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QUANTITATIVE STUDIES ON THE LOCALIZATION OF THE CHOLINERGIC RECEPTOR PROTEIN IN THE NORMAL AND DENERVATED ELECTROPLAQUE FROM *ELECTROPHORUS ELECTRICUS*

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ABSTRACT

Electroplaques dissected from the electric organ of *Electrophorus electricus* are labeled by tritiated α_1-isotoxin from *Naja nigricollis*, a highly selective reagent of the cholinergic (nicotinic) receptor site. Preincubation of the cell with an excess of unlabeled α-toxin and with a covalent affinity reagent or labeling in the presence of 10^{-4} M decamethonium reduces the binding of [^{3}H]α-toxin by at least 75%. Absolute surface densities of α-toxin sites are estimated by high-resolution autoradiography on the basis of silver grain distribution and taking into account the complex geometry of the cell surface. Binding of [^{3}H]α-toxin on the noninnervated face does not differ from background. Labeled sites are observed on the innervated membrane both between the synapses and under the nerve terminals but the density of sites is approx. 100 times higher at the level of the synapses than in between. Analysis of the distance of silver grains from the innervated membrane shows a symmetrical distribution centered on the postsynaptic plasma membrane under the nerve terminal. In extrasynaptic areas, the barycenter of the distribution lies ~0.5 μm inside the cell, indicating that α-toxin sites are present on the membrane of microinvaginations, or *caveolae*, abundant in the extrajunctional areas. An absolute density of 49,600 ± 16,000 sites/μm^2 of postsynaptic membrane is calculated; it is in the range of that found at the crest of the folds at the neuromuscular junction and expected from a close packing of receptor molecules.

Electric organs were denervated for periods up to 142 days. Nerve transmission fails after 2 days, and within a week all the nerve terminals disappear and are subsequently replaced by Schwann cell processes, whereas the morphology of the electroplaque remains unaffected. The denervated electroplaque develops some of the electrophysiological changes found with denervated muscles (increases of membrane resting resistance, decrease of electrical excitability) but does not become hypersensitive to cholinergic agonists.

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Autoradiography of electroplaques dissected from denervated electric organs reveals, after labeling with [^{3}H]α-toxin, patches of silver grains with a surface density close to that found in the normal electroplaque. The density of α-toxin binding sites in extrasynaptic areas remains close to that observed on innervated cells, confirming that denervation does not cause an increase in the number of cholinergic receptor sites. The patches have the same distribution, shape, and dimensions as in subneural areas of the normal electroplaque, and remnants of nerve terminal or Schwann cells are often found at the level of the patches. They most likely correspond to subsynaptic areas which persist with the same density of [^{3}H]α-toxin sites up to 52 days after denervation. In the adult synapse, therefore, the receptor protein exhibits little if any tendency for lateral diffusion.

KEY WORDS acetylcholine receptor · quantitative autoradiography · electroplaque · α-toxin · denervation · receptor protein · electrophysiology

The electric organ from *Electrophorus electricus* has been widely used during the past 10 years as a source of biochemical material for the characterization, isolation, and purification of the cholinergic receptor protein (for reviews, see references 17, 73, 43, and 67). The rather simple anatomy of its elementary units, the electroplaques (57, 60), makes it also a convenient system in which to study the localization, at the subcellular level, of characteristic synaptic proteins such as the enzyme acetylcholinesterase (9) and the cholinergic (nicotinic) receptor protein (AChR) (12–14).

The α-toxins from snake venoms (50, 86), because of their high-binding specificity and the low reversibility of their complex with the AChR, constitute excellent reagents of the physiological receptor site for acetylcholine both *in situ* (20, 51) and in vitro (20, see also references in 39 and 18). In a previous work the α-toxin from *Naja nigricollis* was used to demonstrate the almost exclusive localization of the receptor protein on the innervated face of the *Electrophorus* electroplaque by both indirect immunofluorescence (14) and autoradiography with the light microscope (13). These early observations were subsequently confirmed by high-resolution autoradiography and briefly reported (12).

In this paper we present an extensive and detailed analysis of the localization of the cholinergic receptor protein on the innervated face of the *Electrophorus* electroplaque. The presence of the receptor protein is revealed by its ability to bind the tritiated α_1-toxin from *Naja nigricollis* and the density of toxin molecules bound per surface area determined by quantitative autoradiography. Under the nerve terminals, up to 50,000 ± 16,000 [^{3}H]α-toxin molecules may bind per μm^2 of subsynaptic membrane, and between the synapses the density is, approximately, 100 times lower. Electrophysiological and anatomical observations indicate that destruction of the spinal neurons entails a rapid degeneration of the nerve terminals. However, neither the distribution nor the pharmacology of the acetylcholine receptor appear to change up to 52 days after denervation, despite the appearance of some typical electrophysiological alterations of the innervated membrane.

MATERIALS AND METHODS

Denervation of the Electric Organ

The operation was performed on fresh animals (1–2 m long) purchased from Paramount Aquarium (Ardsley, N.Y.). The tail was sectioned at ~10 cm from its tip, and a sterile metallic rod was introduced into the rachidian canal to destroy the spinal cord (77). In early experiments, the length of the rod was such that the spinal cord was disorganized over more than two-thirds of the length of the animal; most of the main electric organ was denervated and used for biochemical work. In subsequent studies on single electroplaques, dissected from the organ of Sachs according to Schoffeniels (83), a smaller length of the spinal cord (30–50 cm) was destroyed.

Preparation of [^{3}H]α_1-Isotoxin from the Venom of Naja nigricolis

The α_1-isotoxin (10) was purified from the venom of *Naja nigricollis* by the method of Karlsson et al. (46) and tritiated according to Menez et al. (61). The tritiated toxin was a gift of Drs. Boquet, Menez, Morgat, and Fromageot. The different batches of toxin used had the following specific radioactivities and protein concentrations: (*a*) 14.0 Ci/mmol, 0.7 mg/ml; (*b*) 14.8 Ci/mmol, 0.408 mg/ml; (*c*) 10.5 Ci/mmol, 0.495 mg/ml. All stock

solutions were made in 0.2 M sodium phosphate buffer, pH 7.0, and stored at 4°C (91).

Labeling of Isolated Electroplaque with the Tritiated Toxin

ELECTRON MICROSCOPY: The electroplaque dissected from Sachs' organ was labeled with the [^{3}H]α-toxin in the following manner: the cell was soaked for 90 min in a solution of [^{3}H]α-toxin in physiological Ringer's solution (160 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1.5 mM phosphate buffer, pH 7.0, 1.5% wt/vol glucose when needed). The concentration of toxin used in these experiments ranged between 1.6 and 2 μg/ml (approx. 2–3 $\times$ 10^{-7} M). Then the cell was rinsed at room temperature 12 times for 5 min in 100 ml of Ringer's solution to remove the free toxin and fixed overnight at 4°C in 1–4% glutaraldehyde in Ringer's solution. As controls, electroplaques were routinely preincubated in an excess of unlabeled α-toxin (5 μg/ml) for 90 min, rinsed and exposed to the tritiated toxin as described previously.

SCINTILLATION COUNTING: The [^{3}H]α-toxin-labeled electroplaques were rinsed overnight at 4°C in Ringer's solution, dried on blotting paper, dissolved in 0.1 ml of hyamine hydroxyde (Packard Instrument Co., Inc., Downers Grove, Ill.) and counted in flasks containing 10 ml of a solution of 10 g of 2,5-diphenyloxazole, 0.5 g of *p*-bis(2-5-phenyl oxazolyl) benzene in 2 l of toluene and 1.2 l of Triton X-100 (Rohm & Haas Co., Philadelphia, Pa.). Counting was done in an Intertechnique (Plaisir, France) scintillation counter. Quenching or scattering due to the cell or solvents was negligible.

