AUTORADIOGRAPHIC LOCALIZATION OF ACETYLCHOLINE RECEPTORS IN THE SCHWANN CELL MEMBRANE OF THE SQUID NERVE FIBER

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ABSTRACT

Intact and slit nerve fibers of the squid Sepioteuthis sepioidea were incubated in a 50-nM solution of [125I]α-bungarotoxin in artificial seawater, in the absence and in the presence of d-tubocurarine (10⁻⁴ M). The distribution of the radioactive label was then determined by electron microscope autoradiography. It was found that, in the fibers exposed solely to the radioactive toxin, the label was located mainly at the axon-Schwann cell boundary in the intact nerve fibers or at the axonal edge of the Schwann cell layer in the axon-free nerve fiber sheaths. Label was also present in those regions of the Schwann cell layer rich in intercellular channels. No signs of radioactivity were observed in the nerve fibers exposed to the labeled toxin in the presence of d-tubocurarine. These results indicate that the acetylcholine receptors previously found in the Schwann cell plasma membrane are mainly located over the cell surfaces facing the neighboring axon and the adjacent Schwann cells. These findings represent a further advance in the understanding of the relationships between the axon and its satellite Schwann cell.

KEY WORDS Schwann cells · acetylcholine · receptors · nerve fiber · squid

Recent studies carried out on the giant nerve fiber of the tropical squid Sepioteuthis sepioidea (5, 6) revealed the presence of acetylcholine receptors in the plasma membrane of the Schwann cell. The external application of acetylcholine (10⁻⁷ M) and carbamylcholine (10⁻⁶ M) to the resting nerve fiber causes a long lasting hyperpolarization of the Schwann cells, which can be blocked by the external application of d-tubocurarine (10⁻⁹ M). Eserine (10⁻⁹ M) prolongs the Schwann cell hyperpolarizations produced by acetylcholine (6). It was also found that carbamylcholine increases the relative permeability of the Schwann cell membrane to the potassium ion (6).

Further studies carried out on the same nerve fibers (7) permitted the characterization of the sites of action of acetylcholine in the plasma membrane of the Schwann cell. It was found that the α-toxin derived from the venom of Bungarus multicinctus irreversibly blocks the long-lasting Schwann cell hyperpolarizations after the external application of carbamylcholine to the resting nerve fiber, and that d-tubocurarine protects the Schwann cells against the irreversible action of α-bungarotoxin (7). In addition, [125I]α-bungarotoxin was found to bind in vitro to the plasma membrane preparations isolated from S. sepioidea peripheral nerves (R. Villegas, F. V. Barnola and...
Labeling of the Nerve Fibers

concentration used in the studies reported above, inserted into the axon, and the nerve fiber was cut from most of the neighboring small fibers in less than 10 min; and, finally, control artificial seawater for 5 rain. The other half was left lengthwise over its entire length. The other half was left intact. Both nerve halves were then immersed for 15 rain after decapitation of the animal. The fibers were sected out of the first stellar nerve of S. sepioidea giant nerve fibers. The evidence obtained shows that, both in the intact nerve fiber and in axon-free nerve fiber sheaths, the largest density of [3H]-alpha-bungarotoxin-binding sites is found at the axon-Schwann cell boundary region. Radioactivity is also found in appreciable amounts over the rest of the Schwann cell compartment but not inside the axon.

Preliminary results of this work have been reported elsewhere (8, 9).

**MATERIALS AND METHODS**

**General Procedure**

Giant nerve fibers (280–400 μm in diameter) dissected out of the first stellar nerve of S. sepioidea squids were used. The giant fibers were dissected and freed from most of the neighboring small fibers in less than 10 min after decapitation of the animal. The fibers were tied at both ends with fine threads.

