Freeze-Fracture Studies of Photoreceptor Membranes: New Observations Bearing upon the Distribution of Cholesterol

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ABSTRACT We performed electron microscopy of replicas from freeze-fractured retinas exposed during or after fixation to the cholesterol-binding antibiotic, filipin. We observed characteristic filipin-induced perturbations throughout the disk and plasma membranes of retinal rod outer segments of various species. It is evident that a prolonged exposure to filipin in fixative enhances rather than reduces presumptive cholesterol detection in the vertebrate photoreceptor cell. In agreement with the pattern seen in our previous study (Andrews, L. D., and A. I. Cohen, 1979, *J. Cell Biol.*, 81:215–228), filipin-binding in membranes exhibiting particle-free patches seemed largely confined to these patches. Favorably fractured photoreceptors exhibited marked filipin-binding in apical inner segment plasma membrane topologically confluent with and proximate to the *outer* segment plasma membrane, which was comparatively free of filipin binding. A possible boundary between these differing membrane domains was suggested in a number of replicas exhibiting lower filipin binding to the apical plasma membrane of the inner segment in the area surrounding the cilium. This area contains a structure (Andrews, L. D., 1982, Freeze-fracture studies of vertebrate photoreceptors, *In* Structure of the Eye, J. G. Hollyfield and E. Acosta Vidrio, editors, Elsevier/North-Holland, New York, 11–23) that resembles the active zones of the nerve terminals for the frog neuromuscular junction. These observations lead us to hypothesize that these structures may function to direct vesicle fusion to occur near them, in a domain of membrane more closely resembling outer than inner segment plasma membrane. The above evidence supports the views that (a) all disk membranes contain cholesterol, but the particle-free patches present in some disks trap cholesterol from contiguous particulate membrane regions; (b) contiguous inner and outer segment membranes may greatly differ in cholesterol content; and (c) the suggested higher cholesterol in the inner segment than in the outer segment plasma membrane may help direct newly inserted photopigment molecules to the outer segment.

The binding of the sterol-specific polyene antibiotic filipin produces lesions observable by freeze-fracture (37, 38) and thus may be used as a marker for sterol-containing membranes or membrane regions. This idea was initially pursued independently by us (5) and by another group (11), and is beginning to be applied in a variety of systems (1, 6, 8, 16–20, 22–26, 28, 30, 32–36). In our previous study on the membranes of the base of retinal rod outer segments (ROS) (5),[1]

[1] *Abbreviations used in this paper:* DMF, dimethyl formamide; IS, inner segment; OS, outer segment; PFP, particle-free patches; and ROS, rod outer segment.

retinas were exposed to filipin prior to fixation. Filipin-binding in outer segments (OS) (but not in inner segments [IS]) was confined to particle-free patches (PFP). This suggested that a difference in cholesterol content or distribution might be correlated with a difference in PPF content observed in basal and apical disks. The absence of filipin binding in certain particulate ROS membrane regions could not then be rigorously interpreted, since factors independent of sterol content, such as the permeability of membranes to filipin, could not be evaluated. In a recent review of this technique (31), it has been argued that more dependable results are obtained if fixation occurs prior to or simultaneously with the exposure

to filipin. Although in our earlier study we observed no rearrangement of intramembrane particles (a major artifact observed in reference 31), we wanted to examine the distribution of filipin binding in retinas in which the exposure to filipin and fixation occurred simultaneously. Here we report new observations on the sites of filipin binding in the ROS membranes of toads, northern frogs, and mice. These observations have further implications for the disposition of cholesterol in ROS membranes, the significance of filipin-binding in PFP of OS membranes, and the relations of basal disk membrane and OS plasma membrane.

We also describe the binding of filipin to various membranes or membrane regions of the frog rod and cone IS. These structures include one (described in reference 4) that resembles the membrane ridges associated with active zones of the nerve terminal of the neuromuscular junction. These findings suggest a hypothesis that may explain the vectorial flow of photopigment molecules from the IS to the OS (39).

MATERIALS AND METHODS

We obtained *Rana pipiens pipiens* and *Bufo marinus* respectively from NASCO, Inc. (Ft. Atkinson, WI) and Carolina Biological Supply Co. (Burlington, NC). The former were of northern United States origin (obtained in the Great Lakes region), and the latter from the southwestern United States. Mice used were of the pigmented, C57BL6/J strain. All animals were maintained under a 12 h light: 12 h dark light cycle.