Preparation of Specimens for Electron Microscope Autoradiography

After labeling and fixation overnight in 2% glutaraldehyde, the electroplaques were rinsed in Ringer's solution and postfixed for 60 min in 1% osmium tetroxide, washed rapidly in Michaelis buffer, stained for 60 min in 1% uranyl acetate, dehydrated in acetone, and embedded in Vestopal or Epon. Ultrathin sections were cut with a Reichert microtome, (American Optical Corp., Buffalo, N.Y.) in several parts of each electroplaque, and several electroplaques were dissected at each stage of the analysis which was carried out on several normal and denervated eels. Only the silver-colored sections were selected with a copper ring and layered on collodionized glass slides. The thickness of the sections was determined on some of these slides by interferometry with a Zeiss interferometer microscope.

For autoradiography, sections were stained with lead citrate for 2 to 5 min in a CO_2-free atmosphere. After careful rinsing, the slides were carbonated and covered with L_4 Ilford Emulsion diluted one-third in double-distilled water filtered and boiled. Exposure was made at room temperature in the presence of Actigel (Prolabo, Paris, France). At the time required, the emulsion was developed with Microdol X (Eastman Kodak Co., Rochester, N.Y.) 13.54 g in 100 ml of water prepared just before use. Development was done rigorously under the same conditions at 20°C for 5 min. After fixation and washing, sections were placed on 100-mesh copper grids and observed in a Siemens Elmiskop 101 with constant magnifications of 2,400 or 6,000 controlled with a standard carbon replica. Counts of silver grains were made routinely on prints with a 24,000 final magnification. For each step of the experiment between 100 and 200 synaptic contacts were scanned, including those devoid of silver grains. All the silver grains in a 3-μm wide band on each side of the cell surface were counted.

Estimation of the Yield of Radioactivity Counting for Quantitative Autoradiography Measurements

Tritiated methyl-methacrylate (Amersham Corp., Arlington Heights, Ill.), 500 μCi/g, was sectioned and prepared for high-resolution autoradiography as described above for the electroplaque specimen. For each exposure time, several thousand silver grains were counted. Special care was taken to reduce variability; all the preparations were simultaneously carbon-coated and covered with the same emulsion, and methacrylate standards were run systematically in parallel with the electroplaque specimen. All the autoradiographs were exposed and developed at the same time under the same conditions, using the same Microdol X batch. To control the homogeneous layering of the emulsion over thin sections (89), some preparations were not developed but directly observed with the electron microscope.

Electrophysiology

MOUNTING OF THE ELECTROPLAQUE: Dissected electroplaques were stored at room temperature (for less than a few hours) in Ringer's solution supplemented with 1.5% glucose wt/vol and mounted in a Perspex chamber. Under these conditions, 2.5 mm^2 of the innervated face was exposed to a pool of Ringer's solution (pool *A*) through a window in a Mylar sheet (E. I du Pont de Nemours & Co., Wilmington, Del.). The entire noninnervated face was in contact with another pool of Ringer's solution (*B*). The experiments were performed at room temperature.

ELECTRICAL ARRANGEMENTS: The tips of two KCl-filled micropipettes (R $\approx$10 MΩ) were positioned facing each other closely on both sides of the innervated membrane; signals were led from these electrodes to a Grass P16 differential amplifier (Grass Instrument Co., Quincy, Mass.) to measure the transmembrane potential (inside minus outside); the amplified signal was visualized on Tektronix 564 oscilloscopes (Tektronix, Inc., Beaverton, Oreg.) and recorded on a chart. Stimulating currents generated by Grass S44 or S88 stimulators were applied through photoelectric isolation units (Grass PSIU 6*B*) to two chlorided silver

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wires in pools *A* and *B*. The noninnervated membrane has a much lower resistance than the innervated one, and most of the voltage drop during stimulation occurs therefore at the level of the innervated membrane. Using external electrodes (65), the entire surface of the innervated membrane can be stimulated simultaneously.

ANALYSIS OF NONSYNAPTIC CONDUCTANCES: The conductance of the innervated face of the *Electrophorus* electroplaque is sensitive to both acetylcholine and the electric field. The action potential elicited by rapid changes of potential shows the typical components of the Hodgkin and Huxley model: the specific potential-sensitive conductances for sodium (G_{Na}) and potassium (G_K) and the unreactive potassium-specific leak conductance (G_L) (66). These three components and the equilibrium potential for sodium (E_{Na}) were measured according to Ruiz-Manresa et al. (81) and Ruiz-Manresa and Grundfest (80) during square current waves of 30–50 ms durations (Fig. 12). The reactive sodium conductance was determined at the peak of the action potential (G^p_{Na}). With most of the cells G_K did not inactivate completely during depolarization (10–20% of residual conductance), and G_L was therefore measured in the presence of 5 mM barium chloride in Ringer's solution (81) (no correction for osmolarity). The resistance of the fluid between the two microelectrode tips was compensated on the basis of an equal conductivity for intracellular and extracellular solutions. With about one-third of the cells studied, the plateau and peak values of the potential during square-wave stimulations were measured on V vs. t oscilloscope traces (Fig. 12*A*) and plotted manually against the imposed current (Fig. 12); in the other cases, the V vs. I characteristics were directly visualized on the screen of the oscilloscope (Fig. 12*B*).

RESPONSE TO CHOLINERGIC LIGANDS: Neurally evoked postsynaptic potentials were elicited by a brief hyperpolarizing current pulse (typically 50 μs, ~200 mA/cm^2). The amplitude of the postsynaptic potential (PSP) increased with the intensity of the electrical stimulus as more and more terminals were recruited until eventually an action potential developed. The PSP's and neurally evoked ("indirect") spikes were suppressed by cholinergic antagonists such as d-tubocurarine (dTC). If the intensity of stimulation still increased in the presence of dTC, the innervated face itself responded by a direct spike at the end of the stimulus (anode-break stimulation). Neurally evoked spikes could be distinguished from anode-break spikes by their sensitivity to d-tubocurarine, the presence of PSP's, the synaptic delay, and the facilitation.

The steady-state depolarizations resulting from bath application of cholinergic agonists were followed as described by Higman, Podleski, and Bartels (44).

Chemicals

Decamethonium bromide was purchased from K and K Laboratories (Irvine, Calif.), d-tubocurarine chloride from Sigma Chemical Co. (St. Louis, Mo.), Flaxedil-triiodide from Rhône-Poulenc (Paris, France), and tetrodotoxin (TTX) from Calibiochem (San Diego, Calif.). The other reagents were of analytical grade. All solutions were prepared in double-distilled water.

RESULTS

The Normal Electroplaque from Electrophorus Electricus

GENERAL MORPHOLOGY OF THE CELL AND GEOMETRY OF THE PLASMA MEMBRANE: The characteristic features of the electroplaque morphology are known (31, 57, 60), and only data relevant to the autoradiographic analysis are mentioned here. It shall be recalled that, like a striated muscle fiber, the electroplaque is a giant syncytium (Fig. 1) which contains ~5,000–6,000 nuclei distributed at random beneath the cell surface. The *Electrophorus* electroplaque is highly asymmetrical and receives nerve terminals exclusively on its caudal or "innervated" face. After an extensive branching, the nerve fibers lose their myelin sheath and, trailing on the cell surface, make multiple "en passant" synaptic contacts (11). These contacts are characterized by a gutter-like structure with a local thickening of the plasma membrane (Figs. 3, 7, and 9).

Both faces of the electroplaque show a remarkable increase in the surface of the plasma membrane because of two categories of foldings: (*a*) the large villosities or papillae of Fessard (31) (Fig. 2), and (*b*) small fingerglove invaginations of the plasma membrane, the microinvaginations (Figs. 3 and 4) or caveolae of Luft (57), which penetrate inside the cell. The increase in the cell surface associated with the papillae, as shown in Table I, is close to three on both the innervated and noninnervated faces. Because of the deeper penetration of the caveolae on the rostral face of the cell, they cause an increase in the surface area of the noninnervated membrane by a factor of 30 and of the innervated one by a factor of only 10. The final ratio of 30 found between the "apparent" surface of the innervated membrane (from a face-on view of the cell) and the "real" one resulting from stereological studies fits rather well with the capacitance measurements of Morlock et al. (65): whereas the typical capacity of a plasma membrane lies between 0.8 and 1.2 μF/cm^2 (32), that of the innervated face reaches 20–60 μF/cm^2 of "apparent" surface.

