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Anionic lipid domains: Correlation with functional topography in a mammalian cell membrane

(polymyxin B/plasma membrane/phospholipids/spermatozoa/membrane protrusions)

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ABSTRACT **Polymyxin B was used to explore distribution of anionic phospholipids in sperm plasma membranes by electron microscopy of freeze-fracture replicas. After exposure to Hepes/Tris-buffered polymyxin at 4 mM, phosphatidylcholine liposomes showed no perturbations nor did they fluoresce with dansylated incubation. When phosphatidylethanolamine was included in the liposomes, they became perturbed and fluoresced. Plasma membranes of *Drosophila* larval cells, containing or lacking cholesterol, were also disrupted by polymyxin. The cell membranes of guinea pig sperm were likewise disrupted but in specific functional areas. Fusional membrane domains showed protrusions; the stable membrane of the flagellum revealed diffuse bubbling. Regions of well-defined particle arrays and the postacrosomal segment maintained smooth contours. By fluorescence microscopy, we detected the same heterogeneous binding of the polymyxin dansyl derivative.**

By electron microscopy of freeze-fracture preparations, the cell membrane is a tapestry of different textural domains. In the guinea pig sperm plasma membrane, particle arrays (proteins) observed in freeze-fracture replicas exhibit characteristic patterns on particle surfaces—e.g., the "quilt" of the head, and the zipper and annulus of the tail (1). Recently, labeling sperm cells with filipin, a polyene, has also demonstrated cholesterol in a heterogeneous distribution (2, 3). Like filipin, polymyxin B (PXB) disturbs the contour of membranes containing the lipids that it binds. PXB binds anionic lipids, revealing their concentration distribution. After exposure to PXB, the outer membranes of Gram-negative bacteria erupt in protrusions (4–6) discernable by electron microscopy of thin-section, freeze-fracture, and scanning preparations (7, 8); the number of protrusions is correlated with the PXB concentration (9). Moreover, PXB has a proven affinity for anionic lipids (10–21).

In this report, we demonstrate that PXB binds to a mammalian spermatozoon and insect larval cells, producing protrusions similar to those observed in prokaryotes. Unlike the uniformly disposed eruptions of bacterial membranes, however, various membrane perturbations emboss only specific regions of the sperm membrane.

MATERIALS AND METHODS

Liposomes. Unilamellar and multilamellar vesicles were prepared in D. Papahadjopoulos' laboratory (Cancer Research Institute, University of California, San Francisco) from egg phosphatidylcholine (PtdCho) (Sigma), phosphatidylethanolamine (PtdEtn) (transesterified egg PtdCho), and cholesterol (22). Phospholipids (10 μmol)—PtdCho alone, PtdCho/PtdEtn (50% each), or PtdCho/PtdEtn/cholesterol (molar ratio 1:1:1)—were formed and suspended in 1 ml of 0.1 M Hepes/Tris buffer at pH 7.4. The vesicles were stored under argon at 4°C until used. We incubated treated and untreated vesicles in 25% glycerol for 1–2 hr before freezing, or processed them without glycerol for thin-sectioning as specified (2). Treatment of liposomes consisted of incubation at 37°C in 2 mM PXB (Sigma) in 0.1 M Hepes/Tris buffer (pH 7.4). Additionally, one group of PtdCho/PtdEtn/cholesterol liposomes was incubated for 1 hr in 1.25% glutaraldehyde (pH 7.4, 1% sucrose) in the same buffer and processed for freeze-fracture.

Cell Suspensions. Third-instar *Drosophila melanogaster* larval cells, provided by C. M. Havel (Department of Biochemistry and Biophysics, University of California, San Francisco), were grown in cholesterol-free medium until depleted of the sterol (23). Then the cells were incubated with 4 mM PXB in 0.1 M Hepes/Tris buffer (pH 6.8) at 37°C for 10 min or were replenished with cholesterol and then incubated. Next, they were fixed for 1 hr in 1.25% glutaraldehyde in 0.1 M sodium cacodylate/1% sucrose and prepared for freeze-fracture as above. Control cells were fixed without PXB incubation. The lower pH could slightly change the kinetics of lipid–PXB association.