In most of the experiments reported here one retina from each mouse and frog was exposed to a fixative solution (2.5% glutaraldehyde in either 0.16 M

FIGURE 1   Filipin-induced pits in the disk membranes of mouse ROS. Pits may be seen throughout a substantial length of a longitudinally fractured ROS in a retina exposed to filipin (1 mg/ml in fixative) for 20 h. This region appeared to be closer to the pigment epithelium than to the inner segments. Bar, 0.5 μm. × 46,000.
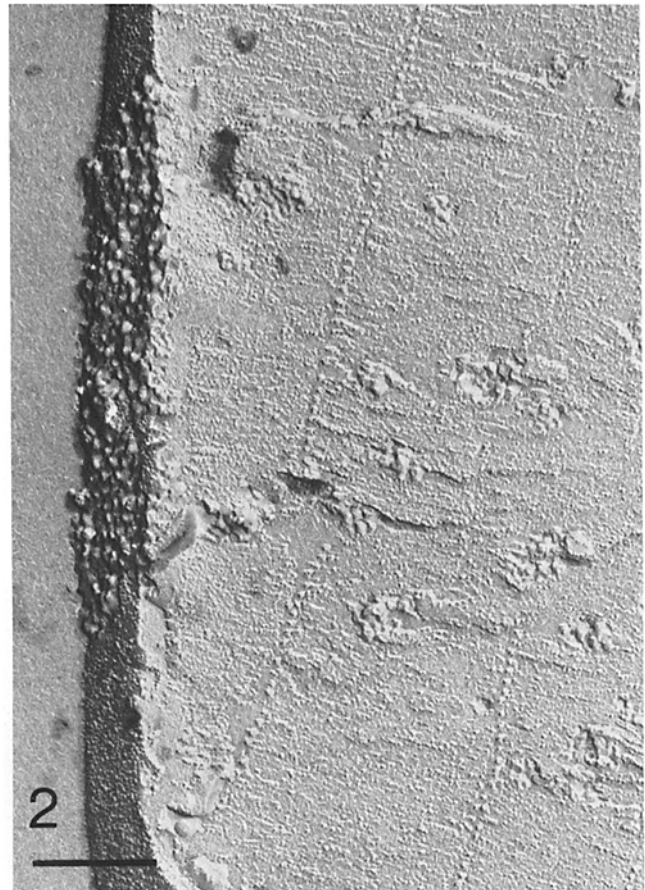

FIGURE 2   Filipin-induced pits in ROS disk membranes of a frog. This region was typical of the entire length of the longitudinally fractured ROS both in this retina, which was sonicated prior to a 10-min exposure to filipin, and also in retinas exposed to filipin for 20 h without sonication. Bar, 0.5 μm. × 35,000.

cacodylate buffer for mice or frog Ringer's [12] for frogs) containing filipin (1 mg/ml, 10% dimethyl formamide [DMF] for mice and 0.2 mg/ml, 2% DMF for frogs) for 20–23 h at room temperature (~22°C). The other retina from each animal was used as a control, i.e. was fixed in a similar solution containing the same concentration of DMF but lacking any filipin.

In another experiment designed to facilitate filipin access to disk membranes, one frog retina and one toad retina were first transferred to fixative immediately following dissection and then sonicated for 15 s in a bath sonicator, after which filipin in DMF was added at a final concentration of 0.5 mg/ml (10% DMF). After 10 min the filipin-containing solution was decanted and fresh fixative added. The filipin incubations were done at room temperature. The remaining retina from each animal was again used as a control by omitting filipin from their otherwise identical treatment. After fixation, retinas were cryoprotected for 1–3 h in 25% glycerol, freeze-fractured on a Balzers BAF-301, and replicas were cleaned as described before (5) and viewed on either a Siemens 1A or JEOL 100-CX electron microscope.

