Proc. Nat. Acad. Sci. USA
Vol. 71, No. 4, pp. 1376–1378, April 1974

Localization of Acetylcholine Receptor by ^{125}I-Labeled α-Bungarotoxin Binding at Mouse Motor Endplates

(electron microscope autoradiography/junctional folds/sternomastoid muscle/neuromuscular junction)

HELEN C. FERTUCK AND MIRIAM M. SALPETER*

Section of Neurobiology and Behavior, School of Applied and Engineering Physics, Cornell University, Ithaca, New York 14850

Communicated by Thomas Eisner, January 10, 1974

ABSTRACT **Exposed sternomastoid muscles of anaesthetized mice were bathed in ^{125}I-labeled α-bungarotoxin until all neurally evoked muscle contractions were eliminated. The distribution of label was then determined by electron microscope autoradiography. It was found that the label was localized at the top of the junctional folds, i.e., at the postjunctional membrane nearest the axon. Since the α-bungarotoxin had fully eliminated the physiological muscle response, these results indicate that the active acetylcholine receptor occupies a limited area of the junctional folds and is not distributed uniformly throughout this membrane. Specialized membrane densities seem to coincide with the labeled regions.**

The neuromuscular junction (endplate) in typical vertebrate striated muscle has the following general structure. The terminal motor nerve fiber lies in a trough or depression of the muscle surface. The axolemma is separated from the postjunctional sarcolemma by a 600-Å cleft (primary synaptic cleft) and the postjunctional membrane is thrown into multiple folds (junctional folds) 0.5 to 1 μm deep. The continuation of the primary synaptic cleft into the depth of the folds is called the secondary cleft. A detailed description of the structure and discussion of the early histochemical literature can be found in Zacks (1) and Csillik (2).

Histochemical studies have indicated that acetylcholinesterase (AChE) is present over the entire depth of the folds (e.g., 1–3). It is generally assumed that the acetylcholine receptor (AChR) is similarly distributed. This assumption plus the evidence of roughly comparable amounts of AChR and AChE-active sites have even given rise to the theory that AChR and AChE form a "mosaic" organization in the junctional fold membranes (2, 4). However, except for very indirect studies with lead staining (2), no demonstration exists regarding the distribution of the acetylcholine receptor along the postjunctional membrane.

The discovery that certain snake venoms combine irreversibly with the AChR (5) has provided a means for localizing and quantifying the receptor. Numerous investigations have since used radioactive snake toxin, mainly α-bungarotoxin, (from the snake *Bungarus multicinctus*) to label receptors at neuromuscular junctions, and have provided values for total binding sites per endplate. Assuming a uniform distribution of the receptor over the junctional folds, the number of receptor sites per μm^2 of membrane has been calculated (4, 6, 7). Porter *et al.* (8) also used [^{3}H]α-bungarotoxin to determine sites per μm^2 of postsynaptic membrane in diaphragm endplates by electron microscope autoradiography. Again, for this tabulation, the authors assumed a uniform distribution of receptors over the junctional folds even though their own data are not fully compatible with this assumption (8, Fig. 2*A*).

The present communication refutes this assumption. We found that the active AChR, as judged by ^{125}I-labeled α-bungarotoxin binding, is concentrated in the region of the junctional folds adjacent to the axonal membrane.

MATERIALS AND METHODS

Biological System. Three mice were used for the results reported here. The exposed sternomastoid muscle of an anaesthetized mouse was bathed in ^{125}I-labeled α-bungarotoxin, while the nerve was stimulated by a suction electrode. Muscle contractions were monitored with a delicate strain gauge and recorded on a two-channel polygraph. Stimulation conditions, chosen to give a maximal tetanic muscle response, were as follows:

The stimulating frequency was 90–100 sec^{-1} (well above mechanical fusion frequencies), and for each animal the stimulating voltage was adjusted for maximum contraction. ^{125}I-Labeled α-bungarotoxin† (2 μM) at 135 Ci/mmol was then applied topically in Krebs' buffer and the nerve stimulation was repeated intermittently (once every 15 min) until the neurally evoked muscle response was eliminated. The muscle was then rinsed in Krebs' solution and fixed with 1.5% glutaraldehyde in phosphate buffer by intravascular perfusion. The tissue was postfixed in 1% OsO_4, embedded in Epon 812, and prepared for electron microscope autoradiography by the flat substrate method of Salpeter and Bachmann (11). Monolayers of Ilford L4 and a modified Kodak NTE emulsion were used.

Autoradiographic Calibration. Although iodine-125 was one of the first isotopes used for electron microscope autoradiography (12), it had not been calibrated for quantitative interpretation of the autoradiograms. In an earlier study we established that the sensitivity with this isotope is higher than with tritium (13). For the present study we tested its resolution by a method similar to that used for tritium (14). We found that for ^{125}I, 1000-Å sections and Ilford L4 emulsion

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; AChR, acetylcholine receptor; HD, half distance.

