−/++	−/++	−/+	peeling/−
Protease	50–60	++/++	−/+	++/++	−/+	peeling/−

* Abbreviations: Incub, Incubation; "capac", capacitated; Acro, Acrosome reaction 20 min post-calcium, acrosomal; Acro cap, acrosomal cap membrane; Prox, proximal; Pa, post-acrosomal region; Es, equatorial segment; +, present; −, absent.
‡ Average of 10 high-power fields (40 ×), from 3–5 different experiments.
§ Intramembranous particle clearings in these regions do not bind either PXB or filipin.
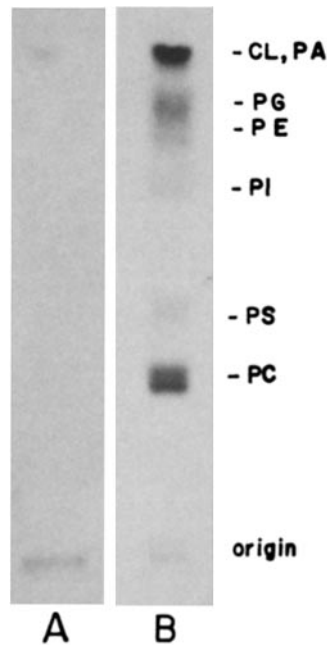‖ Presence of surface coat (sc)/presence of quilt (q).
¶ Clustered.

FIGURE 25 (A) Autoradiograph of a thin-layer chromatogram of chloroform:methanol lipid extracts of sperm incubated for 30 min in ³²PO₄-supplemented TS containing crude trypsin extract. (B) Autoradiograph of a thin-layer chromatogram of chloroform:methanol lipid extracts from sperm capacitated in a ³²P-supplemented medium. The arrowheads indicate co-migration of lipid standards: PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; PI, phosphatidylinositol; PA, phosphatidic acid.
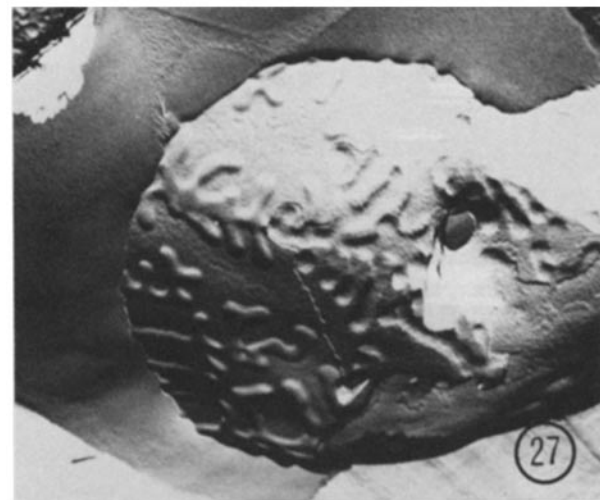
Labels: −CL,PA / −PG / −PE / −PI / −PS / −PC / origin / A / B

Radioactive Phosphate Incorporation

Because the spermatozoon lacks rough endoplasmic reticulum (RER), it was surprising that our preparations could incorporate radioactive phosphate into phospholipids. A slight enhancement of cardiolipin has been reported during capacitation of porcine sperm (16); but this lipid may be synthesized in mitochondria, which contain the largest amounts (27). The synthesis of the other phospholipids has only been conclusively demonstrated in endoplasmic reticulum. However, the presence of just PA and cardiolipin in brief incubations suggests that the synthesis we observed was following the established pathway, which begins with PA.

Protease greatly enhanced PXB-susceptibility, but did not qualitatively change the synthesis of PA or cardiolipin. Because our methods of extraction removed lipid from all of the sperm membranes, it was impossible to ascertain the source of these lipids. Both PA and cardiolipin have been isolated as a small percentage of plasma-membrane lipid in diverse cells (27), although cardiolipin primarily inhabits mitochondrial membranes. Polymyxin B, however, forms volcanic lesions in liposomes composed of PA (25), whereas cardiolipin vesicles respond to PXB with vermiform protrusions; also common in capacitated sperm membranes.

Cardiolipin synthesis occurs in 30-min TS-incubated sperm

FIGURE 26 Phosphatidylcholine-cardiolipin multilamellar vesicles, fixed, glycerinated, and frozen from room temperature. × 34,000.

FIGURE 27 The same vesicle preparation pre-incubated in PXB. × 25,000.

without the addition of protease and without elevation of PXB binding. Thus, an increased rate of anionic-lipid flip-flop outward across the bilayer could account for the enhanced PXB-binding after protease digestion. The inner leaflet, though, may replace its anionic lipid via the synthesis noted here.

Yet it is not unexpected that cardiolipin should contribute to cellular fusional events. Using model lipid systems (14), biophysicists have proposed a possible function for this lipid in fusion. By ³²P nuclear magnetic resonance, x-ray diffraction, and freeze-fracture electron microscopy, cardiolipin has been

observed to form hexagonal arrays at high temperatures or upon the addition of calcium to the suspension (14). These arrays seem particularly attractive in the role of bilayer "destabilizing centers" for the initiation of membrane:membrane fusion. The tiny pores encountered in quick-frozen mast cells during exocytosis correlate with a theoretical model of one small, perpendicularly oriented, rodlike, lipid channel.

CONCLUSIONS

(1) Polymyxin B, a cytochemical probe of anionic phospholipids, revealed a concentration of these lipids in fusigenic parts of the spermatozoan plasma membrane, and a paucity in neighboring but stable domains.

(2) As the membrane prepares for the actual fusional event (i.e., the acrosome reaction), anionic-lipid concentration increases in fusional areas of the membrane. These lipid domains, therefore, are dynamic and form in response to cellular activity.

(3) Crude trypsin extract can augment anionic-lipid concentrations in fusional membrane domains, but not in other regions. Nor does the protease affect β-OH sterol distribution as determined by filipin.

(4) Cell-surface structures do not maintain these lipid domains. The disappearance of the quilt is not sufficient to promote fusion, while the underlying mat remains throughout capacitation.

(5) During incubation in capacitating media, spermatozoa incorporate radioactive phosphate into cardiolipin, PA, and other phospholipids.

(6) Protease does not qualitatively affect the early stages of $^{32}PO_4$ incorporation into PA and/or cardiolipin.

(7) Hence both the synthesis of anionic lipids and their "flip-flop" from interior to exterior plasma-membrane leaflets may be participants in the sequential events leading to capacitation.

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