RESULTS

In contrast to the results of our previously reported short incubations (5), prolonged (20 h) incubations in filipin plus fixative resulted in the appearance of pits in random fracture faces of disks from any region along the length of mouse and frog ROS (Figs. 1 and 2). In each case characteristic filipin-induced pits were observed in the densely particulate areas of both the disk and plasma membranes which are normally free of PFP. Since there is a clear tendency for particles to be excluded from the pits representing filipin-cholesterol complexes (Fig. 3), caution must be exercised in deducing the membrane particle pattern that was present prior to filipin
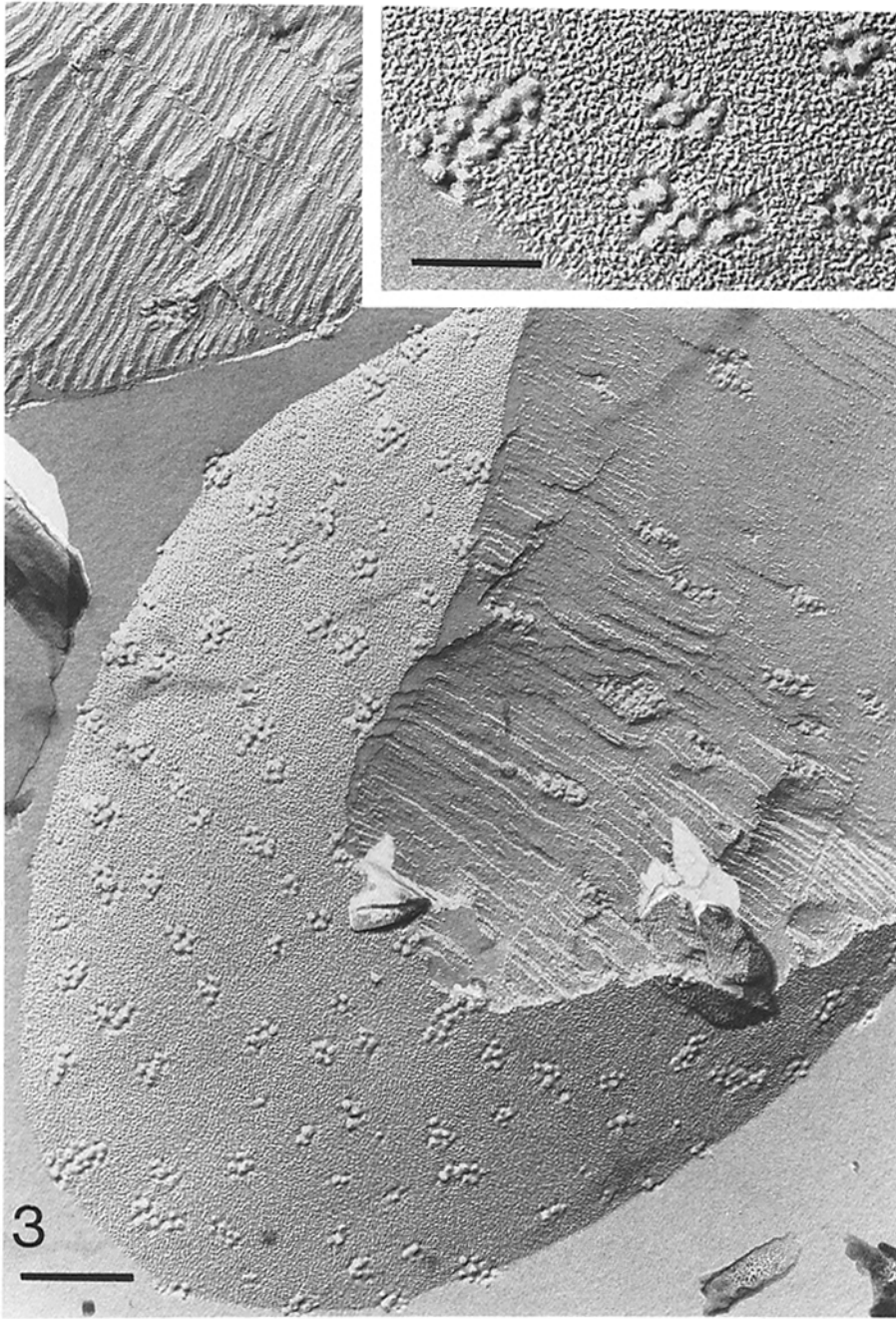
FIGURE 3 The exclusion of particles from clusters of filipin-induced pits in the ROS plasma membrane of a frog, in which controls lacked PFPs. A site in which some mixing of particles and pits occurs is shown at higher magnification in the insert. No PFPs without pits are present. Bar, 0.5 μm. × 28,400. *Insert:* bar, 0.25 μm; × 66,600.

binding. To conclude that binding occurred within pre-existing PFP, it is necessary to observe PFP of similar dimensions in the same region in controls not exposed to filipin. Other observations that aid in this decision are the presence of PFP of normal rounded outline only partly filled with pits, and the nearby presence of PFP devoid of pits. The last two conditions are met in Fig. 4. On the other hand, a close adherence of particles to the perimeter of each cluster of filipin pits, and the occasional trapping of membrane particles in the cluster, are consistent with binding to a particulate membrane region with subsequent particle exclusion.

As suggested in our previous report (5), there was greater filipin binding observed in the plasma membrane of the apical IS than in that of the OS of rods. This is most clearly demonstrated by examination of the filipin binding to the plasma membranes of calycal processes and the immediately

underlying OS of several toad rods (Figs. 5 and 6).

