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Distribution of cytochemically detectable cholesterol in the electric organ of *Torpedo marmorata*

(synapse/membrane/freeze-fracture)

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ABSTRACT **A cytochemical probe for cholesterol, the polyene antibiotic filipin, was applied to aldehyde-fixed samples of the electric organ of *Torpedo marmorata* to identify filipin-binding sites in the various membrane components of the organ and, hence, the probable cholesterol content at these levels. In both thin-sectioned and freeze-fractured samples, filipin–cholesterol complexes appeared numerous and homogeneously distributed on the Schwann cell plasma membrane. On the presynaptic membrane, filipin–cholesterol complexes occurred in patches alternating with unlabeled membrane segments. The postsynaptic, acetylcholine receptor-rich plasma membrane of the electroplax showed no or few filipin–cholesterol complexes in the flat region and upper part of the invaginations (both areas characterized by a lattice of small intramembrane particles); however, the membrane of the bottom part of the postsynaptic invaginations contained several complexes. The ventral, noninnervated plasma membrane of the electroplax showed a moderate, homogeneous filipin labeling. These data suggest that the distribution of cholesterol among membranes of the electroplax is not homogeneous and that the acetylcholine receptor-rich region of the postsynaptic membrane (as characterized by the lattice of small intramembrane particles) may contain little cholesterol.**

The description in structural and functional terms of the acetylcholine receptor (AcChoR) is an important goal of neurobiology, and a great deal of effort has been paid towards this aim (for a recent review, see ref. 1). The most studied sources of AcChoR are the neuromuscular junction and the chemical synapse in electric organs. As far as their "*in situ*", freeze-fracture morphology is concerned, the receptor sites in the postsynaptic membrane of the neuromuscular junction have been identified as loose aggregates of intramembrane particles (IMPs) in both membrane leaflets (2–4), whereas in the electric organ, receptor areas are characterized by a lattice of small (6–7 nm) IMPs on the exoplasmic (E) face and by numerous, usually 8- to 9-nm particles on the protoplasmic (P) face of the postsynaptic membrane (5–9). Although IMPs represent a reliable morphological marker for the distribution of integral proteins (10), none of the lipidic components of the membrane could be related until recently to identifiable deformations of the fracture plane. The use of cytochemical markers for one of the essential membrane lipids, cholesterol (Chol) (11–14), has rendered possible the mapping of Chol distribution in a variety of aldehyde-fixed membranes. With one of these markers, filipin, it has been possible to show that the regions containing AcChoR aggregates in the frog neuromuscular junction (15) and in embryonic amphibian muscle cells (16) are lacking filipin–Chol complexes, thus suggesting a low Chol concentration at these levels. The present study reports the results obtained by filipin labeling of the electric organ of *Torpedo marmorata*.

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MATERIAL AND METHODS

Three female *Torpedo* (a gift from Y. Dunant, Geneva) were anesthetized with tricaine methanesulfonate (MS222, Sandoz, Basle) dissolved in seawater (50 mg/liter) and perfused through the heart for 10 min with 2.5% (vol/vol) glutaraldehyde in 0.1 M cacodylate/HCl. After perfusion, small pieces (volume, <1 mm^3) were cut out from the electric organ, chopped in 40-μm slices with an Oxford Vibratome (Oxford Laboratories, San Mateo, CA), and further fixed from 1 to 48 hr in the same glutaraldehyde solution as used for perfusion but containing 300 μM filipin (a gift from J. E. Grady, Upjohn) and 1% dimethyl sulfoxide (the latter serves to dissolve the filipin).