**Labeling of the Nerve Fibers**

In a first group of experiments, five nerve fibers, each from a different animal, were individually divided into halves. In one of the halves, sharp microscissors were inserted into the axon, and the nerve fiber was cut lengthwise over its entire length. The other half was left intact. Both nerve halves were then immersed for 15 min in a small petri dish containing 3 ml of a 50-nM solution of [3H]-alpha-bungarotoxin (sp act, 2.3 × 10^6 Ci·mol⁻¹) in artificial seawater. For a second group of experiments, six nerve fibers from different animals were also divided in halves (both halves were left intact) and kept immersed for 5 min in control artificial seawater. Then, one of the halves was successively immersed in: 10⁻⁴ M α-tubocurarine seawater solution for 10 min; 10⁻⁴ M α-tubocurarine plus 10⁻⁴ [3H]-alpha-bungarotoxin (sp act, 3.5 × 10⁶ Ci·mol⁻¹) seawater solution for 15 min; 10⁻⁴ M α-tubocurarine seawater solution for 10 min; and, finally, control artificial seawater for 5 min. The other half of the same nerve fiber was similarly treated except for the absence of α-tubocurarine from all the test solutions. The purified [3H]-alpha-bungarotoxin used in these experiments was kindly supplied by Dr. Michael Raftery from the Division of Chemistry, California Institute of Technology. In both groups of experiments, the different test solutions were kept at room temperature (20–22°C) and after the last incubation period each half-fiber was fixed for 2 h in ice-cold 1% osmium tetroxide solution in artificial seawater, buffered to pH 7.6 with Veronal acetate (final osmolality 1.010 mosmol/kg, which is isotonic for the squid tissue). Dehydration was carried out in an ethanol series, starting at 50% concentrations and ending with pure propylene oxide. All the specimens were embedded in Epon 812.

**Processing the Tissue for Electron Microscope Autoradiography**

Pale-gold transverse sections from radioactive and nonradioactive nerves were prepared on a Porter-Blum Sorvall MT2-B ultramicrotome (DuPont Instruments-Sorvall, DuPont Co., Wilmington, Del.) with a diamond knife (TVIC, Caracas, Venezuela) and coated with a monolayer of Ilford-L4 emulsion (Ilford Ltd., Essex, England). The sections were placed on collodion-coated slides, covered with a carbon layer, and the emulsion monolayer was prepared according to the flat substrate method of Salpeter et al. (2). The obtaining of a packed monolayer of silver halide crystals was controlled with the electron microscope.

Emulsion-coated experimental slides exposed for 24 h to radiation (~7 mR·h⁻¹) from the [3H]-alpha-bungarotoxin solution used in the second series of experiments (see above) were processed at the beginning, and others at the end, of the exposure period to assess latent image fading during the exposure time. The slides were allowed to dry for 2 h in an upright position and stored in black plastic boxes containing humicap dessicant (Drialre, Inc., South Norwalk, Conn.) at 4°C. Two emulsion-coated slides with similar sections from nonradioactive nerve fibers were placed in each box to be used as controls. After a 3-wk period, they were developed with Microdol-X (Eastman Kodak Co., Rochester, N. Y.) for 3 min at 24°C.

**Analysis of the Autoradiographs**

The autoradiographs were viewed with a Siemens Elmiskop 1A microscope, and all areas containing grains were photographed at constant magnification (× 10,000). Several grids from different blocks were used for collecting 1.092 and 1.113 grains from the intact and slit fibers, respectively. Prints of the autoradiographs were analyzed at a final magnification of 30,000. Although pictures were taken only from those areas containing developed grains, the area photographed was large relative to the size of the grains and usually included several potential sources. Thus, it was essentially random.
relative to the tissue compartments analyzed. Distribution of label in the autoradiographs was carried out with a resolution (half distance, HD) value of 900 Å, according to Salpeter et al. (3). 1 HD is the distance from a line source in an autoradiographic specimen within which half of the developed grains fall (2). Each fiber was divided into two compartments: axon (or axonal side in the slit fibers) and Schwann cell compartment. The origin was taken as the midline between the axolemma and Schwann cell plasma membrane in the intact fibers or as the axonal surface of the axon-free Schwann cell in the slit fibers.