Guinea pig spermatozoa from ether-anesthetized, mature (>500 g) animals were obtained from the epididymal tails and vasa deferentia. The pooled sperm was either fixed directly, as for the larval cells, or incubated in 0.1 M Hepes/Tris-buffered 4 mM PXB, for 10 min at 37°C (pH 7.4) and then fixed. Cells were then prepared for either freeze-fracture or thin section.

Fluorescence Microscopy. PXB dansyl derivative was prepared as described (5), with dansyl chloride from Sigma. The eluate was tested chromatographically for purity (21). That conjugation had been achieved was confirmed by a shift of the absorption spectrum to the red. Liposomes and sperm in buffered 5 mM PXB dansyl derivative were monitored at 10-min intervals for 1 hr.

OBSERVATIONS

Liposomes. In the presence of PXB, multilamellar liposomes comprised of PtdEtn and PtdCho in equimolar ratio, with or without equimolar addition of cholesterol, developed protrusions on the outer lamellae at physiologic pH and temperature, in 0.1 M Hepes/Tris-buffer (Fig. 1 *A* and *D*). Non-PXB-treated multilamellar vesicles of the same composition, and both treated and untreated vesicles containing egg PtdCho (Fig. 1*B*), were smooth-surfaced, as were the inner layers of all liposomes (Fig. 1 *B–E*). That only outer lamellae of PtdEtn-containing liposomes was perturbed is consistent with observations of bacterial

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Abbreviations: PXB, polymyxin B; PtdEtn, phosphatidylethanolamine; PtdH, phosphatidic acid; PtdCho, phosphatidylcholine.

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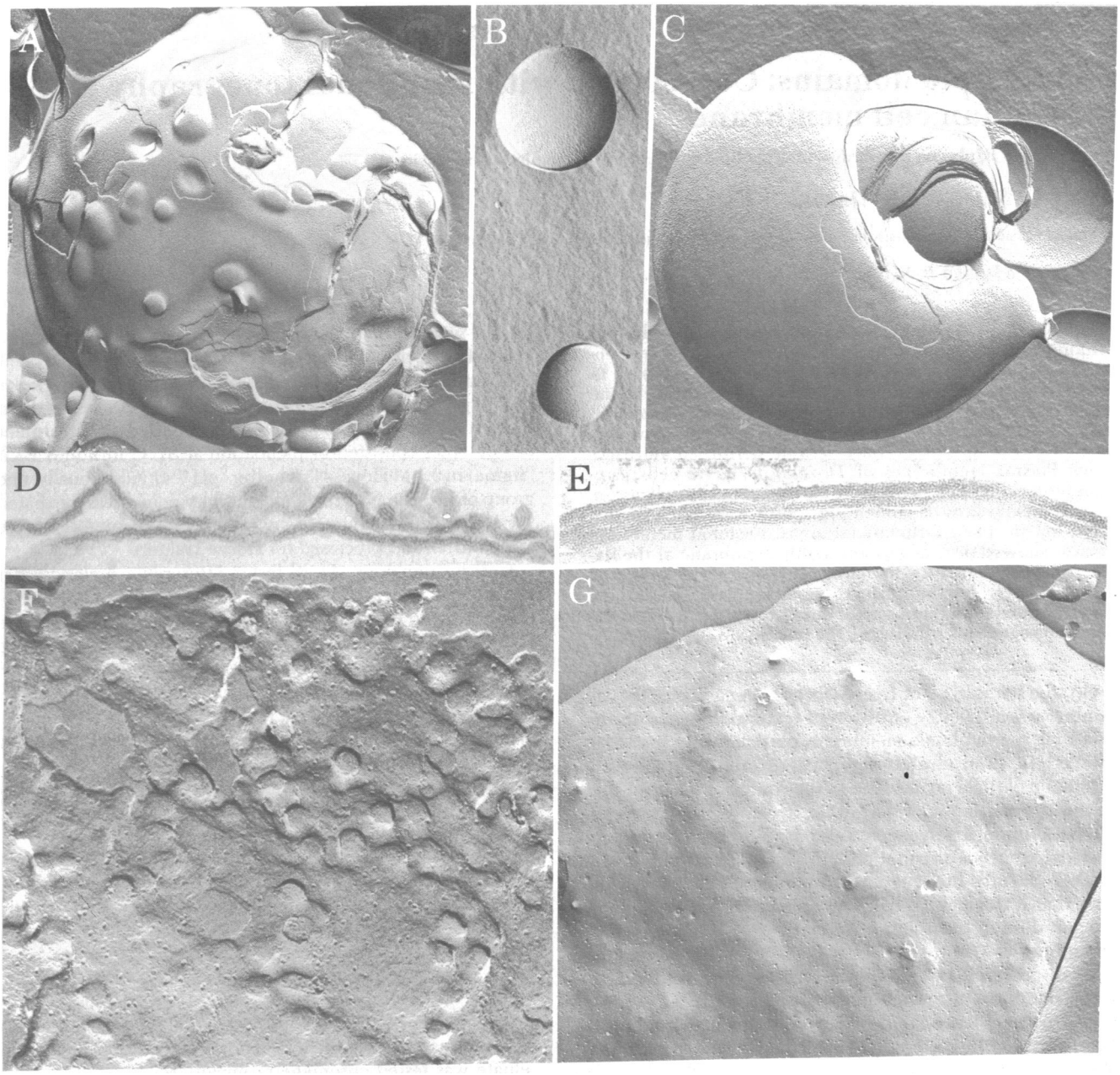