* To whom reprint requests should be sent.

† Mr. Peter M. Ravdin of Cornell University purified and iodinated the bungarotoxin using lactoperoxidase (9) based on the procedure described by Eldefrawi and Fertuck (10). The specificity of the iodinated bungarotoxin was compared with noniodinated toxin by its lethal dose, by the concentration and time taken to inactivate the muscle response, by its localization at the endplate using light autoradiography, and by its competition for ACh receptor sites in *torpedo* electroplax membrane fractions (10).

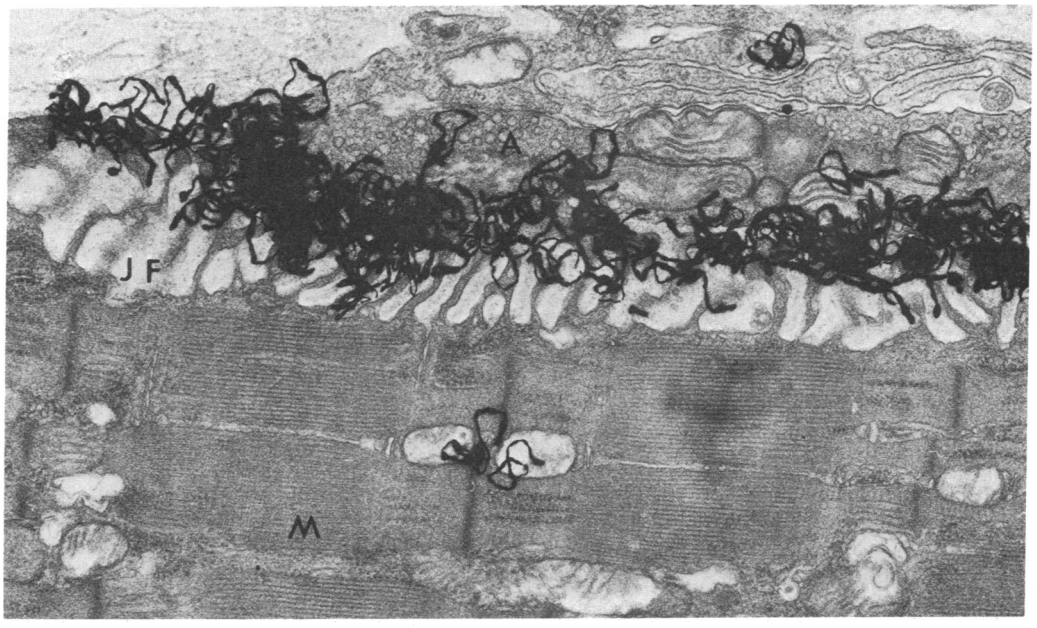

FIG. 1. Electron microscope autoradiogram (Ilford L4 emulsion) of endplate from mouse sternomastoid muscle incubated with ^{125}I-labeled α-bungarotoxin until all neurally evoked muscle contractions were blocked. The autoradiogram is overexposed (i.e., the emulsion saturated with developed grains) in order to dramatize the illustration that the label is not uniformly distributed throughout the postjunctional membrane but is concentratcd near the axonal interface. *JF*, junctional folds; *A*, axon; *M*, muscle. ×21,000.

developed in Gold latensification–Elon ascorbic acid (Gold-EAS) (15), the half-distance (HD) value for ^{125}I (i.e., the distance from a line source within which 50% of the grains fell) is about 900–1000Å; and with Kodak-NTE developed with Dektol, the HD value is about 600Å (Salpeter and Fertuck, in preparation). The resolution with ^{125}I is thus about 30–40% better than with tritium under the same autoradiographic conditions.

RESULTS

Figs. 1 and 2 are electron microscope autoradiograms of endplates labeled with ^{125}I-labeled α-bungarotoxin. The grains form a narrow band centered on the top surface of the junctional folds. In spite of the deliberate overexposure of the autoradiogram in Fig. 1, which causes loss in resolution, the depths of the junctional folds are clearly unlabeled below the