Chopping the tissue proved to be useful to facilitate filipin diffusion. Filipin-treated blocks were then soaked in 0.1 M cacodylate buffer containing 30% (vol/vol) glycerol for 2 hr, frozen in Freon 22 cooled with liquid nitrogen, fractured, and replicated as described (17) in a Balzers freeze-fracture device, type 301 (Balzers Ltd., High Vacuum Company, Balzers, Liechtenstein). Other blocks of filipin-treated material were postfixed in 1% osmium tetroxide/0.1 M Veronal acetate buffer, treated with tannic acid (Mallinckrodt, code 1764) dissolved in 0.05 M cacodylate buffer (pH 7.0) for 15 min to enhance contrast (18), and stained in block with 0.5% uranyl acetate in cacodylate buffer for 20 min. Blocks were then dehydrated in ethanol and embedded in Epon. Thin sections cut with a diamond knife were further stained with uranyl acetate and lead citrate. Thin sections and freeze-fracture replicas were observed in a Philips EM 300 electron microscope (Eindhoven, The Netherlands).

Quantitative analysis was carried out on freeze-fracture pictures of the electric organ membranes. The number of filipin-induced deformations of the fracture plane (protuberances and pits) and the surface of the exposed membrane region were recorded with the aid of an electronic pen and a graphic tablet (Tektronic, type 4953) connected to a microprocessor system (IMSAI, type 8080), which was programmed to calculate the number of filipin–Chol complexes per μm^2 of membrane in either the P or E fracture face. Only flat areas of the respective membranes were evaluated. The mean numbers of filipin–Chol complexes per μm^2 of each membrane region (Schwann cell; presynaptic membrane; postsynaptic membrane, flat and infolded zones; and dorsal membrane of the electric organ) were compared with the Student unpaired *t* test.

RESULTS

Freeze-Fracture. The criteria for the identification of the various membrane faces in *Torpedo* electric organ *in situ* (Fig. 1A) have been described (8). In suitable replicas, the following

Abbreviations: AcChoR, acetylcholine receptor; Chol, cholesterol; IMP(s), intramembrane particle(s); P, protoplasmic; E, exoplasmic.

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plasma membranes can be recognized from the ventral to the dorsal side of the electric organ (Fig. 1*B*): Schwann cell, nerve presynaptic terminal, postsynaptic (ventral, innervated) membrane, and basal (dorsal, noninnervated) membrane. In the postsynaptic membrane, flat and infolded regions can be further distinguished. The binding of filipin to Chol results in the formation of multimolecular filipin–Chol complexes, which appear either as protuberances or pits on the fracture plane. By their large size (20–30 nm), these deformations are easily distinguishable from the much smaller (5–12 nm) IMPs.

The following distribution of complexes was observed in the different membrane faces (Figs. 1*B* and 2 *A–D*). In the Schwann cell, the plasma membrane appeared to be homogeneously and densely labeled with filipin–Chol complexes, either on the side facing the extracellular space or on the side applied to the nerve terminal. The number of complexes found in each fracture face of this membrane is shown in Table 1. The presynaptic plasma membrane of *Torpedo* differed from its counterpart in the neuromuscular junctions because it lacks the IMP-free bands known as active zones (20–22). Whereas IMPs appeared homogeneously distributed in this membrane, filipin–Chol complexes formed irregular aggregates with no consistent pattern (Fig. 2*B*). When the total number of filipin–Chol complexes was evaluated in each leaflet of this membrane, without separating richly labeled from poorly labeled areas, it appeared lower than in the Schwann cell membrane but higher than in the postsynaptic membrane (Table 1). In fracture orientations that exposed the presynaptic cytoplasm (Figs. 2*B* and 3*B*), the synaptic vesicles were revealed and often appeared deformed by filipin–Chol complexes (Fig. 3*B*). In the postsynaptic membrane, two distinct regions could be recognized on the basis of their IMP pattern and their labeling with filipin. The flat postsynaptic membrane between the infoldings and the upper portion of the latter showed the distinctive lattice of closely packed 6- to 7-nm IMPs