FIG. 1. (*A*) Freeze-fracture replica of a PtdCho/PtdEtn/cholesterol liposome incubated in PXB and then fixed in glutaraldehyde. Conspicuous antibiotic-induced bubbles (0.8 μm in diameter) decorate the outer membrane. (×26,000.) (*B*) PtdCho vesicles incubated in PXB do not show protrusions. (×21,000.) (*C*) Fractured PtdCho/PtdEtn/cholesterol non-PXB-treated liposome; note smooth lamellae. (×29,000.) (*D* and *E*) Thin sections through the specimens depicted in *A* and *C*, respectively. All the untreated bilayers and only the inner bilayers of PXB-treated vesicles show linearity, whereas the outer treated membranes form 0.8-μm-diameter blebs. (×70,000.) (*F*) Portion of a *Drosophila* larval cell, depleted of cholesterol, after PXB incubation. Multiple 0.8-μm protrusions cover the plasma membrane. (×55,000.) (*G*) Untreated, cholesterol-depleted *Drosophila* larval cell, fractured through the plasma membrane and shadowed from above. A few truncated microvilli dot the otherwise smooth surface. (×22,000.)

membranes—i.e., that PXB interacts with the outer membrane and does not readily penetrate it.

Thin sections also showed disruption of the multilamellar structures (compare Fig. 1 *D* to *E*) by PXB. The outermost membrane erupted in protrusions of the same size as those seen in fractures. When a dansyl chloride fluorescent probe was conjugated to PXB, the PtdEtn-containing multilamellar vesicles fluoresced, demonstrating the binding of the antibiotic.

Drosophila larval cells grown in cholesterol-deficient medium were exposed to PXB before and after cholesterol replenishment. Membranes known to contain no cholesterol (Fig. 1 *F* and *G*) and cholesterol-saturated membranes were equally perturbed by the antibiotic (23).

Spermatozoa. The spermatozoon is an extensively specialized mammalian cell with three structural divisions: (*i*) the head, which in guinea pig sperm is overlain by the crystalline pattern of the quilt [here the plasma membrane covers the acrosomal cap, the equatorial segment of the acrosome, and the postacrosomal portion of the nucleus (Fig. 2*A*)]; (*ii*) the midpiece of the tail, arrayed with strands of particles, contains mitochondria, the flagellar root, centrioles, and the cytoplasmic droplet; and (*iii*) the principal piece, the continuation of the flagellum, which is separated from the midpiece by a densely particulate annulus and is embroidered down its length by two straight lines of transmembrane particles, the zipper.