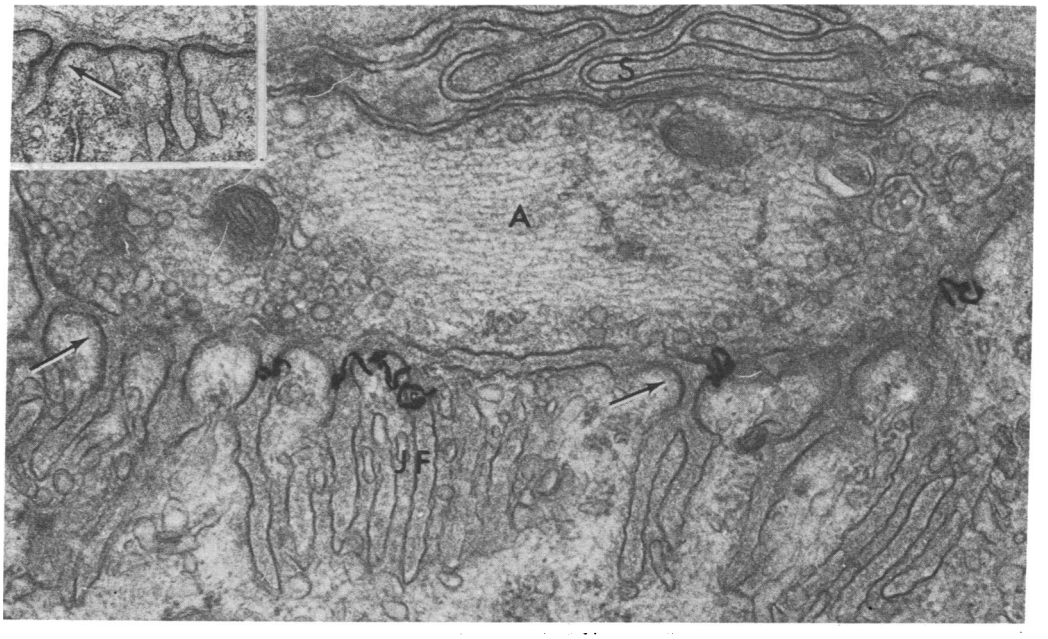

FIG. 2. Electron microscope autoradiogram of endplate labeled as in Fig. 1, but coated with the higher resolution emulsion Kodak NTE and not overexposed. Note the subneural location of the developed grains, again concentrated at the postjunctional membrane nearest the primary cleft and not distributed throughout the folds. ×37,500. *Inset:* Section after lead citrate staining (the autoradiograms are not lead-stained), emphasizes the suggestion of increased postsynaptic membrane densities near the muscle surface and dipping partly down the folds (*arrows*) which may be related to the receptor specializations. *S*, Schwann cell; *JF*, junctional folds; *A*, axon. ×21,000.

row of developed grains. Overexposing of autoradiograms makes grain counting impossible, but has the advantage of showing unequivocally at a glance the distribution of radioactivity.

These results with ^{125}I-labeled α-bungarotoxin are in marked contrast with those obtained after labeling of AChE with [^{3}H]diisopropylfluorophosphate, where the radioactivity is distributed over a broad band coincident with the junctional fold region (16, 17). Although no evidence is at present available that even the AChE is uniformly distributed over the whole junctional fold membrane, it is clearly distributed over a much wider zone than the AChR.

Postjunctional specializations in the form of increased membrane densities can be seen after lead staining along the upper surface of the postjunctional membrane and dipping partly into the folds (*arrows*, Fig. 2 *inset*). In view of our results, the possibility must be entertained that these segments of electron-dense membrane represent the ACh receptive surface.

DISCUSSION

High-resolution ^{125}I electron microscope autoradiograms provide a clear demonstration that the AChR (α-bungarotoxin-binding sites) are not uniformly distributed throughout the depth of the junctional folds but are concentrated at the upper surface near the axonal membrane. The exact width of the labeled band still needs to be determined.

Salpeter and Eldefrawi (7) have recently estimated an average AChR density of 7×10^3 sites per μm^2 of postjunctional membrane for vertebrate endplates, based on previously published data on sites per whole endplate and on the assumption of a uniform distribution of receptor throughout the junctional folds. However, if the AChR sites are not uniformly distributed, the above estimates must be revised upward. In the sternomastoid endplate the area of the presynaptic axonal membrane is only about 1/6 that of the postjunctional fold membrane. The postsynaptic sarcolemma, which is parallel and apposed to the axonal membrane, has an even smaller surface area since it is interrupted by the secondary cleft, which follows the membrane invagination. However, the electron-dense membrane regions dip partly down into the folds and may constitute as much as $^1/_4$ the total surface area of the postjunctional membrane. If the receptor were restricted to these parts of the postjunctional membrane, the estimate for the sites per μm^2 could then be increased 4- to 6-fold and approach a monolayer of receptor, comparable to the 33,000 sites per μm^2 given by Bourgeois *et al.* (18) for *Electrophorus* electroplax. The nonuniform distribution of the receptor at the postjunctional membrane could provide the "regions of high receptor density" which Katz and Miledi (19, p. 572) suggest may be needed to account for the flat-topped miniature endplate potentials seen in their studies.