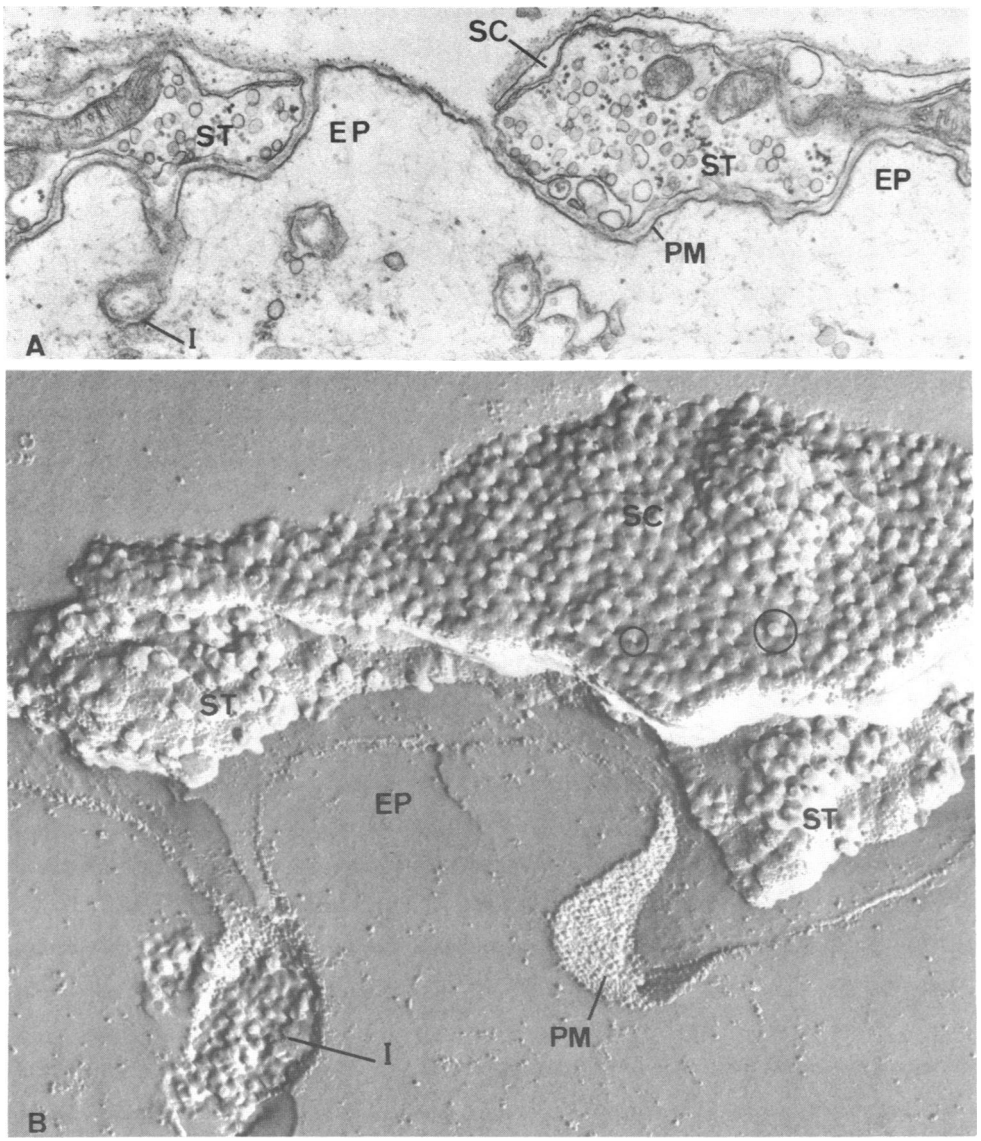

FIG. 1. (*A*) Thin section of the ventral side of the electroplax showing the main components of the synapse. SC, Schwann cell; ST, presynaptic terminal with synaptic vesicles; PM, postsynaptic membrane; EP, electroplax cytoplasm; I, postsynaptic infolding. (×16,200.) (*B*) Freeze-fracture replica of the ventral side of the electroplax after filipin treatment to reveal membrane Chol. The Schwann cell membrane is heavily and homogeneously labeled with filipin–Chol complexes. These appear either as protuberances (large circle) or pits (small circle) on the fracture face and are easily distinguished from the much smaller IMPs. The presynaptic terminal membrane (ST) shows an irregular labeling, while the flat postsynaptic membrane (PM) with its characteristic lattice of small particles appears unlabeled. The bottom part of a postsynaptic infolding (I) whose P leaflet has been exposed shows by contrast numerous filipin–Chol complexes. EP, cross-fractured electroplax cytoplasm. (×58,500.)