Due to the clear functional and morphological delineation

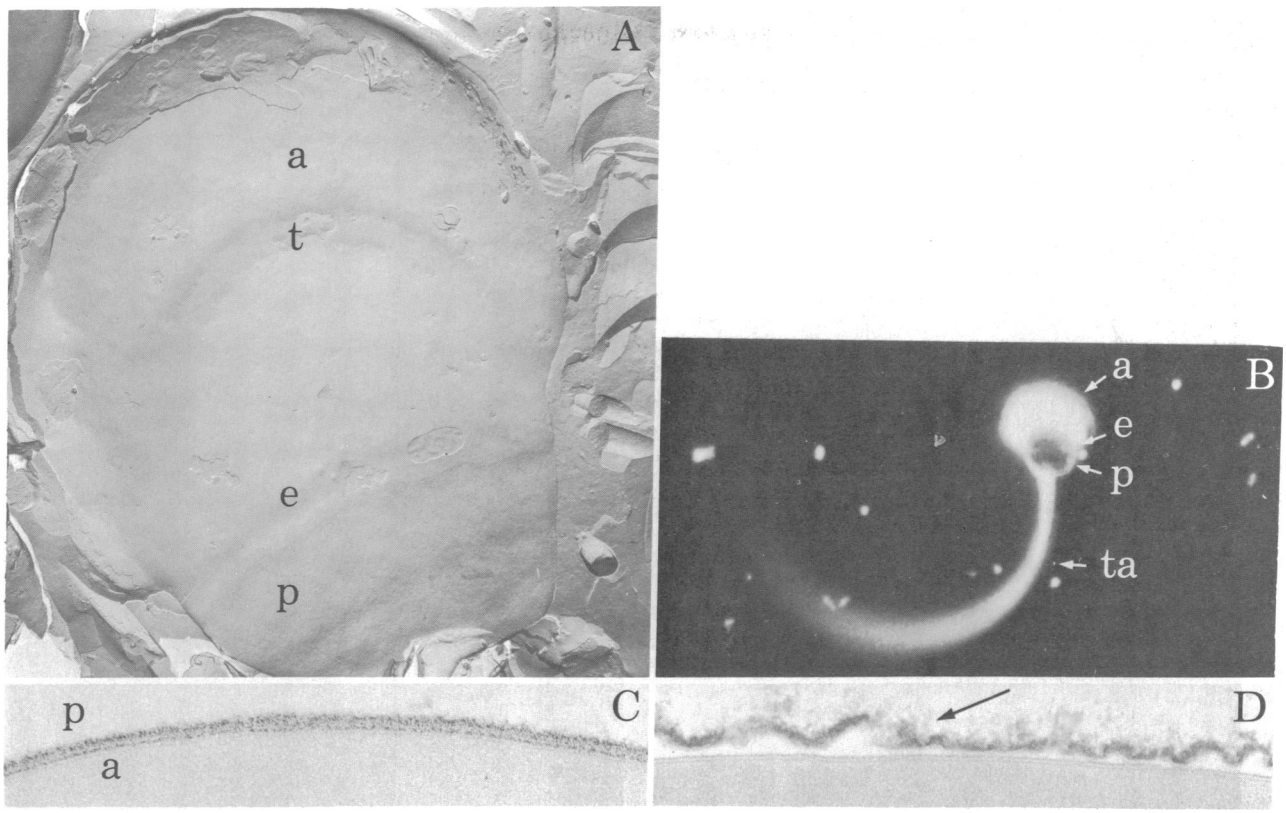

FIG. 2. (*A*) Plasma membrane of a control sperm head, exhibiting undisrupted contours. a, Acrosomal cap; t, position corresponding to the tip of the nucleus; e, equatorial segment; and p, postacrosomal segment. (×6000.) (*B*) Fluorescent acrosomal cap (a) of a spermatozoon incubated in PXB dansyl derivative. Both the equatorial (e) and postacrosomal (p) segments remain dark; the tail (ta) fluoresces faintly. (×1000.) (*C*) Section through the acrosomal cap of a control spermatozoon, showing plasma membrane (p) and outer acrosomal membrane (a). (×78,000.) (*D*) Section through the acrosomal cap of a sperm cell incubated in PXB. The plasma membrane (arrow) is crinkled; the acrosomal membrane remains flat. (×92,500.)