Another noteworthy observation was that of filipin binding to the periciliary plasma membrane in the apex of the rod inner segment. This region contains linear particle arrays, which are radially oriented around the base of the cilium (4) (Fig. 7). These structures are reminiscent of particle arrays observed in the presynpatic nerve terminal of the frog neuromuscular junction (21). Filipin was not observed to be bound to this area when the reaction intensity was relatively low throughout the rod inner segment plasma membrane (Fig. 8), but did bind to this area in an instance in which the reaction intensity was generally high (Fig. 9). Binding was also observed in a similar structure in a cone inner segment displaying generally moderate filipin binding (Fig. 10), but to a much lesser extent than to surrounding contiguous membrane.
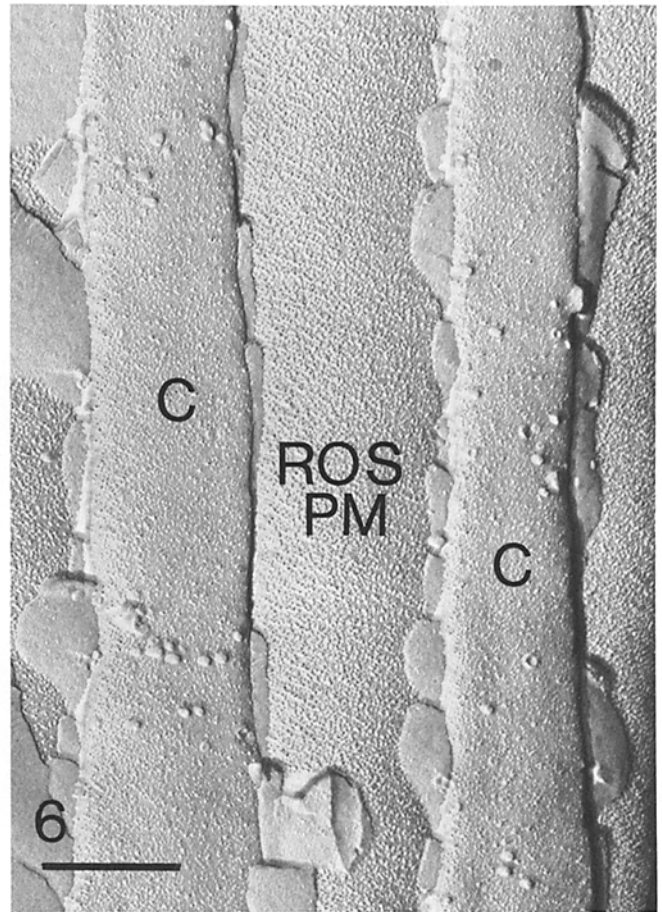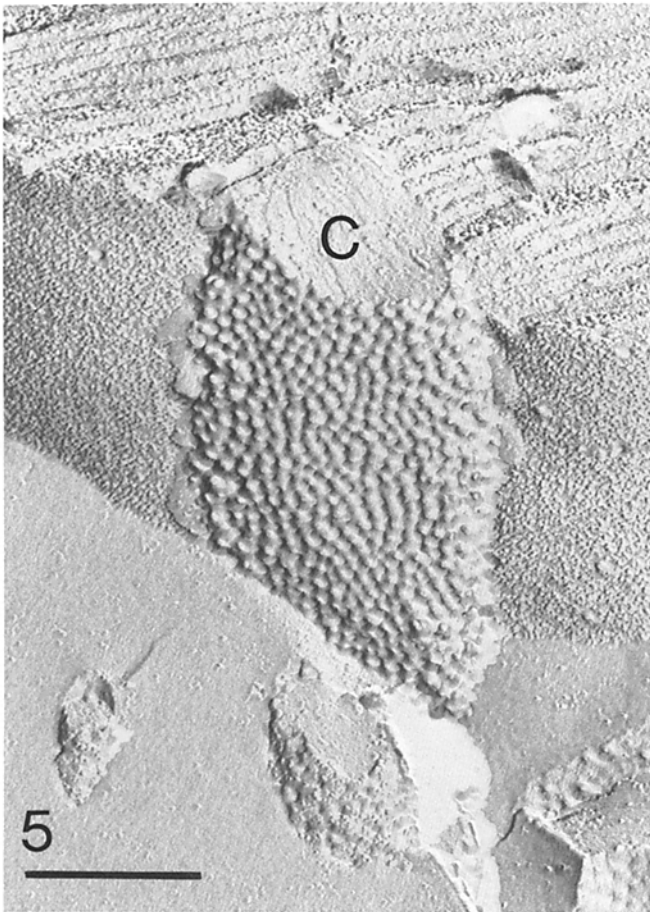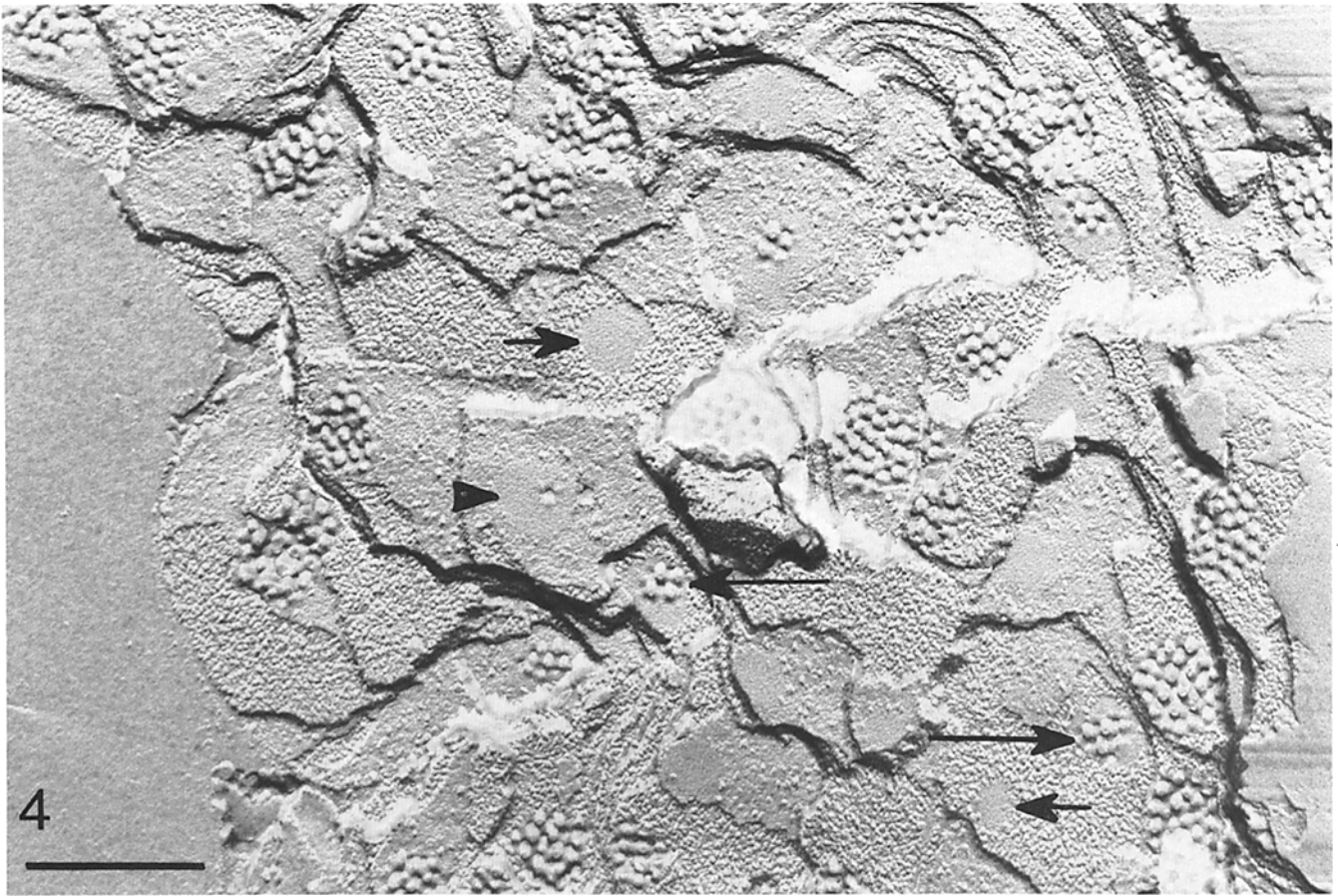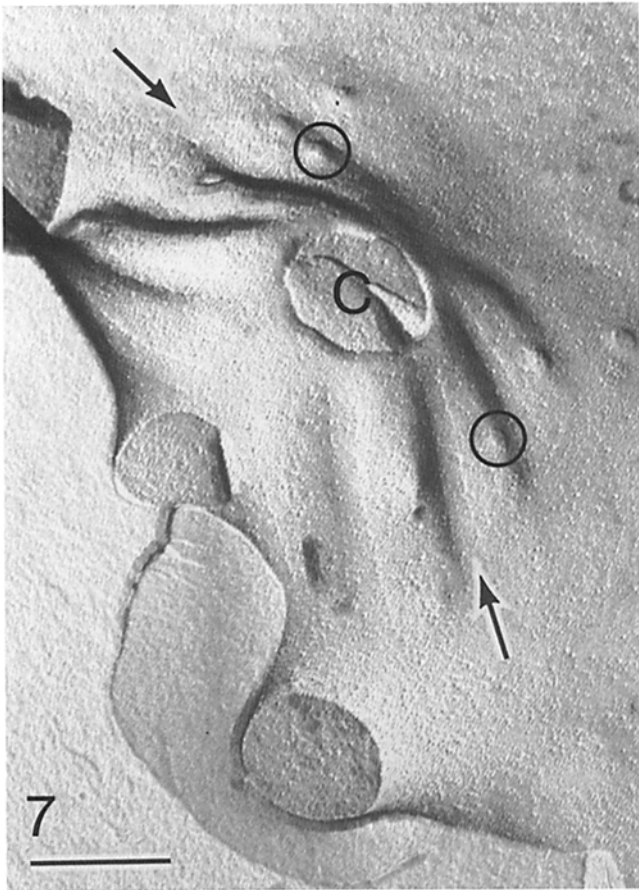
Figure 7 Particle rows located on shallow ridges in the P-face of the plasma membrane at the apex of a frog rod inner segment (two of which are indicated by arrows). These structures are radially oriented around the connecting cilium (C), and resemble the active zones on the nerve terminal of the frog neuromuscular junction. Note also the presence close to these ridges of "dimples" (two of which are circled) which suggest sites of exo- or endocytosis. Bar, 0.5 µm. × 35,900.
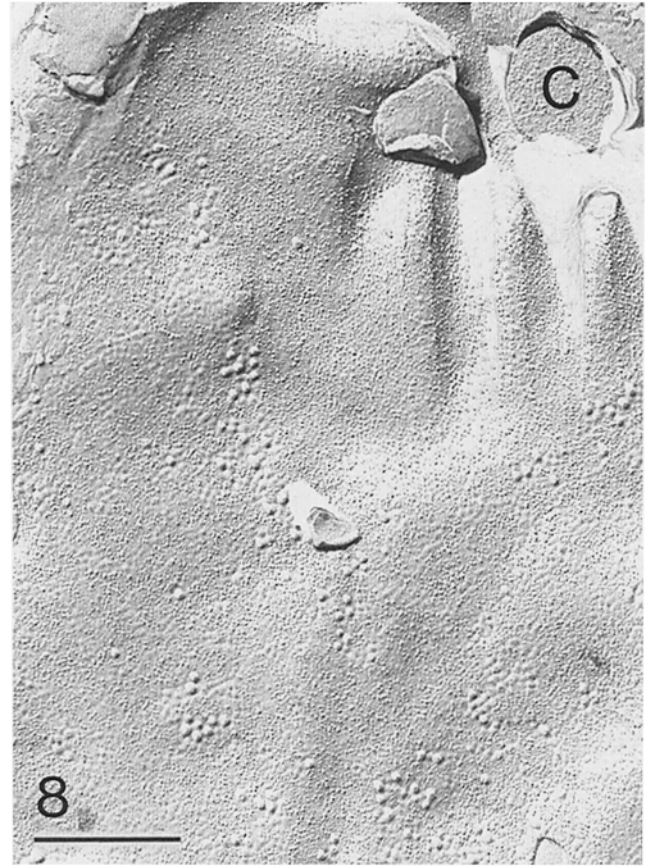
Figure 8 Moderate filipin binding to the apical plasma membrane of a frog rod inner segment. Note the absence of pits in the region occupied by the membrane specializations near the connective cilium (C). Bar, 0.5 µm. × 52,000.