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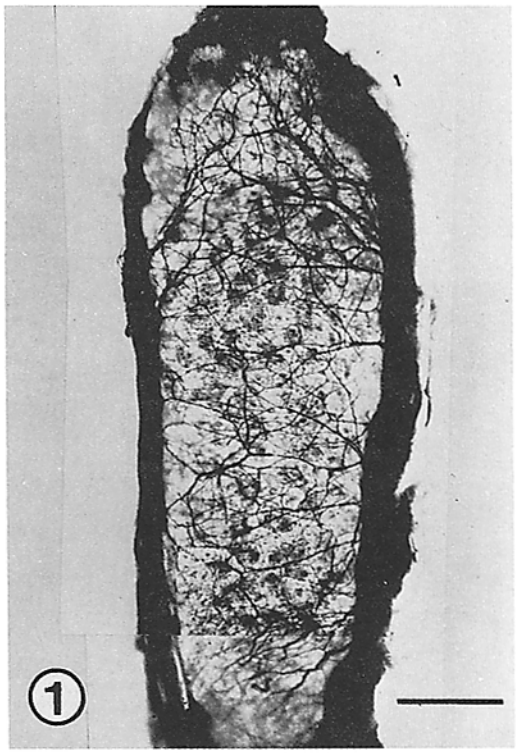

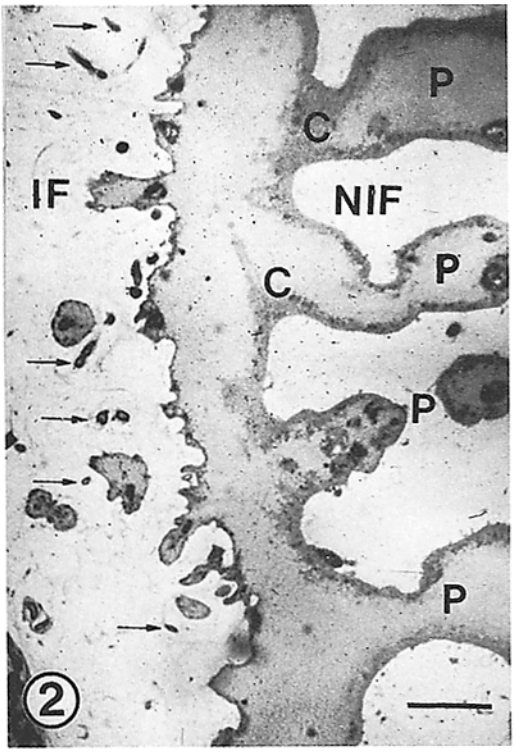

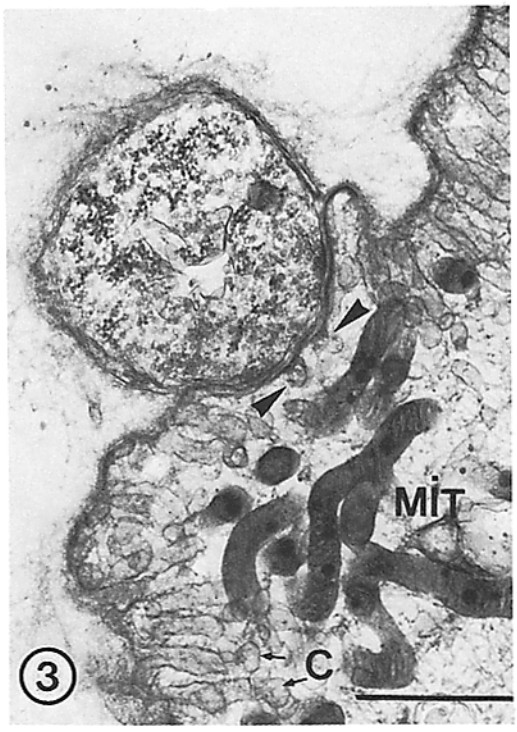

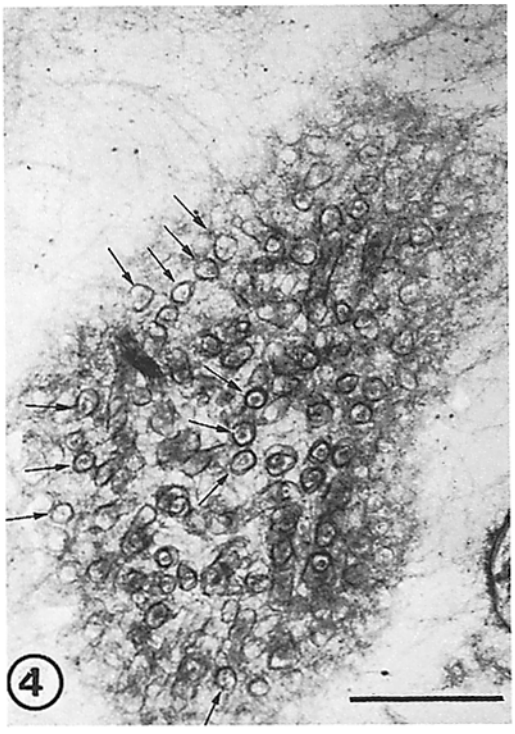

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TABLE I

Geometry of the Plasma Membrane of Electrophorus Electroplaque

	Mean depth of caveolae*	Mean diameter of caveolae	Mean density of caveolae per μm^2 of cell surface‡	Surface increase due to microinvaginations	Surface increase due to papillae§	Total surface increase
	μm	μm				
Innervated face	0.72 ± 0.15 (87)	0.18 ± 0.02 (150)	20 ± 5 (26)	10	2.9 ± 0.2 (14)	30
Noninnervated face	2.2 ± 0.6 (122)	0.20 ± 0.02 (50)	21 ± 4 (30)	30	3.0 ± 0.7 (14)	90

Values are given ± standard deviation; the number of determinations is given in parentheses.

* Estimated on sections perpendicular to the cell surface (see Fig. 6).

‡ Estimated on sections tangential to the cell surface (see Fig. 7). The average contribution of the caveolae to the total surface of the cell was determined on electron micrographs by measuring the mean diameter of the cylindrical invaginations on thin sections and their mean depth and density per surface area on thick sections (0.5 μm).

§ The increase in cell surface associated with papillae was estimated with a curvimeter on micrographs taken with a light microscope at low magnification (see Fig. 2).

DISTRIBUTION OF THE [^{3}H]-α-TOXIN BINDING SITES ON THE SURFACE OF ELECTROPHORUS ELECTROPLAQUE ANALYSED BY HIGH-RESOLUTION AUTORADIOGRAPHY: Examination of a significant number of electron micrographs (>600 pictures were scanned and 3,000 synaptic contacts examined) has confirmed our preliminary observation (13) that silver grains are abundant on the innervated surface of the electroplaque, both between the synapses and under the nerve terminals (Fig. 5), but rare on its noninnervated membrane. Autoradiographs from serial sections of the innervated membrane reveal that the density of grains falls abruptly (in <2,000 Å) at the boundary of the synaptic contacts (Figs. 5 and 7) and that no significant clustering of silver grains takes place on the innervated membrane outside the synapses. In a previous work (13) we have shown, and here we confirm, that the density of silver grains is approx. 10 times smaller between the synapses than under the nerve terminals, and that on the noninnervated membrane the density of silver grains is about one order of magnitude smaller than in the extrasynaptic areas of the innervated membrane.

SPECIFICITY OF TOXIN-LABELING: To determine which fraction of the silver grains counted do belong to the tritiated toxin molecules bound to the cholinergic receptor site, the following controls were run:

FIGURE 1 Micrograph of a single isolated electroplaque from *Electrophorus electricus* (5-10 by 1-3 by 0.2 mm, from the organ of Sachs). The rich innervation is revealed after fixation with osmium tetroxide. × 13.7. Bar, 1 mm.

FIGURE 2 Micrograph of a semithin transverse section of an electroplaque, stained with toluidine blue. (*P*) papillae. At this magnification, only the caveolae (*C*) of the rostral non-innervated face (*NIF*) are visible; they penetrate deeply inside the cell. Only the caudal innervated face (*IF*) receives the nerve terminals. Arrows: myelinated axons. × 370. Bar, 30 μm.