of each part, sperm cells are uniquely suitable for investigation of the relationship of function to topographical differences in lipid concentrations.

Guinea pig sperm taken from the tails of the epididymides and vasa deferentia and treated with the dansyl derivative of PXB fluoresced vividly in the fusigenic acrosomal cap region and faintly in the stable principal piece (Fig. 2*B*). The postacrosomal region and midpiece remained dark. In freeze-fracture and thin-section preparations, PXB-treated sperm revealed protrusions not seen in control cells (Fig. 2*C*) but characteristically engendered by the agent (Figs. 2*D* and 3*A*). Perturbed areas corresponded in location to those that fluoresced (Fig. 3*B*).

PXB created several types of membrane-bilayer perturbations in different zones. Crenulations and small protrusions adorned 99% of the acrosomal caps (Fig. 3 *B* and *D*). At first restricted to the tip of the acrosomal cap, the protrusions gradually descended toward the equatorial segment in the wake of the melting quilt pattern. When sperm were incubated before fixation, principal-piece membranes were occasionally bubbled down their length. Conspicuously damaged principal-piece membrane, with clumped particles and irregularly shaped particle-free patches, showed no increase in the density of surface bulges. Unperturbed areas of membrane consisted of tracts covered with dense particle arrays—e.g., the quilt (Fig. 3 *C* and *D*), the midpiece strands, the annulus, and the zipper. The portion that arches over the top of the nucleus, as well as the postacrosomal segment, also remained untouched (Fig. 3 *B* and *C*). Neither the acrosomal membrane nor the nuclear envelope was altered by PXB. Thin sections of the head and principal piece revealed the same membrane perturbations observed in fracture (Fig. 2 *C* and *D*).

Barring the cytoplasmic droplet, the midpiece, too, retained its smooth contours and typical protein arrays after incubation with PXB.

Sperm continued to swim for more than 1 hr in medium containing the PXB dansyl derivative if not subjected to ultraviolet irradiation. This duration of incubation, however, proved to be unnecessary because the distribution of fluorescence did not change after the first 10 min in the medium. Addition of 0.4 ml of 10 mM calcium chloride to 1 ml of sperm or liposome suspension reduced the fluorescence of both liposomes and sperm.

DISCUSSION

PXB binds anionic phospholipids. When exposed to the antibiotic, entire outer membranes of these bacteria form protrusions (5–9). Only in specific areas, however, are the plasma membranes of spermatozoa susceptible to the effects of PXB. In contrast, the whole membrane is affected by this agent in *Drosophila* larval cells and liposome membranes containing anionic lipids. The heterogeneous distribution of PXB receptors on the surface of the highly specialized gamete implies that these lipids are not free to diffuse laterally throughout the membrane. In addition, fluorescence-labeling techniques substantiate freeze-fracture evidence of the heterogeneity of binding availability. Previously, investigations utilizing filipin proved that the plasmalemma is indeed capable of forming protrusions in membrane regions lacking susceptibility to PXB: these regions lose their smooth contours when there is poly-

