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Acetylcholine Receptors

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Acetylcholine receptors

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α-Bungarotoxin is one of a class of proteins, isolated from snake venoms, which antagonize the action of acetylcholine at vertebrate neuromuscular junctions and 'electroplaques' of electric fish. α-Bungarotoxin blocks acetylcholine action irreversibly and may be labelled with either ^{125}I or ^{3}H. This irreversible binding is used as the basis of an *in vitro* assay for acetylcholine receptors, whether in intact tissue, membrane fragments or solubilized preparations. Acetylcholine receptors from *Torpedo* and denervated skeletal muscle have been solubilized and substantially purified using affinity chromatography. The distribution of acetylcholine receptors in several tissues has been determined, and an auto-immune response, induced by injection of purified *Torpedo* receptors, has been studied.

Introduction

The neuromuscular junctions of vertebrate skeletal muscle and the closely related electroplaques in the electric organs of certain fish, *Torpedo*, *Narcine* and *Electrophorus*, are chemical synapses which operate by the release of acetylcholine from the pre-synaptic nerve terminal. After its release, the acetylcholine diffuses across the synaptic gap and acts on a component of the post-synaptic membrane, the so-called acetylcholine receptor. The combination of acetylcholine with the receptor opens a membrane gate for about 1 ms (Katz & Miledi 1972) and during this time certain cations, mostly sodium, move inwards, causing a depolarization of the muscle membrane. Actylcholine is then destroyed by the enzyme acetylcholinesterase. For some years, this enzyme was the best characterized chemical component of these synapses, but more recently, progress has been made on the characterization of the aceylcholine receptor. The following is a brief and necessarily incomplete account of some work in this area.

Identification of receptors

The method now widely employed to recognize acetylcholine receptors in tissue, both intact and fragmented, as well as in solubilized preparations, is that which employs the irreversible, or in some cases slowly reversible, binding of particular constituents of certain snake venoms. The earliest to be used was the so-called α-fraction of *Bungarus multicinctus* venom, α-bungarotoxin (Chang & Lee 1963; Lee, Tseng & Chiu 1967; Changeux, Kasai & Lee 1970; Miledi, Molinoff & Potter 1971; Raftery, Schmidt, Clark & Wolcott 1971) but this has been followed by the extensive use of similar fractions from a variety of other snake venoms. They are all proteins between 59 and 74 amino acid residues in length with several disulphide cross bridges. Their molecular masses vary between about 6400 and 8000 and they possess certain invariant regions whose role in their toxicity has yet to be defined.

As circumstantial evidence for considering α-bungarotoxin to be a specific acetylcholine antagonist, the following points should be mentioned.

(*a*) α-Bungarotoxin blocks neuromuscular transmission, abolishing the action of acetylcholine on the post-synaptic membrane. Its irreversible binding to intact tissue is largely restricted to the end-plate in normally innervated muscle and there is a parallel between acetylcholine sensitivity and toxin binding in denervated muscle. Its binding is slowed by curare and acetylcholine, although protection by the latter is a mixture of competitive and desensitization effects (Chang & Lee 1963; Miledi & Potter 1971).

(*b*) There is the recent evidence of Katz & Miledi (1973) concerning the effect of α-bungarotoxin on the elementary ionic channel opened by acetylcholine. α-Bungarotoxin acted in a manner similar to curare by reducing the population of vacant receptors without altering the properties of those remaining unoccupied.

(*c*) Material can be isolated in some degree of purity which binds both acetylcholine and α-bungarotoxin in addition to a range of agonists and antagonists (Eldefrawi & Eldefrawi 1973; Moody, Schmidt & Raftery 1973).

On the basis of this evidence, toxin binding sites and nicotinic acetylcholine receptors are usually considered as identical, but there are, nevertheless, toxin binding sites which apparently have no affinity for acetylcholine (see p. 556).

In order to utilize these toxins it is necessary to label them radioactively and the methods so far employed fall into one of three categories:

(i) Iodination using either ^{125}I or ^{131}I (Lee *et al.* 1967; Miledi *et al.* 1971; Berg *et al.* 1972; Clark *et al.* 1972; Fambrough & Hartzell 1972; Vogel, Sytkowski & Nirenberg 1972).

(ii) Tritiation by catalytic dehalogenation of iodinated derivatives in the presence of tritium gas and a catalyst (Menez *et al.* 1971).

(iii) Modification of surface groups on the toxin molecule; for example acetylation of amino groups using tritiated acetic anhydride (Barnard, Wieckowski & Chiu 1971; Chang, Chen & Chuang 1973).

We use the first two methods, employing α-bungarotoxin labelled with either ^{125}I or ^{3}H.

One particular point which deserves mention at this stage is the effect of chemical modification of the toxin on its biological activity. For example, progressive acetylation of amino groups at first reduces and then almost completely abolishes the toxicity of α-bungarotoxin (Chang *et al.* 1973). Iodination leads to progressive incorporation of two iodine atoms and these mono- and di-iodinated products may be separated by ion exchange chromatography (Vogel *et al.* 1972). They bind to receptors more slowly than native α-bungarotoxin and since this property is independent of the means of iodination (Green, Miledi, Perez & Vincent, unpublished observations), it most probably reflects a specific alteration rather than non-specific damage.