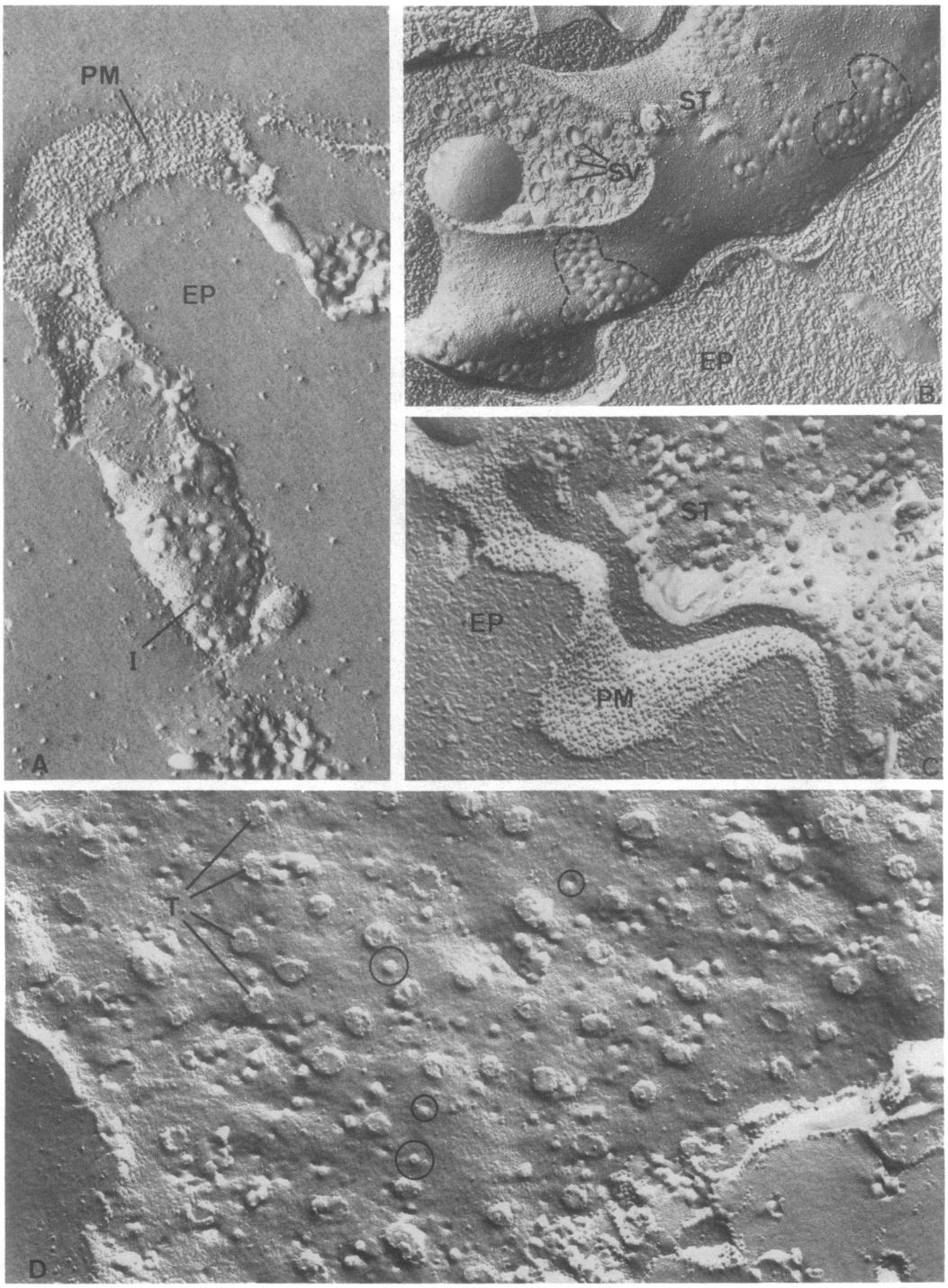

FIG. 2. (*A*) Freeze-fracture replica showing a lack of filipin-labeling on the flat postsynaptic membrane (PM), a low degree of labeling on the upper part of a postsynaptic invagination (I), and several filipin–Chol complexes on the bottom part of the invagination, from which the E leaflet is revealed. EP, electroplax cytoplasm. (×58,500.) (*B*) Freeze-fracture replica of the presynaptic terminal (ST) showing the presence of discrete regions of filipin labeling in the presynaptic membrane. Two labeled regions are delimited by dotted lines. SV, unlabeled synaptic vesicles (see Fig. 3*B*); EP, electroplax cytoplasm. (×28,800.) (*C*) Freeze-fracture replica of the synaptic region showing the contrast between the labeling of the presynaptic terminal membrane (ST) and the lack of filipin–Chol complexes on the lattice of small IMPs of the postsynaptic membrane (PM). EP, electroplax cytoplasm. (×46,800.) (*D*) Freeze-fracture replica of the dorsal, noninnervated face of the electroplax. This face (from which the E leaflet of the membrane has been exposed) shows the neck of the numerous tubular invaginations present at this level (T), a low number of IMPs, and a moderate degree of filipin labeling [filipin–Chol complexes appear either as protuberances (large circles) or as pits (small circles), both of which were pooled for the quantitative evaluation shown in Table 1]. (×58,500.)

