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IMMUNOHISTOCHEMICAL LOCALIZATION OF THE ACETYLCHOLINE RECEPTOR IN THE MAMMALIAN NEUROMUSCULAR JUNCTION

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Abstract

Through the use of biotinylated α- bungarotoxin, the nicotinic acetylcholine receptor has been localized in mammalian motor end plates at light and electron microscopic levels. The receptor is located in the primary post-synaptic membrane of the neuromuscular junction. Neither the presynaptic membrane nor the secondary postsynaptic membrane, which is thrown into junctional folds, contains any traces of the nicotinic acetylcholine receptors. This localization is at variance with that of acetylcholinesterase, which is present in both primary and secondary postsynaptic and the presynaptic membranes. Consequently, subneural apparatuses visualized by α-bungarotoxin differ in fine details from those seen in acetylcholinesterase-stained specimens at both light and electron microscopic levels. Accordingly, theories suggesting the identity of acetylcholinesterase with the acetylcholine receptor cannot be sustained.

Key words: Acetylcholine receptor, nicotinic, neuromuscular junction, α-bungarotoxin, acetylcholinesterase, immunocytochemistry, electron microscopy

Introduction

Ever since LANGLEY's (1907) revolutionary hypothesis that a chemical substance mediates transmission of nerve impulses to the striated muscle, the acetylcholine receptor has played a central role in theories of chemical transmission. Recent molecular biological studies revealed that the nicotinic acetylcholine receptor, displaying a pentameral $2\alpha+\beta+\gamma+\delta$ structure (Fig. 1) which, as an allosteric protein (CHANGEUX, 1990), changes its shape after binding acetylcholine, has a funnel-like structure, the central channel of which the subserves the outward and inward motion of various cations (CHANGEUX et al., 1987).

The superfamily of ligand-gated ion channel neurotransmitter receptors includes GABA and glycine receptors in addition to the acetylcholine receptor (BARNARD, 1992). It is characteristic of this superfamily that the receptor is composed of five homologous subunits arranged around the central ion channel (UNWIN, 1993). Acetylcholine binding sites are located at specific subunit interfaces (SARGENT, 1993).

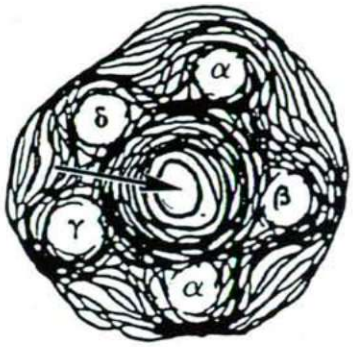

Fig. 1. Molecular structure of the nicotinic acetylcholine receptor. In a virtual cross-section, the central ion channel (arrow) is surrounded by five subunits (Greek letters) of the receptor. Adapted with permission from UNWIN (1989).

Immunohistochemical visualization of the acetylcholine receptor is a puzzling problem, partly because of the multitude of subunits which exhibit different immunogeneities, and partly because the century-old observation of denervation supersensitivity has been traditionally ascribed to the spread of acetylcholine receptors on the surface of the postsynaptic cell. With this dual aspect of the relevant problematics in mind, an attempt has been made to locate the nicotinic acetylcholine receptor in mammalian striated muscle, through the use of biotinylated α-bungarotoxin, at both light and the electron microscopic levels. The results of studies aiming to localize the acetylcholine receptor in denervated motor end plates will be published later.

Material and Methods

Investigations were performed on 22 young adult rats (200-250 g body weight) and on tissues from 2 young Macaca fascicularis monkeys, obtained by courtesy of the Section of Neurobiology, Yale University Medical School, New Haven, CT, USA. Care of the animals complied with the Albert Szent-Györgyi Medical University "Guidelines for Ethics in Animal Experiments". Animals in deep anesthesia were subjected to transcardial perfusion fixation with ZAMBONI's picric acid-formaldehyde solution containing 0.1% glutaraldehyde; this was preceded by a brief flush of 125 ml 0.1 M phosphate buffered saline, pH=7.4, at room temperature. After perfusion, the flexor digitorum brevis muscle was excised and post-fixed in glutaraldehyde-free ZAMBONI's solution for 24 hours. For light microscopic purposes, 30 µm thick frozen sections were obtained on a cryostat; for electron microscopic purposes, 50 µm thick vibratome sections were prepared. Both frozen and vibratome sections were incubated according to the free-floating technique, with biotinylated α-bungarotoxin (Molecular Probes, Inc., USA) as primary serum, which reacts with the α subunit of the nicotinic acetylcholine receptor (SCHOEPFER et al., 1990; CLARKE, 1992).