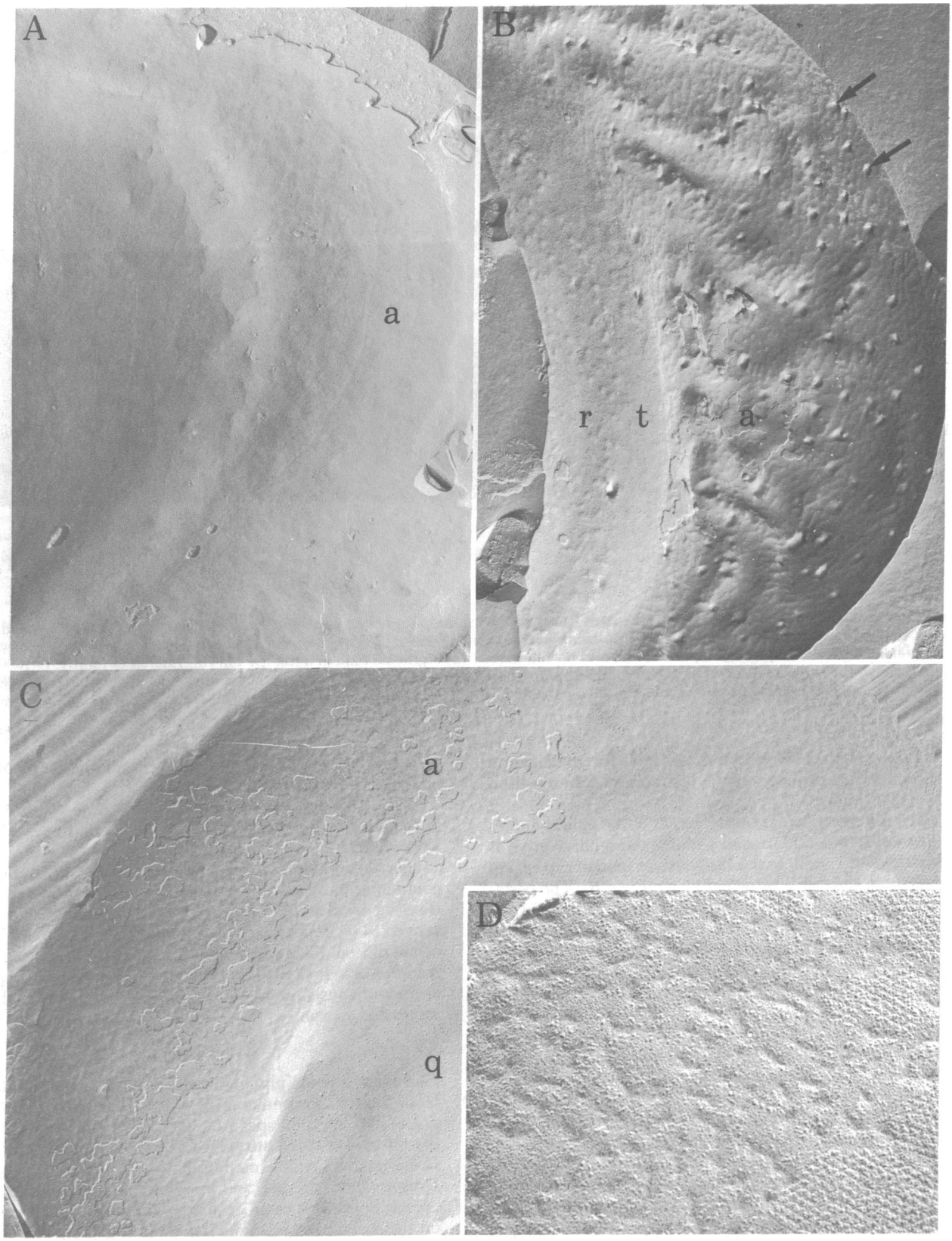

FIG. 3. (*A*) Freeze-fracture replica of a normal acrosomal cap (a) in a non-PXB-treated sperm cell. (×14,500.) (*B*) Extensively perturbed plasma membrane of a PXB-treated sperm head, with acrosomal cap (a) studded with blebs (arrows). Overlying the tip of the nucleus (t), the membrane remains smooth; the plasmalemma over the remainder of the acrosome (r) is lightly crenulated. (×6000.) (*C*) Lightly crinkled plasma membrane over the acrosomal cap (a) and, proximally, an undisturbed quilted area (q). The degree of perturbation ranged from that shown here to the widespread wrinkles and blebs with loss of the quilt pattern illustrated in *B*. (×16,000.) (*D*) Higher magnification of the crenulation amid the disintegrating quilt. Two round areas are devoid of quilt, particles, or crinkles. These circular patches may have been free of anionic lipids. (×85,000.)