The density of grain distribution was recorded after the method of Salpeter et al. (2, 3) as follows: (a) The midpoint of each silver grain was determined by placing over the pictures a plastic lamina which has a series of circles of different size. The center of the smallest circle enclosing the grain was punctured with a needle. (b) A lattice with uniformly spaced points was superimposed over the micrographs. The lattice points were used to provide a relative measure of the area of each compartment. (c) The distance from each midpoint grain and lattice point to the origin was measured using HD units. This was done up to 4.5 HD units toward each side of the origin. Each measurement was designated as (+) or (−) depending on whether it was over the axon (or axonal side in the slit fibers) or Schwann cell compartment, respectively. (d) The results were tabulated as the number of developed grains per number of lattice points within each "HD subcompartment." The first HD subcompartment straddles the origin, i.e., we collected all the grains and points from −0.5 to +0.5 HD, then those from +0.5 to +1.5 HD, etc.). (e) The standard deviation for each HD subcompartment was estimated from the formula:

\[ SD = \frac{G}{P} \sqrt{\left( \frac{1}{\sqrt{G}} \right)^2 + \left( \frac{1}{\sqrt{P}} \right)^2}, \]

where \( G \) equals number of grains and \( P \) equals number of lattice points. This formula combines the independent errors due to statistical sampling of a limited number of grains and points. It is based on the fact that \( 1/\sqrt{N} \) is the fractional error in a Poisson distribution of mean \( \lambda \) (1). All the results were normalized to give a density over the origin (first HD subcompartment) equal to unity. Once normalized, the experimental data were compared with the appropriate universal density curves provided by Salpeter et al. (3) for radioactive sources of different shape.

**RESULTS**

The densities of silver grains over nonradioactive control sections were similar to the background in the experimental autoradiographs, indicating that there was no positive chemography. No evidence of latent image fading was detected. In the first group of experiments developed, grains due to the presence of \([^{125}I] \alpha\text{-bungarotoxin} \) were located mainly toward the axon-Schwann cell boundary in the intact nerve fibers (see arrowhead in Fig. 1) or toward the axonal edge of the Schwann cell layer in the axon-free nerve fiber sheaths (see arrows in Fig. 2). Grains were also found over the rest of the Schwann cell layer, including the region close to the basal membrane (arrow in Fig. 1A). Occasionally, rows of grains could be seen after the tortuous pathway of a Schwann cell channel as seen in Fig. 3. As much as 55% of the grain centers found within the Schwann cell layer were recorded as located directly over the channels.

Similar results were obtained in the second group of experiments with the nerve halves exposed solely to the radioactive toxin. No signs of radioactivity were observed in the autoradiographs of the nerve halves preincubated in \( 10^{-4} \) M \( \alpha\text{-tubocurarine seawater} \), exposed to the radioactive toxin in the presence of \( 10^{-4} \) \( \alpha\text{-tubocurarine} \), and finally postincubated in \( 10^{-4} \) \( \alpha\text{-tubocurarine seawater} \) solution to wash away the unbound toxin.

The normalized grain density distributions for both the intact nerve fibers and the axon-free nerve fiber sheaths are shown in the histograms of Figs. 4 and 5, respectively. In both cases, the highest density of grains is located within the 900 Å-wide region which included the origin of the autoradiographs, i.e. the axonal surface of the axon-free Schwann cells in the slit fibers or the midline between the axolemma and Schwann cell plasma membrane in the intact fibers (0 in Figs. 4 and 5), decreasing toward the Schwann cell and the axon (or axonal side in the slit fibers) compartments. When the normalized experimental values were compared with the normalized universal grain density curves for radioactive sources of different shapes (2, 3), it was found that they best fitted the curve expected for a line source located at the origin of the autoradiographs (see the smooth curve superimposed on the histograms of Figs. 4 and 5). There is a close correspondence between experimental values and expected distribution within the axon (or axonal side in slit fibers) compartments. However, all the experimental values for the Schwann cell compartment were significantly higher (>2 SD) than those expected if all the radioactivity were confined to the origin.

**DISCUSSION**

The present results show that the developed grains...
due to the presence of $[^{125}\text{I}]\alpha$-bungarotoxin, specifically bound to the nerve fiber, were located mainly along the axon-Schwann cell boundary in the intact nerve fiber, and along the axonal edge of the Schwann cell layer in the axon-free nerve fiber sheaths. Grains were also observed over the rest of the Schwann cell layer and accumulated specially over those regions rich in extracellular channels. About half of the grains recorded (midpoint of each silver grain) were located over the channels. The fact that no trace of radioactive label could be detected in the fibers treated with $\beta$-tubocurarine indicates that the $[^{125}\text{I}]\alpha$-bungarotoxin binds mainly to the cholinergic sites of the cell membrane, as has been discussed in a previous work (7). These experimental findings confirm the presence of acetylcholine receptors in the Schwann cell plasma membrane (5-7), and further indicate that they are located on the cell surfaces facing the neighboring axon and the adjacent Schwann cells.