on the E leaflet and the numerous 8- to 9-nm particles on the P leaflet (Fig. 2 *A* and *C*). In these differentiated membrane regions, filipin–Chol complexes were in low numbers in both membrane leaflets (less than 20 complexes per μm^2; Table 1). However, in the deeper part of the infoldings, the 6- to 7-nm IMP arrays were less marked or absent (Fig. 2*A*) (8) and, in these areas, a significantly higher number of filipin–Chol complexes could be detected. A comparable amount of filipin–Chol

Table 1. Number of filipin-Chol complexes (protuberances and pits) per μm^2 of different membrane regions of the electric organ.

					Electroplax					
	Schwann cell		Presynaptic membrane		Dorsal face		Ventral face infoldings (bottom)		Ventral face flat regions	
P face	457 ± 17	*	122 ± 18	*	46 ± 6	NS	50 ± 8	*	11 ± 1	
	$n = 18$		$n = 18$		$n = 18$		$n = 24$		$n = 12$	
E face	414 ± 33	*	115 ± 17	*	73 ± 13	NS	64 ± 5	*	18 ± 5	
	$n = 14$		$n = 12$		$n = 12$		$n = 29$		$n = 12$	

Unlike IMPs, which represent material (integral proteins) embedded in the bilayer and distributed unequally between the two leaflets in most freeze-fractured, aldehyde-fixed membranes [usually more particles remain with the P (protoplasmic or cytoplasmic) leaflet than with the E (exoplasmic or extracellular leaflet)], filipin–Chol complexes represent deformations of the entire membrane structure (see thin section) and appear in roughly comparable numbers in both leaflets (see also ref. 19). The apparently large differences between P and E faces of the electroplax membrane are not significant. Data are mean ± SEM; n, number of faces evaluated; *, significant differences ($P < 0.001$); NS, not significant.

complexes was found in the dorsal, noninnervated plasma membrane of the electric organ (Fig. 2*D*), either in the particle-rich P face, or in the particle-poor E face (Table 1).