Further procedures of the immunohistochemical reaction were performed either according to the PAP method, or by the avidin-biotin technique, with the ABC kit of Vector Laboratories. The specificity of the antiserum was ascertained by incubation of control sections in antiserum-free normal goat serum or by omitting one of the components of the ABC kit. In none of the control experiments was any reaction encountered.

Incubation of the free-floating Vibratome or cryostat sections was carried out under constant movement in glass vials, either in a Pelco rotator or on a horizontal shaker.The immunohistochemical reaction

was visualized with diaminobenzidine (DAB) + hydrogen peroxide, or with the nickel-DAB technique. Dehydration of the sections in an ascending series of alcohols and processing in xylenes was performed on sections dried to gelatine - coated ("subbed") slides. Sections were coverslipped with Permount.

Vibratome sections subjected to the immunohistochemical reaction were used for electron microscopic purposes as follows: after osmication and dehydration, sections were applied to slides pretreated with Liquid Release and flat-embedded in Araldite ACM. Relevant parts of the sections were excised under the light microscope and applied to prepolymerized Epon blocks. Ultrathin sections, 30 nm thick, were obtained on an LKB ultrotome using diamond knifes, stained with uranyl acetate and lead citrate and studied on a JEOL JEM 1010 transmission electron microscope.

Acetylcholinesterase was visualized at the light microscopic level, by means of GEREBTZOFF'S (1959) modification of the KOELLE (1950) acetylthiocholine iodide method, employing ethopropazine inhibition (10-6 to 10-8 M) in order to inactivate non-specific esterases and butyryl(pseudo)cholinesterase. Electron microscopic demonstration of acetylcholinesterase was performed by the copper-uranyl-thiocholine method described by CSILLIK and KNYIHÁR (1968).

Results

Under the light microscope, biotinylated α-bungarotoxin visualizes structures identical with COUTEAUX's (1947, 1955) subneural apparatus (Fig. 2). In this respect, there was no difference between rat and monkey muscles; however, the latter were conspicuously larger than the former, most probably because of the differences in the sizes of the related striated muscle fibers innervated by the neuromuscular junctions. However, in contrast with the patterns obtained with acetylcholinesterase staining techniques (CSILLIK, 1967), which are invariably characterized by the spiny appearance of the borderlines of the acetylcholinesterase-positive gutters (Fig. 6a), the outlines of the subneural apparatuses visualized with α-bungarotoxin (Fig. 4) are completely smooth. In other words, COUTEAUX's "organites", which have been shown (CSILLIK et al., 1966) to be light microscopic equivalents of the junctional folds, failed to contain the nicotinic acetylcholine receptor. In cross-sections, especially in the large motor end plates of primates (Fig 3), the acetylcholine receptor outlines the gutter underlying the motor nerve terminal.

At the level of electron microscopy, α-bungarotoxin outlined the primary postsynaptic membrane of the neuromuscular junction (Fig. 5). Earlier studies (CSILLIK, 1993) revealed that the primary postsynaptic membrane is identical with the crests of the junctional folds. In contrast, the secondary postsynaptic membrane, i.e. the junctional folds themselves, does not contain any acetylcholine receptor. The transition between the intensely reacting primary postsynaptic membrane and the immunoreaction-free secondary postsynaptic membrane is clearly demarcated. Neither did the presynaptic membrane exert any acetylcholine receptor immunoreactivity.

In light microscopic specimens, the acetylcholinesterase reaction outlines the organites of subneural apparatuses (Fig. 6a). Accordingly, neuromuscular junctions "stained" by the acetylcholinesterase technique exhibit a "spiny" or "thorny" appearance, which differs markedly from the smooth outlines of those "stained" for the acetylcholine receptor. At the level of electron microscopy (Fig. 6b), the end-product of the acetylcholinesterase reaction outlines both the pre- and the postsynaptic membranes, including the entire extents of the junctional folds.