One may argue that the receptors are in reality distributed throughout the postjunctional membrane and that in our study the α-bungarotoxin did not penetrate to the depth of the folds. However since the incubation in ^{125}I-labeled α-bungarotoxin was terminated only when the muscle was no longer able to contract in response to nerve stimulation, we have to conclude that at least the receptors responsible for ACh-induced muscle contraction are located in the membrane adjacent to the axon.

Although many questions remain unanswered, we feel justified in publishing these initial findings at the present time, since they clearly challenge some widely held views regarding the distribution of the AChR, its relation to AChE, and the nature of the junctional folds at the vertebrate motor endplate.

We thank Maria Szabo and Mary Johnson for technical assistance, Len Fertuck for providing a computer program for the assessment of the HD values, and Peter Ravdin for the ^{125}I-labeled α-bungarotoxin. This work was supported by USPHS Grant no. NS 09315.

1. Zacks, S. I. (1964) *The Motor Endplate* (W. B. Saunders Co. Philadelphia).
2. Csillik, B. (1965) *Functional Structure of the Post-Synaptic Membrane in the Myoneural Junction* (Academiai Kiado, Publishing House of the Hungarian Academy of Sciences, Budapest).
3. Friedenberg, R. M. & Seligman, A. M. (1972) "Acetylcholinesterase at the myoneural junction: Cytochemical ultrastructure and some biochemical considerations," *J. Histochem. Cytochem.* **20**, 771–779.
4. Barnard, E. A., Wieckowski, J. & Chiu, T. H. (1971) "Cholinergic receptor molecules and cholinesterase molecules at mouse skeletal muscle junctions," *Nature* **234**, 207–209.
5. Chang, C. C. & Lee, C. Y. (1963) "Isolation of neurotoxins from the venom of *Bungarus multicinctus* and their modes of neuromuscular blocking action," *Arch. Int. Pharmacodyn.* **144**, 241–257.
6. Fambrough, D. M. & Hartzell, H. C. (1972) "Acetylcholine receptors: Number and distribution at neuromuscular junctions in rat diaphragm," *Science* **176**, 189–191.
7. Salpeter, M. M. & Eldefrawi, M. E. (1973) "Sizes of endplate compartments, densities of acetylcholine receptor and other quantitative aspects of neuromuscular transmission," *J. Histochem. Cytochem.* **21**, 769–778.
8. Porter, C. W., Chiu, T. H., Wieckowski, J. & Barnard, E. A. (1973) "Types and locations of cholinergic receptor-like molecules in muscle fibres," *Nature New Biol.* **241**, 3–7.
9. David, G. S. (1972) "Solid state lactoperoxidase: a highly stable enzyme for simple, gentle iodination of proteins," *Biochem. Biophys. Res. Commun.* **48**, 464–471.
10. Eldefrawi, M. E. & Fertuck, H. C., "A rapid method for the preparation of [^{125}I]α-bungarotoxin," *Anal. Biochem.*, in press.
11. Salpeter, M. M. & Bachmann, L. (1964) "Autoradiography with the electron microscope: A procedure for improving resolution, sensitivity and contrast," *J. Cell Biol.* **22**, 469–477.
12. Kayes, J., Maunsbach, A. B. & Ullbert, S. (1962) "Electron microscope autoradiography of radioiodine in the thyroid using the extranuclear electrons of ^{125}I," *J. Ultrstruct. Res.* **7**, 339–345.
13. Fertuck, H. C. & Salpeter, M. M. (1974) "Sensitivity in EM autoradiography, for ^{125}I," *J. Histochem. Cytochem.* **22**, 80–87.
14. Salpeter, M. M., Bachmann, L. & Salpeter, E. E. (1969) "Resolution in electron microscope radioautography," *J. Cell Biol.*, **41**, 1–20.
15. Salpeter, M. M. & Szabo, M. (1972) "Sensitivity in electron microscope autoradiography. I. Effect of radiation dose," *J. Histochem. Cytochem.* **20**, 425–434.
16. Salpeter, M. M. (1967) "Electron microscope radioautography as a quantitative tool in enzyme cytochemistry. I. The distribution of acetylcholinesterase at motor endplates of a vertebrate twitch muscle," *J. Cell Biol.* **32**, 379–389.
17. Salpeter, M. M., Plattner, H. & Rogers, A. W. (1972) "Quantitative assay of esterases in endplates of mouse diaphragm by electron microscope autoradiography," *J. Histochem. Cytochem.* **12**, 1059–1068.
18. Bourgeois, J. P., Ryter, A., Menez, A., Fromageot, P., Boquet, P. & Changeaux, J.-P. (1972) "Localization of the cholinergic receptor protein in *Electrophorus* electroplax by high resolution autoradiography," *FEBS Lett.* **25**, 127–133.
19. Katz, B. & Miledi, R. (1973) "The binding of acetylcholine to receptors and its removal from the synaptic cleft," *J. Physiol. (London)* **231**, 549–574.