DISCUSSION

The prolonged exposure of northern frog, toad, and mouse retinas to a fixative solution containing the sterol-specific polyene antibiotic filipin has shown that filipin binding is possible in particulate areas of ROS membranes that lack particle-free patches. The overall abundance of filipin binding appeared similar along the entire length of the ROS observed, excluding the basal disks. Moreover, basal disks may have the same total amount of filipin binding, although it is concentrated in their PFP. Any tendency for filipin to extract cholesterol should not be markedly reduced by glutaraldehyde fixation, since cholesterol has no primary amine moiety avail-

able to react with glutaraldehyde. The observation of filipin binding in particulate membrane regions after a long exposure to a filipin-containing fixative solution thus argues against the possibility of a selective extraction by filipin of cholesterol from particulate membrane regions of ROS disks. Therefore, the failure of filipin to bind to particulate regions of the ROS membranes containing PFP is probably due to a lower local concentration of cholesterol in membrane surrounding PFP rather than to cholesterol extraction or other artifacts.

Lateral phase separations in membrane lipids can produce PFP in some other membrane systems and have been found to influence filipin binding to membrane sterols, but not in a consistent way. Ohki et al. (27) found that filipin binding to sterols in phosphatidylcholine bilayer membranes doubled upon lowering the temperature through the phase transition and continued to increase at even lower temperatures. Sekiya et al. (35) studied the effects of lipid phase separation of filipin

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Figure 4 Filipin-induced pits in ROS disk membranes of a toad. Pits in the P-face of these membranes are confined to PFP (long arrows), but not all PFPs have pits (short arrows). In the E-face, note that there are smooth regions resembling P-face PFPs some of which contain pits (arrowhead). Bar, 0.5 µm. × 46,000.