FIGURE 3 Semithin section (0.5 μm in section) perpendicular to the cell surface at the level of a synaptic contact. The caveolae (*C*) are sparser under the nerve ending (thick arrows; ~0.1 microinvagination per micrometer of membrane profile) than in the extrasynaptic areas (~1.5/μm). Taking into account the typical geometry of the plasma membrane, the subsynaptic membrane would represent only 1.4-2% of the total surface for the plasma membrane on the innervated face (~3,000 synaptic contacts counted). Branched mitochondria (*MIT*). × 21, 150. Bar, 1 μm.

FIGURE 4 Semithin section tangential to the cell surface in the extrasynaptic area of the innervated face. Orifices (arrows) of the caveolae are distributed evenly on the surface of the cell. × 20,400. Bar, 1 μm.

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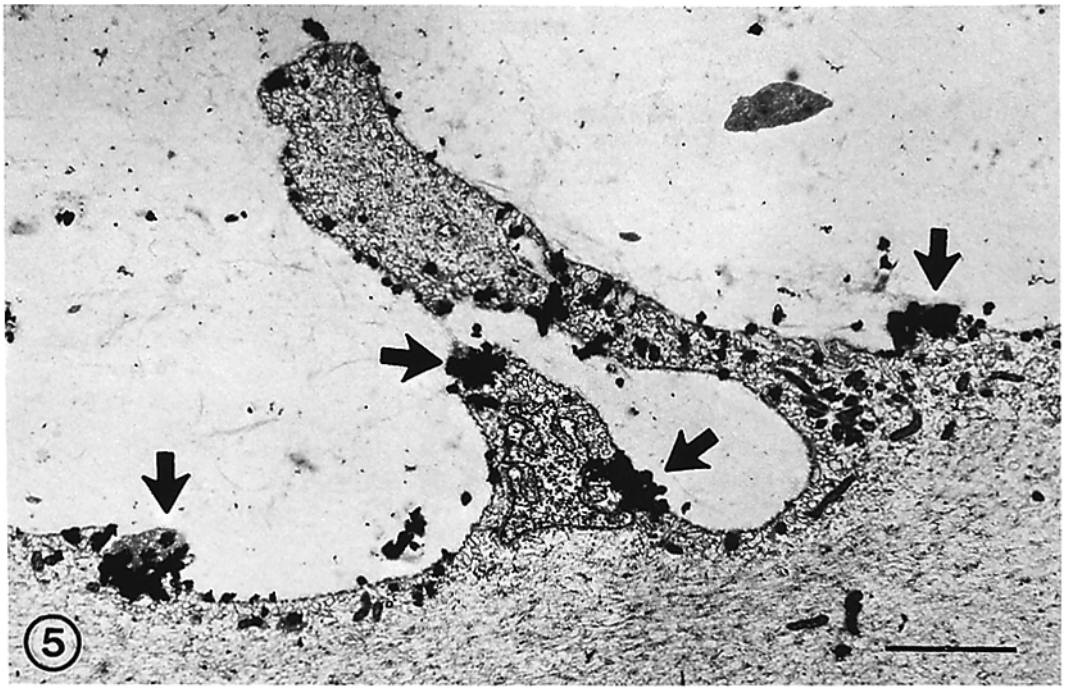

FIGURE 5 Low magnification autoradiograph of the innervated face of a normal electroplaque labeled with the [^{3}H]α-toxin from *Naja nigricollis* and overexposed for one year. All the clusters of silver grains (arrows) lie in subsynaptic areas. Silver grains are present also in extrasynaptic areas. × 4,300. Bar, 4 μm.

(*a*) Electroplaques were preincubated with an excess (5 μg/ml) of unlabeled toxin before the labeling with the tritiated toxin; then the autoradiographs were developed and analyzed in parallel with normally labeled electroplaques. The data collected in Table II show that, under the nerve terminals, preincubation with the unlabeled toxin eliminates most of the grains but that 25% of them persist between the synapses, i.e., correspond to nonspecifically bound toxin molecules. No significant effect of preincubation was noticed on the labeling of the noninnervated face. The grains present on this face were therefore considered as representing exclusively nonspecific binding. From these data, and taking into account the geometry of the cell surface, one concludes that on the whole cell ~75% of the grains belong to toxin molecules bound to the cholinergic receptor sites.

(*b*) The labeling of single isolated electroplaques was followed by scintillation counting (see Materials and Methods) as a function of the time of incubation with the [^{3}H]α-toxin and with or without prelabeling with either the nonradioactive α-toxin or with two nicotinic effectors: a covalent affinity reagent, 10^{-4} M *p*-trimethylammoniumbenzenediazoniumdifluoroborate (TDF) (21), and an agonist, 10^{-4} M decamethonium. The specific binding of toxin being essentially over after 10 min of incubation (11), under the standard conditions of labeling all the toxin sites available react with the labeled toxin. Preincubation with the unlabeled toxin or with the two cholinergic reagents tested reduces the labeling by ~75–78%. In close agreement with the autoradiographic studies, the amount of nonspecifically bound [^{3}H]α-toxin does not exceed one-fourth of the total radioactivity fixed by the cell.

LOCALIZATION OF THE TOXIN-BINDING SITES ON THE PLASMA MEMBRANE: The distance of the grains from the cell surface was measured and histograms were drawn. Fig. 11 shows that under the nerve terminals the grains appear distributed in a symmetrical manner on both sides of the postsynaptic plasma membrane. The distance at which half of the maximal number of grains are counted is 1,000 Å. Despite the fact

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TABLE II

Counting of Silver Grains in Various Areas of the Plasma Membrane Estimated by High-Resolution Autoradiography with or without Preincubation of the Electroplaque with Unlabeled α-Toxin

Denervation time	Cell surface localization		Number of silver grains per μm of cell contour: Without preincubation with unlabeled α-toxin	Number of silver grains per μm of cell contour: After preincubation in unlabeled α-toxin	Nonspecific adsorption of tritiated α-toxin after preincubation in unlabeled α-toxin
days					%
0	Innervated face	Subsynaptic	3.0 ± 1.0 (106; 14)	0.2 (208; 10.5)	3
		Extrasynaptic	0.3 ± 0.2 (106; 14)	0.15 (208; 10.5)	24.5
	Noninnervated face		0.16 ± 0.03 (106; 14)	0.15 (208; 10.5)	100
8	Innervated face	Under Schwann cells	2.5 ± 0.2 (87; 14)	0.23 (87; 14)	9
15	Innervated face	Free cluster	1.7 ± 0.6 (153; 10.5)	0.039 (153; 10.5)	2.3
		Extrasynaptic	0.11 ± 0.06 (153; 10.5)	0.024 (153; 10.5)	21.8
28	Innervated face	Under Schwann cells	2.1 ± 0.7 (109; 14.8)	0.27 (165; 10.5)	8.4
		Extrasynaptic	0.14 ± 0.05 (109; 14.8)	0.056 (165; 10.5)	25.8

The dissected electroplaques were incubated in *Naja nigricollis* tritiated α-toxin (1 μg/ml) with or without preincubation in unlabeled toxin (5 μg/ml). The results are expressed in silver grains per μm of cell contour (caveolae not taken into account). The first number in parentheses is the time exposure of the autoradiograms in days, and the second number is the specific radioactivity of the tritiated α-toxin used (Ci/mmol).
The horizontal lines separate three independent autoradiography experiments. For the estimation of the nonspecific adsorption, the differences in time exposure, specific radioactivities of toxins and geometry of the electroplaques are taken into account.

that the width of the synaptic cleft is of the same order of magnitude (700 Å), this observation strongly supports the view that the toxin-binding sites, i.e. the cholinergic receptor sites, are located almost exclusively along the synaptic cleft on the surface of the postsynaptic membrane (Fig. 7).

In the extrasynaptic areas, the barycenter of the distribution of silver grains is located inside the electroplaque as far as 0.46 μm from the external contour of the cell (Fig. 11). This distance is close to half of the mean depth of the caveolae, which suggests that toxin sites are present on both the external surface of the plasma membrane and on that of the caveolae. It is difficult to ascertain whether differences in density exist between these two membrane compartments.