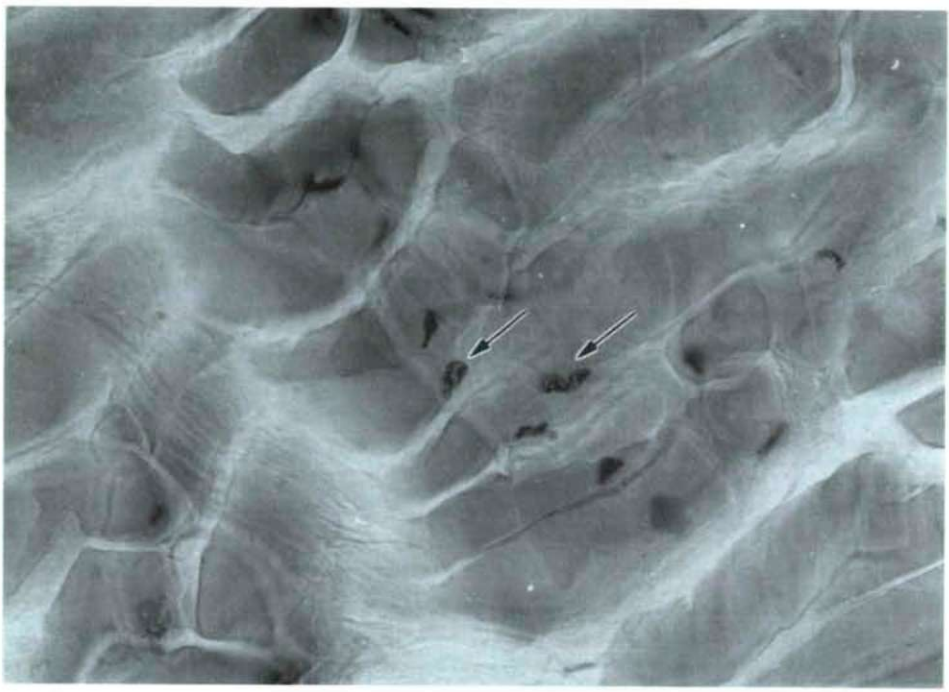

Fig. 2. Localization of the nicotinic acetylcholine receptor in the flexor digitorum brevis muscle of the rat. Arrows indicate some of the motor end plates displaying acetylcholine receptor immunoreactivity. × 100.

Discussion

Structural analysis of cholinergic impulse transmission was initiated by the histochemical demonstration of the enzyme hydrolyzing acetylcholine, i.e. acetylcholinesterase (KOELLE and FRIENDENWALD, 1949; KOELLE, 1950). Due to its evasively small size and diffusibility, the transmitter itself could not be detected with histochemical techniques (CSILLIK, 1975), except for the indirect evidence provided by the radioautographic localization of hemicholinium (CSILLIK et al., 1970; KNYIHÁR and CSILLIK, 1970). Before the advent of immunohistochemical techniques, localization of the enzyme synthesizing acetylcholine, i.e. choline acetyltransferase, and that of the protein binding acetylcholine, i.e. the acetylcholine receptor, were tantalizing questions of neurohistochemistry. Whereas the localization of choline acetyltransferase, at both light and electron microscopic levels, could be achieved flawlessly by using mono- and polyclonal immune sera directed against this enzyme protein (LEVEY et al., 1982; MESULAM et al., 1983), the fine structural identification of the nicotinic acetylcholine receptor remained a partially unaccomplished problem.