In addition, the in vitro assay of the binding of $\alpha$-bungarotoxin to the isolated nerve plasma membranes, carried out by R. Villegas, F. V. Barnola, and J. Villegas (see reference 7) has shown that the plasma membranes isolated from $S$. sepioidea peripheral nerves bind $[^{125}\text{I}]\alpha$-bungarotoxin. The binding to the fraction consisting mainly of the Schwann cell plasma membrane was found to be of the order of $2.6 \times 10^{-12}$ mol/mg of membrane protein (7). Furthermore, the electrophysiological studies carried out on the same nerve fibers indicated that $\alpha$-bungarotoxin combines with the same sites in the Schwann cell membrane that carbamylcholine and $\beta$-tubocurarine do. Thus, it was found that in nerve fibers immersed in $(10^{-9}$ M) $\alpha$-bungarotoxin solutions containing $(10^{-5}$ M) $\beta$-tubocurarine there was a complete recovery of the Schwann cell hyperpolarizing response to carbamylcholine $(10^{-4}$ M) with 10 min of reimmersion of the fiber in toxin-free seawater solution, whereas in nerve fibers exposed solely to $10^{-9}$ M $\alpha$-bungarotoxin there were no signs of recovery of the Schwann cell hyperpolarizing response to carbamylcholine $(10^{-9}$ M) even after 70 min of reimmersion of the fiber in the toxin-free seawater solution (7).

The resolving power of the method employed
in the present study (900 Å) does not permit finding out whether the sources of radioactivity at the axon-Schwann cell boundary are located solely on the Schwann cell plasma membrane. Nevertheless, taken together with the experimental findings cited above, the results of the present work give a clear indication of the distribution pattern of the

Figure 2 Autoradiograph of a cross section of a slit nerve fiber. Grains are seen located at the axonal edge of the Schwann cell layer in the axon-free cell boundary (arrows). Sc, Schwann cell; BM, basal membrane; and AS, axonal side. × 20,000.

Schwann cell receptors for acetylcholine. Thus, the binding sites for [125I]α-bungarotoxin appear concentrated along the axon-Schwann cell boundary and at the lateral surfaces of the Schwann cell delimiting the intercellular channels.

On the other hand, previous ultrastructural and histochemical studies carried out on these nerve

Figure 3 Portion of an autoradiograph of a cross section of an intact nerve fiber showing several grains aligned along a Schwann cell channel (arrows). A, axon; Sc, Schwann cell; and BM, basal membrane. × 30,000.

Figure 4 Histogram (bars) of the density distribution of silver grains in the autoradiographs of cross section of intact nerve fibers incubated with [125I]α-bungarotoxin. Positive (+) and negative (−) values of X-axis indicate the axon and Schwann cell compartment, respectively, and 0 (origin of the autoradiograph) indicates the center of the axolemma-Schwann cell space. The distance from 0 is measured in HD units (1 HD is 900 Å). The experimental grain density was normalized to one at the origin. The smooth curve superimposed on the histogram represents the expected distribution if the radioactivity were confined to a line source (3). The experimental values over the axon compartment are in close correspondence with the curve. However, those over the Schwann cell compartment are higher than the expected densities by more than 2 SD, which indicates that such a compartment is also labeled.

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On the other hand, previous ultrastructural and histochemical studies carried out on these nerve
fibers have shown the presence of acetylcholinesterase enzymatic activity distributed mainly along the axolemma (10). In addition, recent studies carried out on the same nerve fibers indicate the presence of acetylcholine in extracts obtained from isolated giant nerve fibers (8). The presence of acetylcholine receptors in the Schwann cell membrane, of acetylcholinesterase enzymatic activity mainly on the axolemma, and of acetylcholine in the nerve extracts obtained from these fibers has been related to the mechanism responsible for the long-lasting Schwann cell hyperpolarizations after the conduction of nerve impulse trains by the axon (4-7, 10). Thus, the present autoradiographic localization of the acetylcholine receptors at the axon-Schwann cell boundary and at the Schwann cell channels represents a further advance in the understanding of the mechanism responsible for the axon-Schwann cell interactions previously described.

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