Thin Section. Filipin–Chol complexes induced characteristic deformations of the plasma membrane visible in thin-sectioned material. At sites of filipin–Chol complexes, the membrane leaflets took on a scalloped appearance, which contrasted with the smooth appearance of the unlabeled membrane. Thin-section images of the electric organ treated with filipin confirmed the distribution of filipin–Chol complexes detected by freeze-fracture in the different regions of the synapse (Fig. 3*A*): homogeneous labeling of the Schwann cell membrane and patchy labeling of the presynaptic terminal, in which the synaptic vesicles may appear also deformed by filipin–Chol complexes (Fig. 3*A*). In the postsynaptic region, thin sections showed a distribution of filipin–Chol complexes comparable to that observed in freeze-fracture—i.e., little or no filipin-induced deformations in the flat or in the upper part of the infoldings and an increased number of deformations at the bottom part of the postsynaptic invaginations (Fig. 3*A*).

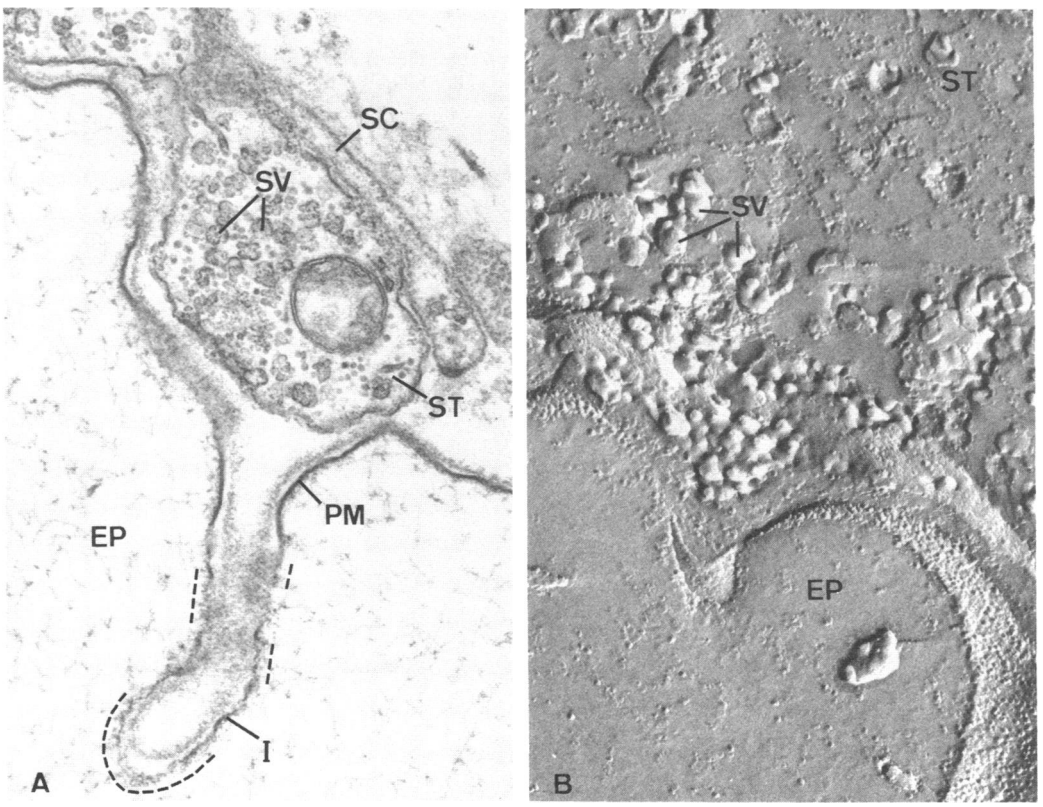

FIG. 3. (*A*) Thin section of the synaptic region after filipin treatment. The membrane segments where filipin binding occurred are deformed by filipin–Chol complexes and take on a scalloped appearance. Such segments are visible on the Schwann cell (SC) membrane; on parts of the presynaptic terminal (ST) membrane, including on the membrane limiting synaptic vesicles (SV); and in the bottom part (indicated by the dotted lines) of a postsynaptic infolding (I). On the contrary, most of the flat postsynaptic membrane (PM) appears unmodified. A limitation of the thin-section approach compared with the freeze-fracture approach to assessment of filipin binding is that the identification of filipin–Chol complexes is difficult in tangentially cut membrane segments. (×27,900.) (*B*) Freeze-fracture replica of a region comparable to that in *A*, showing a presynaptic terminal (ST) containing many synaptic vesicles (SV) deformed by filipin–Chol complexes. Compare the freeze-fracture appearance of unlabeled vesicles of another terminal in Fig. 2*B*. (×57,600.)