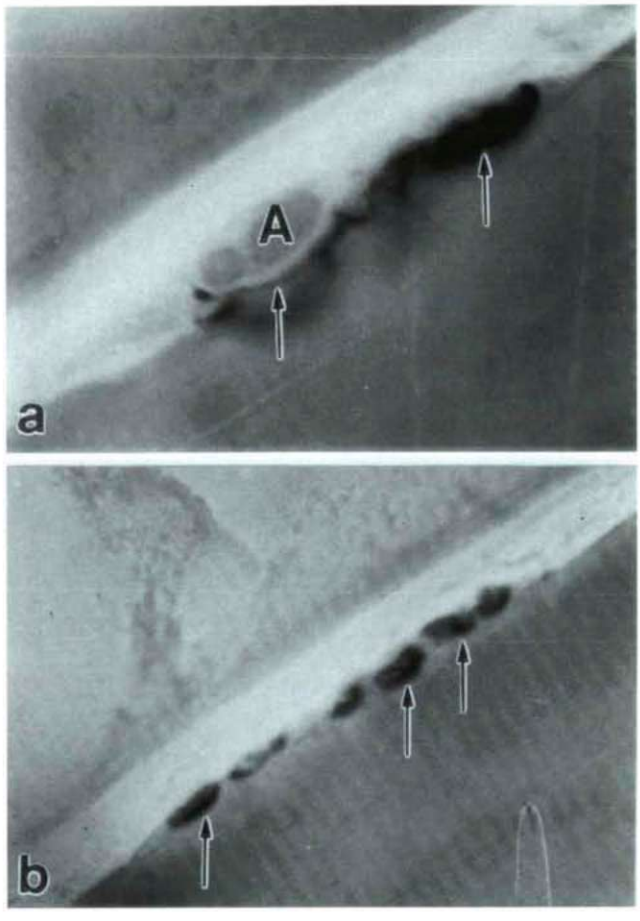

Fig. 3. Cross-sections of the subneural apparatus in the monkey flexor digitorum muscle. Note that the barely visible axon terminals (A) do not exert any acetylcholine receptor immunoreactivity, whereas the postsynaptic membranes which appear as cross-sections of gutters (arrows) contain high amounts of the acetylcholine receptor. × 2500

It is known that α-bungarotoxin blocks neurotransmission at the neuromuscular junction in a "remarkably potent, specific and persistent manner" (CLARKE, 1992). Binding of α-bungarotoxin to the α subunit of the nicotinic acetylcholine receptor in the neuromuscular junction (and to α7 in the central nervous system) is well established (SCHOEPFER et al., 1990; CLARKE, 1992; SARGENT, 1993). According to LEE, who first purified α-bungarotoxin from elapid snake venom (CHANG and LEE, 1963) and later characterized it (LEE et al., 1967; LEE, 1972), the toxin has a high affinity and specificity for the acetylcholine receptor in skeletal muscle. Binding of α-bungarotoxin to the acetylcholine receptor is virtually irreversible (MILEDI and POTTER, 1971).