Figures 5 and 6 Greater filipin binding to the plasma membranes of calycal processes than to the ROS plasma membrane in the toad retina. In Fig. 5, the intensity of the reaction to the plasma membrane of a calycal process (C) is high, but very few pits are evident in the adjacent ROS plasma membrane. In Fig. 6, the intensity of the filipin binding to the calycal processes (C) is much lower, and again few pits are present in the plasma membrane of the adjacent ROS (ROS PM). Bars 0.5 µm. × 46,400 (Fig. 5); × 35,500 (Fig. 6).

binding to pellicle membranes of ergosterol-replaced *Tetrahymena pyriformis* cells with freeze-fracture electron microscopy. They found that filipin-induced pits were *excluded* from the phase separation-induced PFP observed at 22°C, but were *confined* to particle-free regions at 15°C. Feltkamp and van der Warden (13) found that fixation with glutaraldehyde prevented the redistribution of filipin-cholesterol complexes observed upon chilling of unfixed mouse leukemia cell nuclear membranes. Some redistribution seemed to occur, however, in tissue fixed at 0°C and then exposed to filipin at 37°C. Friend (15) has described the binding of filipin to guinea pig sperm membranes during capacitation in vitro and immediately after the acrosome reaction. Filipin-induced pits were interspersed among the intramembrane particles and were excluded from PFPs which developed during this process. Thus the relationship between PFP and filipin binding cannot be generalized, but must be evaluated in individual membrane systems.

As in the previous study (5), we again encountered variability both within and among experiments in the amount of filipin binding observed in comparable ROS membrane regions. Variables other than the concentration of membrane cholesterol could be responsible, including the activity and the concentration of filipin delivered to the surface of the various membranes. The local filipin concentration in the medium may vary according to the thickness and persistence of a gel normally present about both IS and OS (14).

FIGURE 10   Filipin binding in the plasma membrane of a frog cone inner segment. Of special interest is the presence of membrane specializations (arrows) near the connecting cilium (C) resembling those seen in the rod inner segment, and of filipin binding within these structures as well as elsewhere within the inner segment plasma membrane. Bar, 0.5 μm. × 51,000.

Our observations tend to support one of the several hypotheses we advanced earlier (5), namely that disk and plasma membrane PFP may present a more favorable environment for cholesterol, whereas, in the absence of PFP, cholesterol may be found dispersed among the particles. A molecular model that is consistent with this behavior and is based upon three previously well documented phenomena is that PFP are membrane regions of locally reduced fluidity that preferentially concentrate sphingomyelin and cholesterol. First, spontaneous segregation of different classes of membrane phospholipids into domains of different fluidity results in a preferential association of rhodopsin with the more fluid lipids (7). Second, the saturation of the hydrocarbon chains of ROS sphingomyelin (2) makes it reasonable to suppose that it may tend to segregate into relatively less fluid, rhodopsin-deficient membrane regions. Finally, sphingomyelin preferentially attracts cholesterol with an avidity sufficient to overcome the general preference of cholesterol for relatively more fluid membrane regions in systems exhibiting lateral phase separations (10).