DISCUSSION

The treatment of *Torpedo* electric organ with filipin, a cytochemical probe inducing recognizable deformations at membrane sites containing Chol (11–14), results in a differential labeling of the various parts of the synapse; whereas the Schwann cell covering the presynaptic terminal shows a dense and homogeneous distribution of filipin–Chol complexes, the other membranes of the electric organ have different degrees of labeling. The labeling is patchy in the presynaptic terminal and low in the region of the postsynaptic membrane thought to contain the AcChoRs; however, it is present, although in lower concentration than in presynaptic membranes, in the bottom part of the postsynaptic infoldings and in the dorsal, noninnervated membrane of the electroplax. A recent study involving quantitation of filipin–Chol complexes in relationship to experimentally inserted Chol in membranes of cultured cells (23) has shown that the number of filipin–Chol complexes formed is directly proportional to the Chol concentration in the system evaluated. By assuming the same accessibility of the filipin probe to all of the plasma membranes of the electric organ and identical local conditions for filipin–Chol complex formation, extrapolation of these conclusions to our data suggest that Chol is present in a low concentration in AcChoR-rich areas of the electroplax membrane and in discrete regions of the presynaptic terminal. These results extend and complement a recent study made with the same probe on another well-characterized cholinergic synapse, the neuromuscular junction (15). Points of similarity between the present and previous studies are represented by the homogeneous dense filipin labeling of the Schwann cell, the absence of filipin–Chol complexes in patches of the presynaptic terminal, and the low filipin labeling in the regions of the postsynaptic membrane thought to be rich in AcChoR. Thus, the conclusions derived from the study of the neuromuscular junction after filipin treatment—i.e., that presynaptic active zones and AcChoR-rich postsynaptic areas may represent membrane regions poor in Chol—seem to hold for *Torpedo* cholinergic synapse as well; however, the lack of "active zones," in a morphological sense, in the presynaptic membrane of *Torpedo* makes it impossible to determine whether Chol-poor areas do correspond to the presynaptic bands of the neuromuscular junction. By contrast, the finding that the postsynaptic infoldings of the electric organ show a heterogeneous labeling may be correlated with the observation that, in the electric organ of a closely related species, *Narcine*, the infoldings are also heterogeneous in their membrane structure and α-bungarotoxin-binding properties; the bottom of the infoldings appears limited by a thin plasma membrane and shows little toxin binding as compared to the upper part of the synaptic infolding and to the noninfolded postsynaptic membrane, which show both a thick limiting membrane and numerous binding sites for α-bungarotoxin (24, 25). If interpreted as due to a low Chol content, filipin labeling data on the AcChoR-rich areas of the electric organ appear in discrepancy with the biochemical analysis of membrane fragments enriched in AcChoR, which, on the contrary, indicate a high Chol content (26–28). At present, the reason for this discrepancy has no univocal explanation. Aside from the fact that we cannot rule out completely other causes than a low Chol concentration to account for our cytochemical results [for example, the extreme density of protein or the marked cytoskeletal web (29) characterizing the AcChoR-rich membrane, or both, could prevent or decrease filipin–Chol complex formation], the discrepancy also may be sought in the different spatial resolution of the two techniques. Whereas filipin-labeling *in situ* appears to detect boundaries in sterol concentration within a few hundred nanometers (as evidenced in the heterogeneous labeling of the invaginations), purified postsynaptic membrane preparations are likely to include both labeled and unlabeled regions of this membrane. Therefore, a filipin-labeling study of such preparations should help to progress towards the integration of both types of data. The recent isolation of three subpopulations of receptor-containing membranes in the electroplax (30) is another promising approach towards this goal.

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