QUANTITATIVE ESTIMATION, FROM THE AUTORADIOGRAPHIC DATA, OF THE ABSOLUTE DENSITY OF CHOLINERGIC RECEPTOR SITES PER SURFACE AREA OF PLASMA MEMBRANE: The estimation of a number of toxin molecules from a number of silver grains hinges upon a major difficulty: the determination of the efficacy of the counting procedure.

In a first series of experiments the number of grains was followed as a function of the time of exposure of the autoradiographs. Fig. 6 shows that the number of grains in subsynaptic areas, taken as reference, deviates slightly from a straight line after 45 days of exposure. At day 45, grains are numerous and never overlap. The standard deviation does not exceed 15%. One finds, for instance, 1.48 ± 0.2 (SD) grains per μm of cell profile. This curve was used to standardize results obtained after different exposure times.

The yield of the counting was finally estimated by the method of Salpeter and Szabo (82), using tritiated methacrylate with a known specific radioactivity. The variation in the number of grains against the time of exposure (top of Fig. 6) fits closely with the curve obtained by those authors. After 45 days of exposure, under conditions identical to those used with the labeled electroplaque, one finds an efficacy of 12 ± 2.5 (SD) silver grains per 100 disintegrations, again a value close

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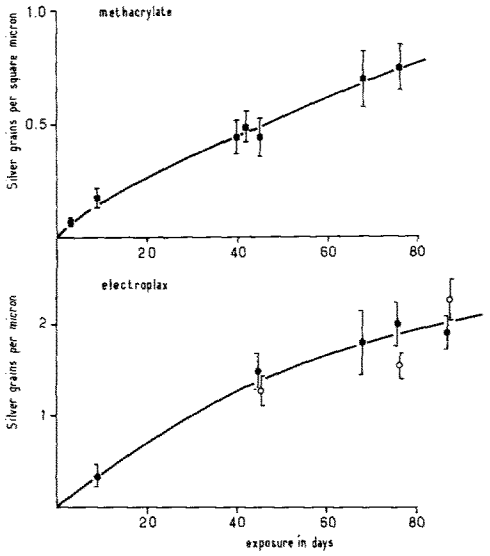

FIGURE 6 Variation in the number of silver grains as a function of the time of exposure, done in parallel with normal (●) and denervated (○) electroplaques labeled with *Naja* tritiated α-toxin and with tritiated methacrylate of known specific radioactivity (■). The ordinate represents the number of silver grains per square micron of thin methacrylate section (500 μCi/g; this analysis concerned 45,614 silver grains counted on 136 silver sections, 600 Å thick, taking four electronmicrographs per section) or per μm of postsynaptic membrane of normally innervated electroplaque and after 8 days of denervation. In the denervated electroplaques, only the synaptic gutters freely exposed to the extracellular medium (as shown in Fig. 9) were used for the present quantitative analysis. Electroplaques were labeled with the same tritiated α-toxin (14 Ci/mmol): a total of 2,605 silver grains was counted on 1,863 μm of plasma membrane representing 677 synaptic contacts observed on silver sections 600 Å thick. The background estimated on the electroplaque autoradiographs was 0.1–0.2 silver grains per 100 square micron of thin section until 87 days of exposure over regions excluding the cellular structures.

to that found by Salpeter and Szabo (82).

Taking into account the thickness of the section, time of exposure, efficacy of counting, specific radioactivity of the labeled toxin, nonspecific adsorption, and, considering that under the present experimental condition all the toxin sites are occupied (11), one finds a density of 49,600 ± 16,000 sites for the α-toxin per square micrometer of postsynaptic plasma membrane. To make this estimation, it was assumed that the plane of the section was always strictly perpendicular to the plasma membrane; in other words, the surface counted was taken as the product of the contour length by the thickness of the section. It can be demonstrated that the density of sites proposed should therefore be slightly overestimated but only by a small percentage.

Taking into account the geometry of the cell surface (Table I) and the nonspecific labeling (Table II), one finds that in the extrasynaptic areas of the innervated face the density of receptor sites is approx. 100 times smaller than under the nerve terminals or 370 ± 260 sites per square micrometer of plasma membrane.

The total number of sites per electroplaque of average size would then be close to 2.7×10^{11} with a ratio of the subsynaptic to the extrasynaptic sites close to 1.5. This value of the total number of sites found by autoradiography fits rather well with the $(2.0 \pm 0.5) \times 10^{11}$ sites given by direct scintillation counting with the tritiated [^{3}H]α-toxin, or the 1×10^{11} sites found by Karlin et al. (45), with a covalent affinity labeling reagent. The values are also in agreement with those offered by studies on homogenates of the electric organ from *E. electricus* (47, 91): the 10–30 nmol of toxin sites per kg of fresh electric organ represent about $1.5–4.5 \times 10^{11}$ sites per single cell of an average wet weight of 10 mg.

The Denervated Electroplaque from Electrophorus Electricus

GENERAL MORPHOLOGY: 2 days after denervation, the synaptic vesicles appear disorganized and, simultaneously, the postsynaptic potentials disappear (see below). Then the presynaptic membrane progressively detaches from the cell surface (Fig. 8), leaving the cleft substance and surface coat attached to the postsynaptic membrane (Fig. 9). From 4 days after denervation, the degenerating nerve terminals leave the synaptic gutters; on day 8, only 0.1% of the surface of the innervated membrane (instead of 1.7%) is still occupied by nerve terminals which disappear completely after a fortnight. After the regression of the nerve terminals, Schwann cells emit numerous digitations which impinge upon the synaptic gutters. Such contacts become frequent 15 days after denervation (Fig. 10).

Up to 52 days after denervation the electroplaque itself does not show any important ultrastructural modification, and therefore appears as a rather stable structure as compared to the early signs of atrophy observed on the denervated skeletal muscle (35) or the appearance of new structures such as the contractile-like elements observed by Gautron (36) in the cytoplasm of the

Torpedo marmorata electroplaque. However, 142 days after denervation, the electroplaque shows signs of degeneration.

AUTORADIOGRAPHIC STUDIES ON THE DENERVATED ELECTROPLAQUE: Single electroplaques were dissected from electric organs 8–52 days after denervation, labeled with the tritiated α-toxin and processed for autoradiography along with normal electroplaques. In all the autoradiographs taken of denervated electroplaques, patches with a high density of silver grains are observed on the plasma membrane of the caudal face. These patches appear freely exposed to the extracellular medium (Fig. 9). Their frequency, the shape and the dimensions of the underlying gutterlike structures, the thickening of the plasma membrane and the number of caveolae per μm of membrane contour are identical to those described in the case of normal synapses (Figs. 3 and 7).

The distribution of silver grains across these membrane structures (histogram, Fig. 11) and the density of silver grains per μm of contour length also do not change, from 8 to 52 days after denervation, and are the same as in the subsynaptic membrane of normal junctions (Fig. 11).

The patches of silver grains observed by autoradiography still occupy 1.4–2% of total surface of the caudal face 28 and 52 days after denervation (Fig. 10). The ratio of the densities of silver grains in the patches and outside the patches does not vary significantly up to 52 days after denervation (12). The nonspecific adsorption of the tritiated α-toxin remains in the same range before and after denervation (Table II) whereas on the noninnervated face of the electroplaque the density of silver grains never exceeds the nonspecific labeling. These results were complemented by direct measurements of the total number of toxin sites both on isolated denervated electroplaques and on crude homogenates of denervated electric organ.

An excellent agreement exists between these different series of results. In particular, and at variance with what is classically found with skeletal muscle, up to 152 days after denervation the total number of toxin sites is still 2×10^{11} sites per electroplaque as in the normal cell; in crude homogenates the specific activity remains close to 10–20 nmol of [^{3}H]α-toxin sites per gram of protein, as in the normal electric organ (62). These data indicate a remarkable stability of the distribution and density of [^{3}H]α-toxin sites after denervation on both formerly sub- and extrasynaptic areas of the electroplaque membrane, without any tendency to lateral diffusion from the high density clusters or to an increase in the number of sites in the extrasynaptic areas.

ELECTROPHYSIOLOGY OF THE DENERVATED ELECTROPLAQUE: Electrophysiological experiments were undertaken to check the effectiveness of denervation and to monitor eventual changes in the physiological properties of the excitable membranes (sensitivity to cholinergic effectors or to electrical fields).