The magnitude of filipin binding to ROS disk membranes may appear surprising at first in light of the fact that these membranes have relatively little cholesterol compared to many biological membranes. It must be appreciated, however, that in the frog there are approximately three to four molecules of cholesterol for every rhodopsin molecule (calculated
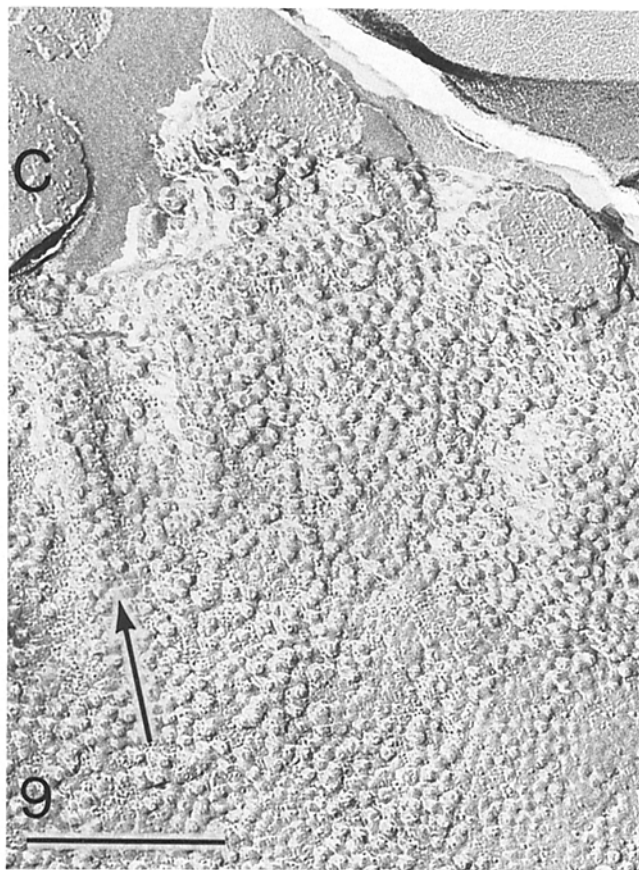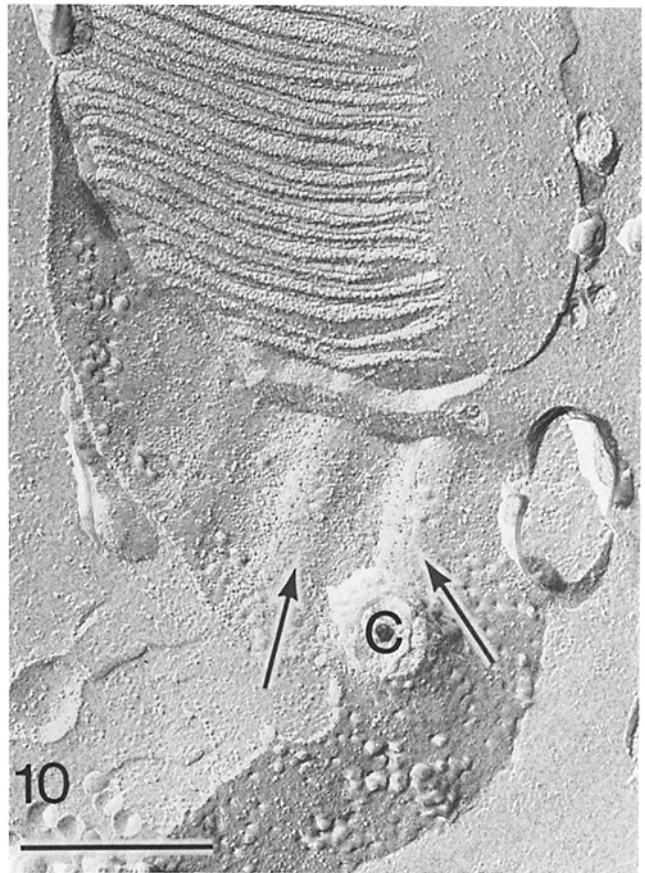

FIGURE 9   Heavy filipin binding to the plasma membrane at the apex of a frog rod inner segment. Note the presence of filipin complexes throughout the membrane, including the structure (arrow) near the connecting cilium (C), similar to that seen in Fig. 10. Bar, 0.5 μm. × 52,500.

from data from reference 3 and from Dr. R. Wiegand, personal communication). Since the particles seen in freeze-fracture replicas of disk membranes include four to five rhodopsin molecules (9), there are about 12–25 cholesterols present in disk membranes for each particle appearing in a freeze-fracture replica. Although it is not precisely known how many cholesterol and filipin molecules are involved in the formation of the 25–30 nm diameter "pit" seen in freeze-fracture replicas, Elias et al. (11) have proposed a model in which four molecules of each are involved. It is, therefore, reasonable to expect that a substantial number of filipin-induced pits may be generated in ROS disk membranes.

A revealing observation was that there could be a markedly different intensity of filipin binding to plasma membranes of the calycal processes of the rod inner segment and of the rod outer segment when these are close together. These membranes are not only physically close, suggesting equal access to applied filipin, but also are topologically continuous through the connecting cilium and apex of the IS. Accordingly, their apparently different cholesterol content implies that there is either a barrier in the membrane to the free diffusion of cholesterol or an active mechanism for its removal from the plasma membrane of the ROS. A difference in lipid composition, as suggested above, may help explain why opsin fails to diffuse throughout the IS plasma membrane after insertion in the periciliary region. We observed structures at this location in frog rod inner segments (see Fig. 7) that resemble the active zones of presynpatic nerve terminals of neuromuscular junctions and may direct the fusion of opsin-containing vesicles to occur in this region (discussed in more detail in reference 4). This region is dramatically revealed with very high resolution scanning electron microscopy (29). With this technique, the ridges appear much deeper and are clearly seen to be radially arrayed around the connecting cilium. Our data suggest that the periciliary region of the IS plasma membrane is compositionally similar to the OS in containing substantially less cholesterol than the surrounding inner segment plasma membrane. It has been shown in artificial membranes containing rhodopsin that the addition of cholesterol by itself can lead to the formation of PFP (7), presumably indicating a relative exclusion of rhodopsin from membrane regions enriched in cholesterol. If rhodopsin is inserted in the periciliary plasma membrane of the IS, then this tendency would favor the observed pattern (29) of lateral diffusion up the connecting cilium to the newly forming disks at the base of the OS over random diffusion throughout the IS.

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