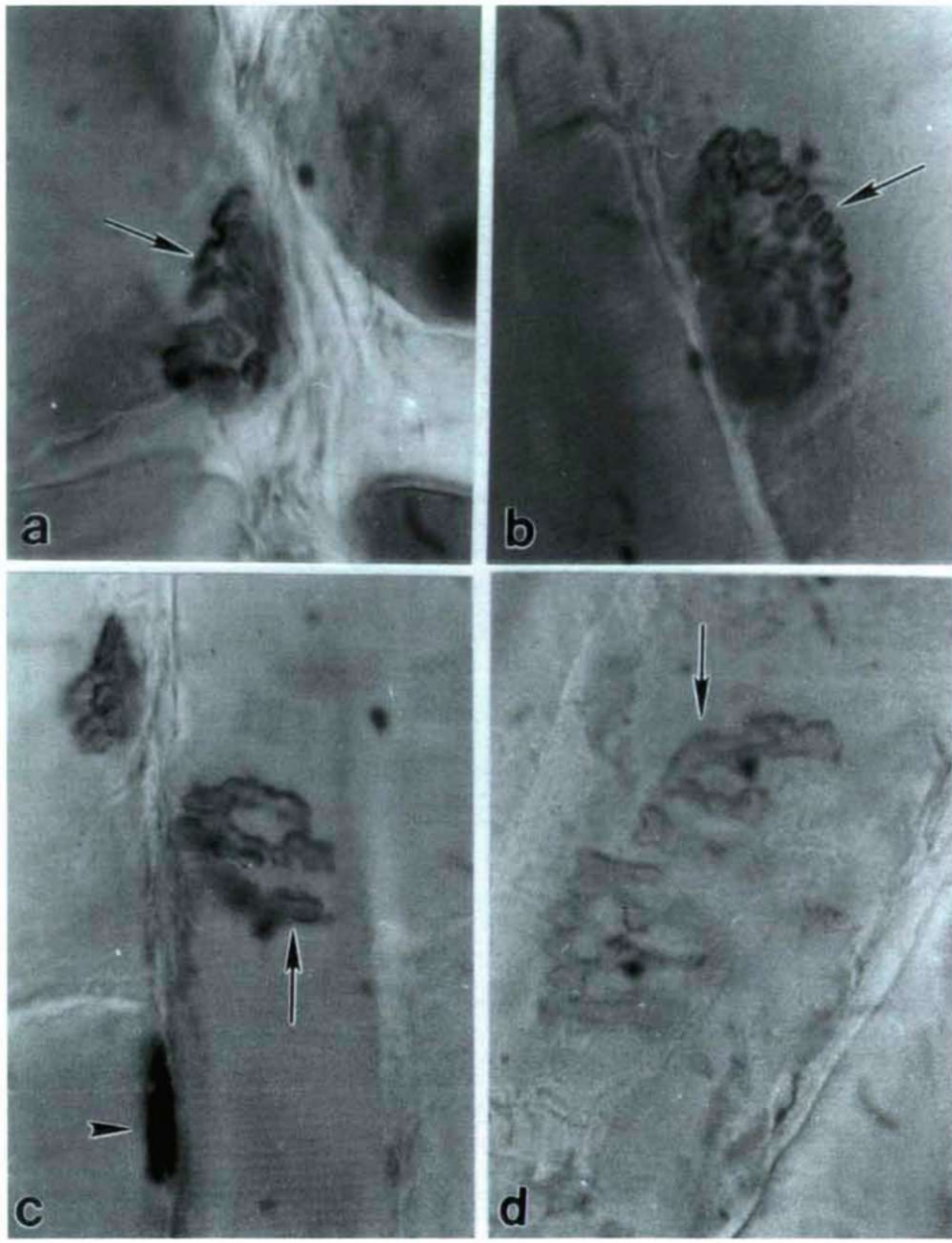

Fig. 4. Localization of the nicotinic acetylcholine receptor in neuromuscular junctions of the flexor digitorum brevis muscle of the rat (arrows). Note the smooth outlines of the subneural apparatuses which display receptor immunoreactivity. Arrowhead in 4c indicates cross-section of a subneural apparatus. Note absence of any reaction of the organites, especially conspicuous in 4d, which depicts a subneural apparatus exerting modest acetylcholine receptor immunoreactivity. × 1000