NEURALLY EVOKED RESPONSE: In the normal electroplaque, strong hyperpolarizing pulses stimulate the nerve terminals without eliciting a "direct" action potential in the electrically excitable membrane of the electroplaque (see Materials and Methods). At low stimulus intensities, a postsynaptic potential can be generated with a delay of approx. 1 ms, and its amplitude increases with increasing intensities. For 50-μs square current pulses, the postsynaptic potential reaches the threshold for the generation of an action potential ("indirect spike") with an intensity of 173 ± 16 mA/cm^2 of the apparent cell surface (mean ± SEM, 12 cells). 1 day after denervation, indirect spikes are still elicited with the normal intensity of stimulation (200 mA/cm^2, mean of five cells) and, as expected for a neurally evoked spike, the response is blocked by 10^{-5} M d-tubocurarine.

In agreement with the anatomical data, the "indirect" spike disappears on the 2nd day after denervation (even with stimulus intensities as high as 600 mA/cm^2), and does not reappear up to 140 days later.

RESPONSE OF THE CAUDAL MEMBRANE TO CHOLINERGIC EFFECTORS APPLIED IN BATH: The development of denervation hypersensitivity in mammalian and amphibian muscles can be monitored upon bath application of acetylcholine (6, 64). This method was used with the denervated electroplaque. Fig. 13*b* and Table III show that neither the maximal response to the agonist decamethonium nor the apparent dissociation constant changes significantly up to 52 days after denervation. The apparent dissociation constant for the antagonist d-tubocurarine (determined from 3 to 52 days after denervation on eight different cells) did not change either.

RESTING MEMBRANE POTENTIAL: At variance with what happens with most skeletal

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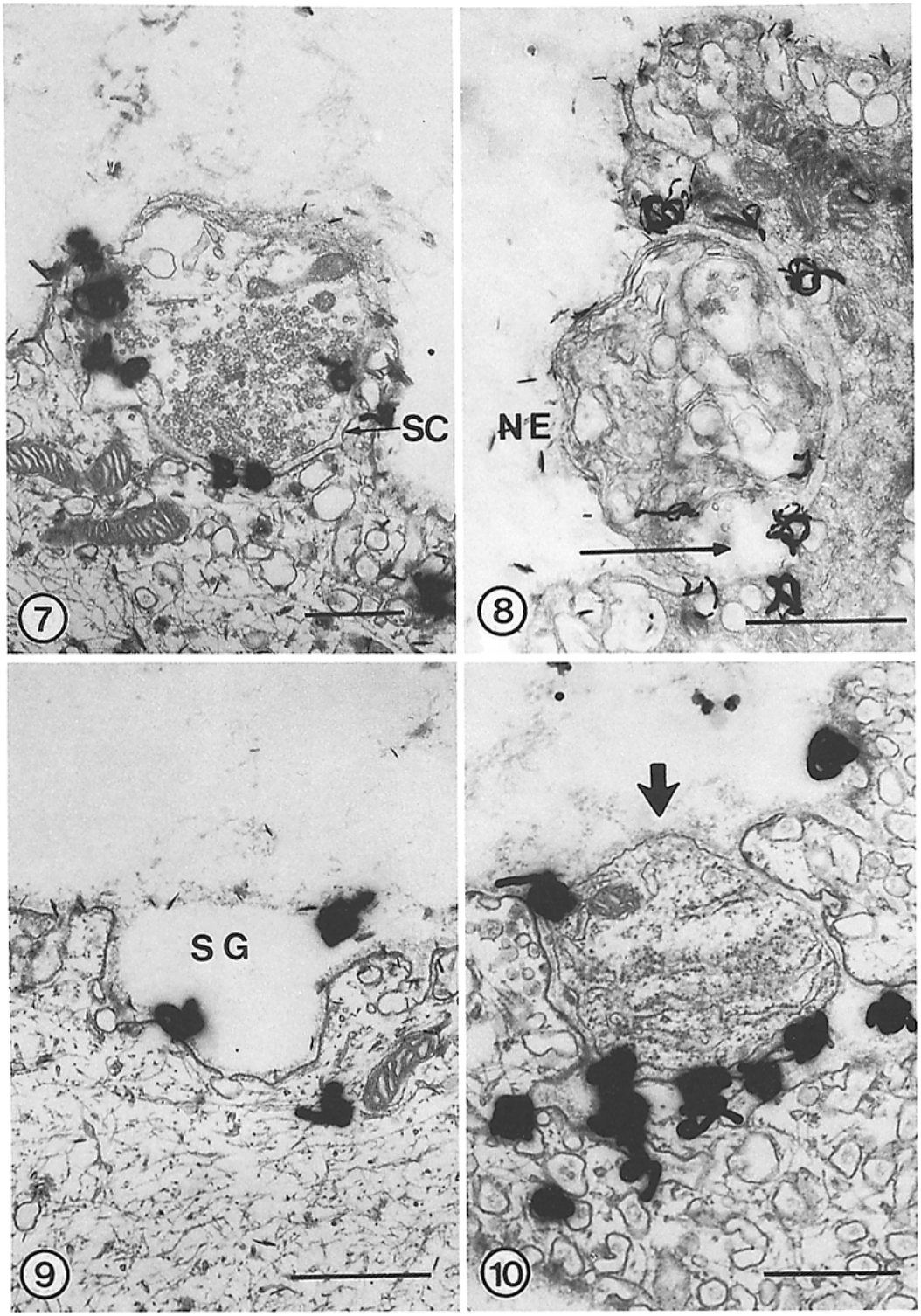

muscles after denervation (90, 54, 2, 49, 87) and in agreement with the early finding of Altamirano (4), the resting membrane potential E_o of the *Electrophorus* electroplaque does not change significantly up to 142 days after destruction of the spinal cord (Fig. 13*a*, Table III). Since E_o reflects essentially E_K, the equilibrium potential for potassium (65, 44), the internal concentration of this ion probably is also constant, in agreement with most of the direct determinations on denervated muscle from frog (41) or rat (58, 49, 24) (see, however, the increase reported in reference 26).

RESTING MEMBRANE CONDUCTANCE: The resting membrane resistance of the electroplaque increases by ~50% after denervation (approx. 9 $\Omega \cdot cm^2$ 10 days after denervation against 6.2 ± 0.5 $\Omega \cdot cm^2$ for 21 normal cells). It is known from the work of Grundfest and his collaborators (65, 81, 80) that the resting conductance has two distinct components, both of them specific for potassium ions—a passive "leak" conductance G_L and a reactive one G_K which inactivates during depolarisation or bath applications of various cations (Ba^{++}, Cs^+, Rb^+). Denervation does not affect G_L but, as found with muscle (66, 64, 2), causes a significant decrease of G_K to about one-half or one-third of its value in the innervated controls (Fig. 13*c*).

ACTION POTENTIAL: In agreement with the early findings of Couceiro and Martins-Ferreira (23), the direct action potential persists after denervation (Fig. 12). The critical depolarization (30-ms pulses) and the sodium equilibrium potential (and therefore probably the internal sodium concentration) did not significantly change (Table III). However, as found with skeletal muscle (75, 25, 38, but see also reference 53) the conductance to Na^+ at the peak of the action potential (G^p_{Na}) decreases within 5 days after the destruction of the spinal cord to less than one-third of its normal value (Fig. 13*d*). In parallel, as a consequence of the decrease of G^p_{Na}, the mean amplitude of the action potential falls by ~20% (112 ± 5 mV for 21 denervated cells against 134 ± 3 mV for 20 normal cells). Graded action potentials have been observed after prolonged denervation (77); they were also sometimes encountered in this study, but with both normal and denervated electroplaques. Finally, Redfern and Thesleff (76) have reported that the action potential of rat skeletal muscle becomes partially resistant to tetrodotoxin in areas of the cell surface that exhibit supersensitivity. Similar experiments done on four electroplaques denervated for 4, 8, 11, or 52 days did not reveal any significant alteration of the sensitivity of the direct action potential to 5×10^{-8} M or to 10^{-7} M tetrodotoxin (Fig. 12).