ene/sterol aggregation (3). Although it may be argued that inhibition of binding, rather than a paucity of anionic lipids, spares the membrane of the spermatozoan midpiece and postacrosomal segment in the presence of PXB, most potential inhibitory effects can be excluded: (*i*) the membrane is available to filipin, and this polyene is capable of insertion; (*ii*) neither cholesterol nor PtdCho (both implicated as binding inhibitors) prevents membrane disruption in *Drosphila* cells or liposomes; (*iii*) moreover, anionic sites remain unmasked in these areas, as evidenced by the binding of colloidal iron hydroxide, a much larger molecule than PXB (24). The distribution of colloidal iron hydroxide, a nonspecific anion label, overlaps that of PXB disturbance. We therefore consider it highly probable that anionic lipids are heterogeneously dispersed in the plane of the bilayer.

PXB-induced membrane disruption is variable in terms of contour. Blebbing and wrinkling may be a result of the deletion of anionic lipids from the membrane. In high concentrations or with long incubation, PXB solubilizes the membrane, denuding the cell and casting vesicles into the surrounding medium. Because of susceptible-membrane variance in form, it is impossible to pinpoint the precise whereabouts of the PXB/lipid complex.

All four of the phospholipid substrates for PXB—phosphatidylglycerol, cardiolipin, PtdEtn, and phosphatidic acid (PtdH)—are present in mammalian sperm, with PtdEtn the greatest in proportion (25–28).

The three major functional regions of the spermatozoon respond quite differently to the antibiotic PXB. Specialized for fusion, the head, where exocytosis of the acrosomal contents occurs over the cap, becomes crenulated as the quilt pattern vanishes. The postacrosomal area of the membrane, the site of subsequent sperm–egg fusion, is seldom disrupted. The midpiece, performing other cellular functions including energy transduction, is perturbed only over the cytoplasmic droplet and not over the mitochondria. Finally, the principal piece, is bubbled infrequently. These differences in perturbation correlating with the functional areas (fusional, energy-generating, and motile) suggest that different amounts and types of anionic lipids participate in these membrane specializations.

Phase separation of lipid bilayers (composed of either a single lipid or a combination of lipids) has been the focus of many studies, but other biophysical properties of lipids may influence lipid heterogeneity. Anionic lipids, carriers of negative charge, are uniquely able to modulate electrical fields, such as surface voltage potential (13, 29–31). When binding anionic lipids, PXB decreases membrane capacitance (20). Gel rather than fluid state of anionic lipids could increase the magnitude of their electrical, ionic, or neutrophilic influence on other molecules in their vicinity. Phase separations of these lipids are affected by pH as well as by ionic strength, temperature, and cholesterol (15, 32–34). With high concentrations of cations, significantly Ca^{2+}, tight aggregations of PtdH can form at physiologic pH and temperature (32). These aggregates squeeze out PtdCho, thus enriching two adjacent areas of membrane in two different lipids. Binding of Ca^{2+} also creates a point of high cationic concentration localized on the membrane.

Well-defined foci of high anionic lipid concentration may serve a completely different purpose in membrane function than that of diffusely high levels of the acidic lipids. In all likelihood, the uniform bubbling on the stable membrane of the spermatozoan principal piece indicates a disseminated but high anionic lipid concentration; the crenulations and distinct protrusions on the fusigenic acrosomal cap may reflect focal sites of aggregations. Diffuse deployment of these lipids may then stabilize the membrane, and focusing of the lipids could help to unite apposing membranes by cationic crosslinking of negative charges. It is especially thought-provoking that either Ca^{2+} or Mg^{2+} can compete with PXB for the same binding sites (13, 14, 19, 35).

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