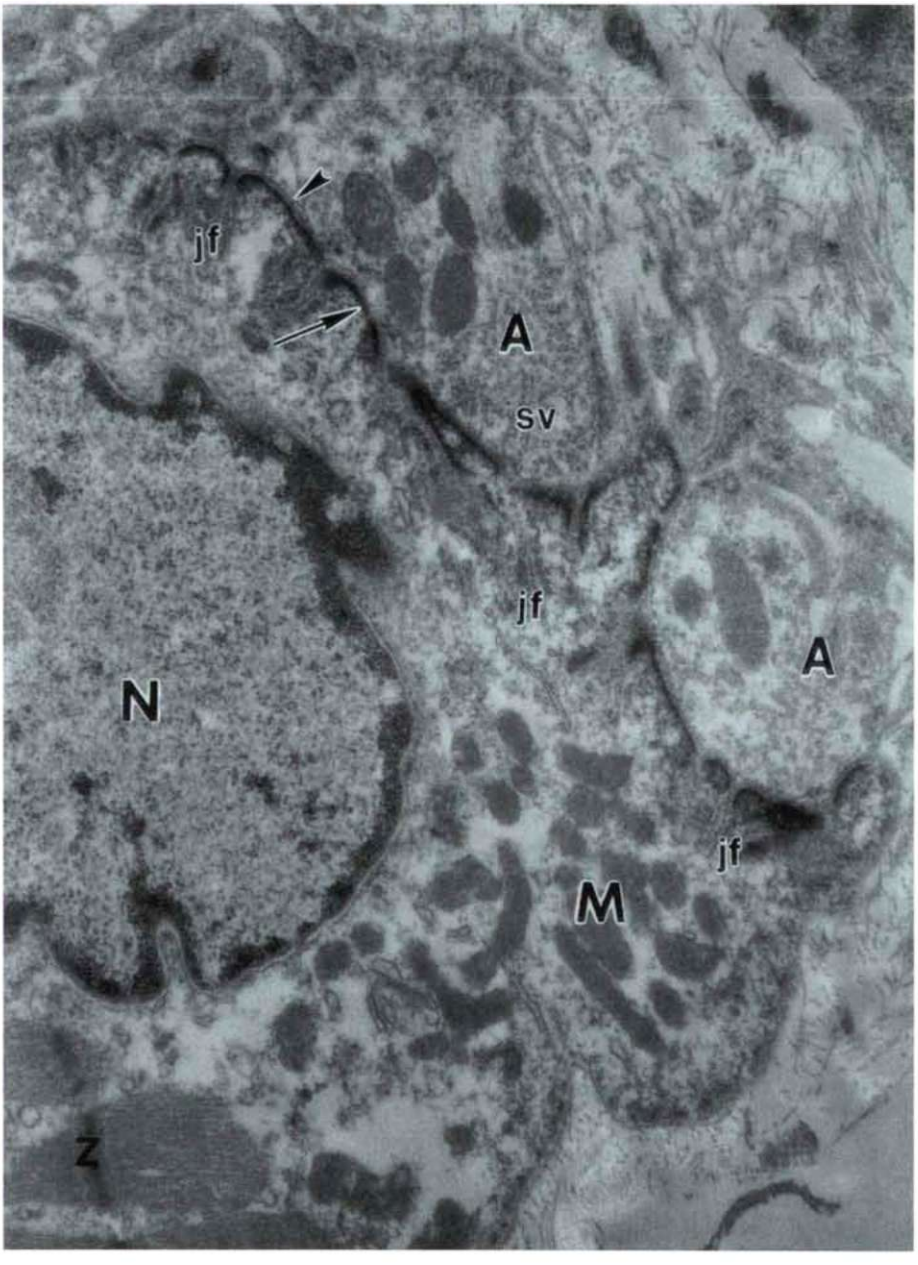

Fig. 5. Localization of the acetylcholine receptor at the electron microscopic level. Flexor digitorum brevis muscle of Macaca fascicularis. Note immunoreactivity of the primary postsynaptic membrane (arrows). Neither the presynaptic membrane (arrowhead) nor the secondary postsynaptic membrane, thrown into junctional folds (JF), exhibits any receptor immunoreactivity. A: cross-section of the axon terminal; sv: synaptic vesicles; N: fundamental nucleus; M: accumulation of siderophilic fundamental mitochondria; Z: Z-line of striated muscle fiber. × 25,000