DISCUSSION

The use of α-toxin from snake venom (for reviews see references 50 and 86) to label the nicotinic receptor site and the development of quantitative autoradiographic methods (82, 29) have led to the measurement of absolute densities of cholinergic receptor sites per area of plasma membrane. The present observations confirm and further extend the previous findings (13, 12) that in the *Electrophorus* electroplaque these sites are almost completely absent from the noninnervated membrane. On the other hand, in the innervated membrane

FIGURE 7 High resolution autoradiographs of [^{3}H]α-toxin-labeled normal electroplaque at the level of the synaptic contacts exposed for one month. Silver grains are localized along the synaptic cleft (*SC*). The synaptic contacts occupy 1.4–2.0% of the total surface of the caudal face of the electroplaque. × 14,900. Bar, 1 μm.

FIGURE 8 Autoradiograph of [^{3}H]α-toxin-labeled electroplaque dissected out 4 days after destruction of the spinal cord. The nerve ending (*NE*) starts to degenerate and to withdraw from the synaptic gutter (arrow). × 24,000. Bar, 1 μm.

FIGURE 9 Typical synaptic gutter (*SG*) devoid of nerve ending observed 8 days after destruction of the spinal cord. These synaptic gutters were characterized by their shape, their thick plasma membrane and the small number of caveolae. Numerous silver grains were observed over such structures. × 20,880. Bar, 1 μm.

FIGURE 10 28 days after denervation, all the synaptic gutters are filled with a Schwann cell process (arrow). The postsynaptic membrane still represents 2% of the total surface of the plasma membrane of the caudal face of the electroplaque. × 20,780. Bar, 1 μm.

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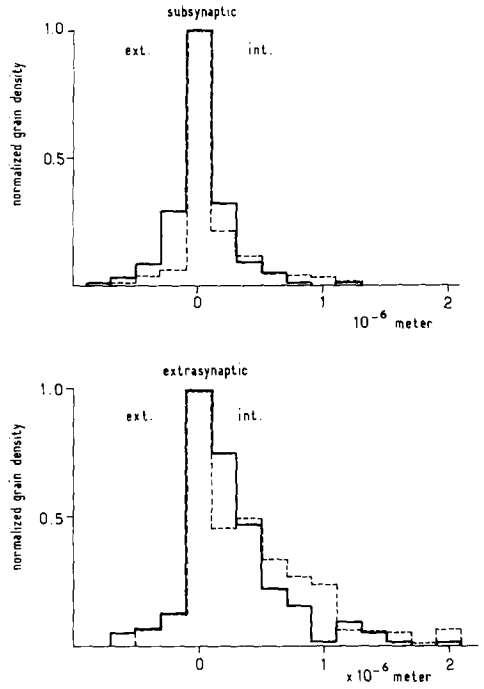

FIGURE 11 Distribution of silver grains across the plasma membrane in subsynaptic (upper histograms), and extrasynaptic (lower histograms) areas of the caudal face from normal (continuous lines) and denervated (dotted lines) electroplaques. On the abscissa is plotted the shortest distance, in micrometer, from the center of each silver grain to the postsynaptic membrane (upper histograms) or to the cell contour (lower histograms). Positive values on the abscissa refer to silver grains present over the cytoplasm. Histograms are normalized to the central class centered on cell surface. The distributions of silver grains across the plasma membrane in former subsynaptic and extrasynaptic areas of an electroplaque 28 days after denervation (dotted lines) can also be obtained 8, 15, and 52 days after denervation.

the receptor sites are present both under the nerve terminals and between the synapses. Their density is approx. 100 times higher in subsynaptic than in extrasynaptic areas, and the extrasynaptic sites represent ~40% of the total number of sites present in the cell.

In extrasynaptic and synaptic areas the silver grains appear to be associated with the plasma membrane; in extrasynaptic areas, they are present in the membrane of the caveolae; the distribution of the α-toxin sites therefore quite closely parallels that of the enzyme acetylcholinesterase (9, see also reference 7). In any circumstance, neither clustering of toxin sites in these extrasynaptic areas nor significant labeling of the Schwann cells or of the nerves terminals were ever observed.

The constancy of the distribution of silver grains along the synaptic cleft after denervation indicates that in the innervated electroplaque almost all the sites labeled are localized in the subsynaptic membrane. The presynaptic sites, if any (52), should represent only a small fraction of the total number of sites present in the synapse (71). The value found for the counting efficiency, despite experimental differences, closely parallels that reported by Salpeter and Szabo (82), Porter et al. (72) and Porter and Barnard (70) with mammalian neuromuscular junction. Quantitative analysis of the high resolution autoradiographs gives a density of 49,600 ± 16,000 cholinergic receptor sites per square micrometer of subsynaptic plasma membrane, assuming a stoichiometry of one [^{3}H]α-toxin site per acetylcholine receptor site (91). Within experimental error, this density is comparable to the 18,000, 20,000–25,000, or 30,500 (±27%) receptor sites per μm^2 of postsynaptic dense membrane found, respectively, by Albu-

TABLE III

Parameter	Control	Denervated
Resting membrane potential E_0 (mV)	80.1 ± 0.8 (79)	81.1 ± 1.3 (36)
"Leak" conductance G_L (mmho/cm^2)	50 ± 2 (20)	54 ± 5 (15)
Characteristics of action potential:		
Threshold (mV)	36 ± 3 (17)	34 ± 3 (17)
Sodium equilibrium potential E_{Na} (mV)	116 ± 13 (14)	119 ± 13 (21)
Response to Decamethonium:		
Maximal depolarisation (mV)	48 ± 1 (18)	46 ± 2 (11)
Apparent affinity $K_{app.}$ (μM)	1.8 ± 0.1 (18)	1.8 ± 0.1 (11)
Apparent affinity, K_{app}, for D-tubocurarine (μM)	0.14*	0.14 ± 0.03 (8)

Electrophysiological parameters of isolated electroplaques unaffected by denervation. The mean values obtained from the number of cells indicated in parentheses, normal or denervated for 1–142 days, are given ± SEM.
* From Kasai and Changeux (47).

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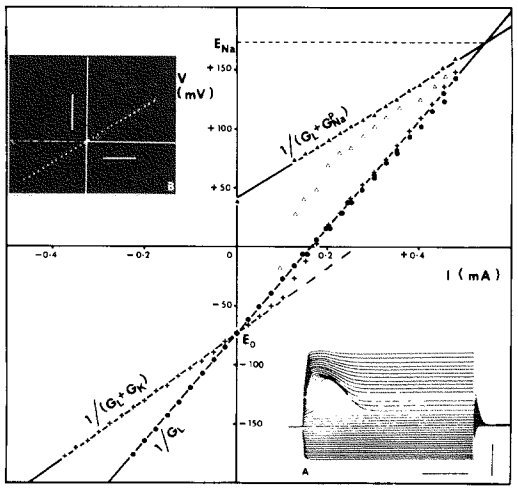

FIGURE 12 Determination of the electrophysiological parameters of an isolated denervated electroplaque. Rectangular current pulses were applied as described in the text, and the potential V across the innervated face was recorded (inset A; calibration bars: ordinate, 100 mV; abscissae, 5 ms). V (inside minus outside) was measured at the plateau (+) and at the peak of the "direct" action potential (▲) and plotted against the intensity I of the stimulating current (counted positive when leaving the cell through the innervated face). The amplitude of the action potential was also measured in the absence of imposed current, after a brief (50 μs) depolarization (▲). The data were analyzed as described by Morlock et al. (65). The experiment was repeated in the presence of 5 mM $BaCl_2$ to insure an accurate determination of G_L (plateau: ●) and, in some cases, during application of 10^{-7} M tetrodotoxin (peak of the spike: △). The cell presented had been denervated for 52 days (inset A: 4 days). In two-thirds of the experiments, V was electronically plotted against I on the screen of an oscilloscope (inset B; cell denervated 24 h before; calibration bars: ordinate, 100 mV; abscissa, 100 μÅ).

querque et al. (1), Porter and Barnard (70), and Fertuck and Salpeter (29), in mammalian neuromuscular junctions. Fertuck and Salpeter (30) discovered that, in over exposed autoradiographs of endplates labeled with ^{125}I-α bungarotoxin, the toxin sites appear concentrated in the crest of the subsynaptic folds beneath the nerve terminals. This observation fits with other results obtained with similar systems but different techniques (8, 74, 78). The subsynaptic membrane of the *Electrophorus* synapse is, in many respects, analogous to the crest postsynaptic membrane of the neuromuscular junction. These two membranes would therefore consist of closely packed receptor molecules. Regular organization of the receptor molecules in these "one-protein" membranes has indeed been observed by both electron microscopy (15, 69) and X-ray diffraction (27).

On receptor-rich membrane fragments isolated from the *Torpedo marmorata* electric organ, Cartaud et al. (15, 16) and Nickel and Potter (69) observed, by both negative staining and freeze etching, 70–90 Å particles with a density of 10,000–15,000 per square micron of membrane (which most likely is subsynaptic). These particles are similar in size and shape to the detergent-extracted purified receptor oligomer from *Electrophorus* (63) or *Torpedo* (28 and 16) electric organs. Their density is compatible with that found by counting the number of toxin sites if one assumes that, in agreement with the known bio-

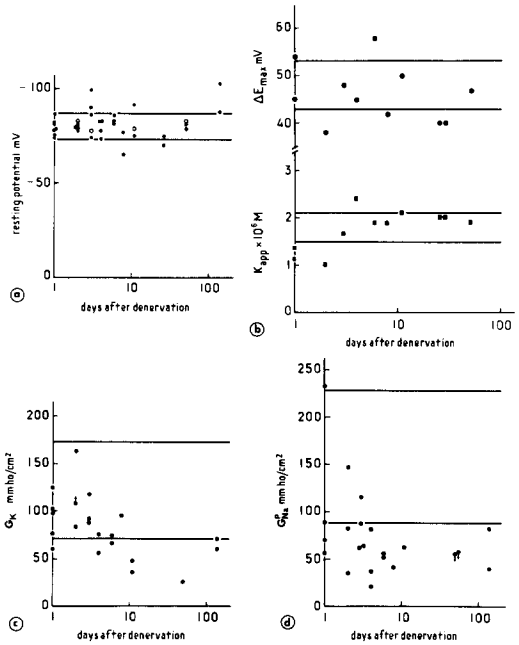

FIGURE 13 Electrophysiological properties of electroplaques after denervation. Each point represents an individual cell. The horizontal lines delimit the range of variation (mean ± SD) on innervated controls. (*a*) Resting membrane potential. Empty circles correspond to electroplaques that exhibited graded action potentials. See also Table IV. (*b*) Apparent dissociation constant (K_{app}) and maximal response (ΔE_{max}) to decamethonium bromide added to the physiological solution. See also Table IV. (*c*) Reactive potassium conductance at rest (G_K). The arrowed circles indicate a minimal value. The mean ± SD for 16 normal cells was 122 ± 51 mmho/cm². (*d*) Sodium conductance at the peak of action potential (G^P_{Na}). Arrowed circles indicate maximal values. The mean ± SD for 16 normal cells was 158 ± 70 mmho/cm².

chemical findings (for a review see Changeux [17]), several, at least three toxin sites are present per receptor oligomer.

The consequences of denervation on the *Electrophorus* electroplaque are, in several respects, similar to those classically found with vertebrate striated muscle, but differences were noticed. In both cases, the nerve terminals show signs of degeneration a few days after denervation (8, 37, 59) and disappear completely after 8 days; the first morphological transformations in the nerve terminal also coincide with the abolition of synaptic transmission (8). Also, in both cases, the degenerating nerve endings become replaced by a Schwann cell which occupies the remnants of the formers endplate. In the *Electrophorus* electroplaque, however, this process takes place with a longer delay than at the neuromuscular junction, and the former subsynaptic membrane remains "naked" for days or even weeks. Important transformations have been reported to occur in the postsynaptic cell after denervation of striated muscle; signs of atrophy appear in the structure of the muscle fibers and a "hypersensitivity" to acetylcholine develops in extrasynaptic areas (see reference in 56).

In the *Electrophorus* electroplaque, no such phenomenon occurs. The dose-response curves to bath-applied cholinergic agonists do not change up to 142 days after denervation, nor does the number of [^{3}H]α-toxin sites per cell as determined by scintillation counting and by autoradiography. The electroplaque is therefore a much more stable structure than the striated muscle fiber. The absence of denervation hypersensitivity in the *Electrophorus* electroplaque might be accounted for by several alternative possibilities: (*a*) the persistence of a residual innervation due to partial destruction of the spinal cord or the existence of a nonmedullar innervation but evidence in favor of this hypothesis was never encountered in the course of our anatomical and electrophysiological investigations; (*b*) the stimulation of the denervated organ by an electric field generated in its nondenervated anterior moiety; these field effects would be sufficient to shut off receptor synthesis in the denervated electroplaques as in the case of the denervated striated muscle, submitted to a direct electrical stimulation (56); although this possibility cannot be completely excluded, it is made improbable by the old observation of Couceiro and Martins-Ferreira (23) that the elimination of a few centimeters of spinal cord in the middle of the animal body prevents the discharge from spreading in the posterior part of the electric organ; (*c*) the absence of a contractile apparatus and the impossibility of creating mechanical energy.

In contrast with this absence of hypersensitivity, the denervated electroplaque undergoes some electrophysiological alterations frequently encountered after denervation of muscles. Most denervated muscles present both hypersensitivity and a drop in resting potential (90, 54, 2, 49), but some exhibit hypersensitivity without depolarization (frog: 68, 53, 48), depolarization without hypersensitivity (locust: 87, 88) or, as in the case of the electroplaque, neither of them (crayfish: 33). The reactive conductance to sodium ions of the electroplaque plasma membrane diminishes drastically, occasioning a reduction in the action potential, as is the case for rat, mouse, and chicken muscles (75, 38, 25), but not for frog muscle (53, 22). The observation that this reduction occurs on the denervated electroplaque in the absence of hypersensitivity supports the conclusion of Colquhoun et al. (22) that the appearance of new acetylcholine receptors in the extrajunctional membrane of muscle fibres is not at the origin of the diminution of electrical excitability. Our few experiments with TTX seem to confirm that the occurrence of partially TTX-resistant action potentials (42, 76, 38) is restricted to mammals (40, 22, 25). Thus, the only change in membrane parameters that seems constantly associated with denervation is a rise in the resting resistance (68, 64, 2, 55, 12, and this work). Albuquerque et al. (3), working on rat "fast" muscle, had already noted that the rises in hypersensitivity and resistance had different time courses. Our experiments indicate that one of them can even be observed in the complete absence of the other. From the presently available data, it seems likely therefore that the appearance of new acetylcholine receptors does not in itself perturb the extrasynaptic membrane.

Finally, one of the most striking results of our autoradiographic studies (12 and this paper) is the persistence of a high density of cholinergic receptor sites at the former endplate long after denervation. This finding, which is consistent with the observation of Frank et al. (34) on the motor endplate, was not unexpected in view of the early findings of Birks et al. (8) that the postsynaptic organization of the lizard endplate remains intact up to 56 and 142 days after denervation but degenerates afterwards with the muscle fiber if reinnervation does not take place. Once accumu-

lated under the nerve terminal, the acetylcholine receptor molecule therefore does not show any tendency (at least in the adult) to lateral diffusion, even in the absence of nerve terminals. (In the case of the *Electrophorus* electroplaque, nothing can be said yet about the turnover of these molecules.) This strong immobilization has also been found in the case of the patches which form spontaneously on myoblasts in culture (5) and in vitro on subsynaptic membrane fragments isolated from the *Torpedo marmorata* electric organ (79). Moreover, in the adult muscle endplate, the rate of degradation of the receptor molecule appears several orders of magnitude slower than that of the extrasynaptic receptor protein in both embryonic and denervated adult muscle fibers. The maturation of the endplate therefore involves a "stabilization" of the receptor molecule which both immobilizes it and renders it resistant to degradation (19). The chemical reactions and the signals involved in the development of this slow process are still unknown, but experiments are in progress (84, 85) which may lead to their identification.

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