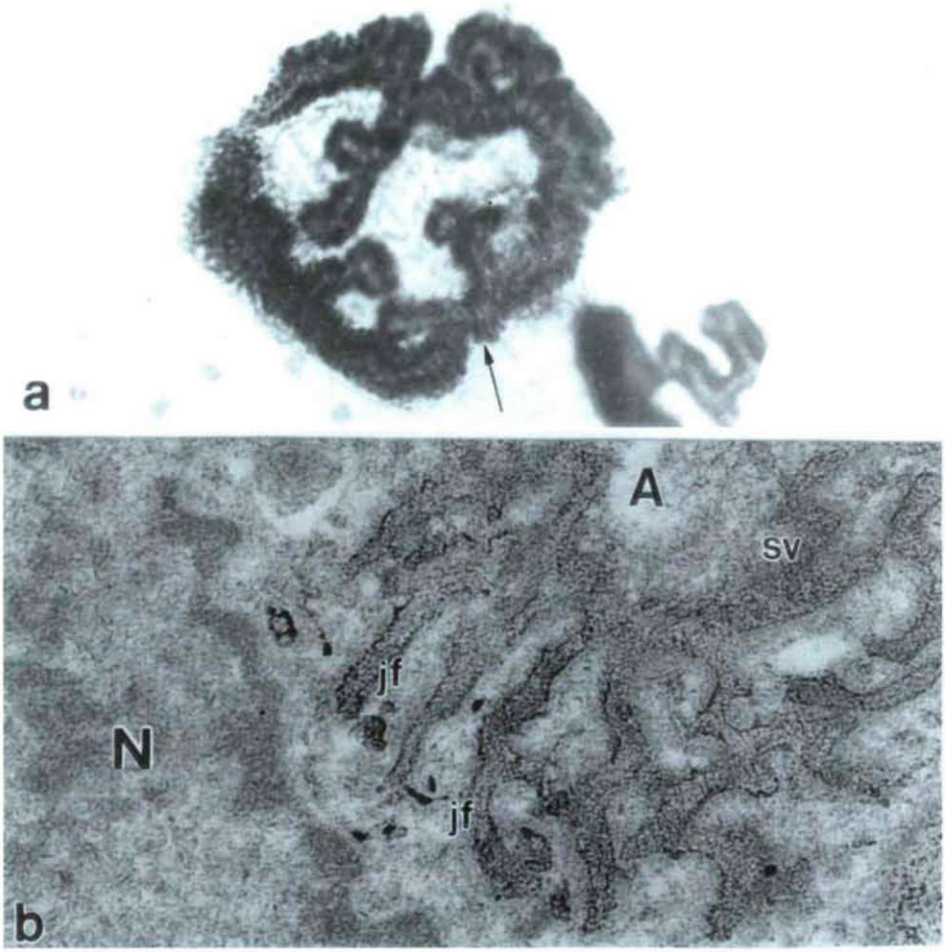

Fig. 6. Acetylcholinesterase activity of motor end plates in the rat gastrocnemius muscle. (a): Spiny appearence of the outlines (arrow)) of the acetylcholinesterase-active subneural apparatus; this is due to the acetylcholinesterase reaction of the organites (light microscopic equivalents of junctional folds). x 1000. (b): Electron microscopic localization of acetylcholinesterase activity in the neuromuscular junction. Note that both the pre- and postsynaptic membranes, including the secondary postsynaptic membrane of the junctional folds (jf), display acetylcholinesterase activity. The synaptic cleft contains traces of the end-product of the acetylcholinesterase reaction. A: cross-section of the terminal axon; sv: synaptic vesicles. N: fundamental nucleus. × 35,000

On the basis of this principle, localization of the nicotinic acetylcholine receptor has been attempted with radioautographic methods, by using 125I-labeled α-bungarotoxin at both light and electron microscopic levels (LEE et al., 1967; HARTZELL and FAMBROUGH, 1972; VOGEL et al., 1972; FISCHBACH and COHEN, 1973; PORTER et

al., 1973; ALBUQUERQUE et al., 1974; FERTUCK and SALPETER, 1974; PORTER and BARNARD, 1975; FERTUCK and SALPETER, 1976). Conjugates of α-bungarotoxin with fluorescent dyes have also been used to locate the acetylcholine receptor of the neuromuscular junction (ANDERSON and COHEN, 1974). Ferritin-labeled α-bungarotoxin was used for electron microscopy (HOURANI et al., 1974) and horseradish peroxidase conjugates of α-bungarotoxin were employed at both light and electron microscopic levels (DANIELS and VOGEL, 1975; JENSEN et al., 1975; LENTZ et al., 1977). The use of biotinylated α-bungarotoxin (AXELROD, 1980) is a relatively novel approach for location of the nicotinic acetylcholine receptor at light and electron microscopic levels (MA et al., 1993; KNYIHÁR-CSILLIK et al., 1995; CSILLIK et al., 1995a, b; 1996).

The present studies indicated that neither the presynaptic nor the secondary postsynaptic membrane, i.e. the junctional folds themselves, contains any nicotinic acetylcholine receptor. In other words, the acetylcholine receptor in the neuromuscular junctions of adult mammals is confined to the the primary postsynaptic membrane. This localization is at striking variance with that of acetylcholinesterase, which is present in both pre- and postsynaptic membranes of the neuromuscular junction, including the depths of the junctional folds. In fact, such an organization is optimal for the role of the acetylcholine molecule in neuromuscular impulse transmission: once released from the axon terminal, it passes the synaptic gap and binds to the acetylcholine receptor in the postsynaptic membrane. Excess acetylcholine, not bound to the receptor, will be effectively hydrolyzed by the enzyme in the synaptic cleft, in the postsynaptic membrane and, if any transmitter occurs in excess amount, in the depths of the junctional folds.

Differential localization of the acetylcholine receptor and the enzyme acetylcholinesterase does not support the theories regarding the identity of this receptor and enzyme (ZUPANCIC, 1967; STALC and ZUPANCIC, 1972). A membrane mosaic consisting of acetylcholinesterase and the nicotinic acetylcholine receptor, as suggested by BARNARD et al., (1971), is plausible in the primary postsynaptic membrane; however, in the case of the junctional folds, i.e. in the secondary postsynaptic membrane, only acetylcholinesterase molecules are present, which excludes any membrane mosaic at this location.

Finally, the question of the presence of acetylcholinesterase and the absence of the nicotinic acetylcholine receptor in the presynaptic membrane (and in the synaptic cleft) should be addressed. The immunohistochemical technique employed here is apparently more specific than the horseradish-labeled compounds used by earlier investigators; LENTZ et al. (1977) for instance, also observed a "faint" reaction presynaptically. In all probability, this was a technical artifact, perhaps due to the substantivity of horseradish peroxidase. On the other hand, the presence of muscarinic acetylcholine receptors on the presynaptic membrane is a viable possibility, which has been suggested by recent pharmacological studies (VIZI and SOMOGYI, 1989).

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