Isolation of receptors from *Torpedo marmorata*

The electric organs of the elasmobranch, *Torpedo*, are composed of stacks of hexagonal cells called electroplaques, each of which is several millimetres in diameter, but only 5–10 μm thick. The ventral surface of each cell is known to be richly innervated by cholinergic nerve terminals (Feldberg & Fessard 1942; Sheridan 1965; Grundfest 1967; Israël & Gautron 1969).

Miniature 'electroplaque' potentials can be recorded which are blocked reversibly by curare and irreversibly by α-bungarotoxin (Miledi *et al.* 1971).

In the isolation of receptors, membrane fragments are prepared as a first step and subsequently solubilized by detergent. Several detergents have successfully been used to solubilize toxin binding activity, and one widely employed is the non-ionic detergent, Triton X-100.

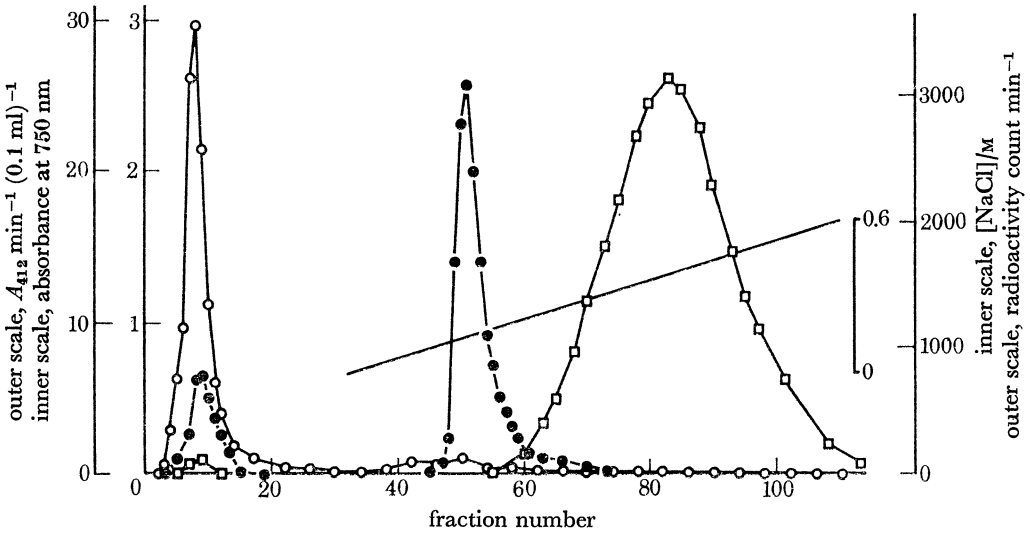

FIGURE 1. The purification of acetylcholine receptors from *Torpedo marmorata* by affinity chromatography on a curare–Sepharose 4B column (0.7 μmol/ml of packed gel). The crude Triton X-100 extract of *Torpedo* membranes (protein concentration about 1 mg/ml) in 0.02 mol/l sodium phosphate buffer, containing 0.1 % Triton X-100 (mass/vol), pH 7.4, applied to a 20 cm × 2.5 cm column and, after washing with starting buffer, eluted with a linear gradient of 0–0.6 mol/l NaCl in the same buffer. Yield of purified receptor was 50–60 %. ●, Esterase; ○, protein; □, α-bungarotoxin binding activity.

TABLE 1.

Torpedo	protein yield/mg / mass of tissue/g	toxin binding activity/nmol / protein/g	recovery of binding activity (%)
initial homogenate	23.5	42	—
membrane fragments	5.6	170	—
detergent extract	1.88	425	85
purified receptor	0.05–0.06	7500	40–50

The recovery of binding activity is related to that in membrane fragments.

The most general approach to the purification of receptors from the solubilized extracts has been by the use of affinity chromatography. In this technique, a material having a known affinity for the receptor is covalently attached to an insoluble support, usually a cross-linked agarose derivative. The materials which have been so attached are agonists and antagonists of acetylcholine action and include curare, reversible toxins similar to α-bungarotoxin, and quaternary ammonium compounds (Olsen, Meunier & Changeux 1972; Karlsson, Heilbronn & Widlund 1972; Schmidt & Raftery 1972; Biesecker 1973; Karlin & Cowburn 1973; Klett *et al.* 1973; Patrick & Lindstrom 1973; Eldefrawi & Eldefrawi 1973; Green *et al.* unpublished). A typical elution profile obtained from a curare column is shown in figure 1.

The purification achieved at each stage is shown in table 1. Each gram of tissue contains,

upon disruption, about 25 mg of protein, 1 nmol of toxin binding activity and about 60 μg of esterase. These figures are approximate, for the number of cells remains more or less the same irrespective of the size of the organ, and this leads in larger fish to a smaller surface to volume ratio with a concomitant decline in the yields per gram of tissue. The membranes themselves contain between 5 and 7 mg of protein. Solubilization in the presence of Triton X-100 produces an extract which contains about 2 mg of protein per gram of tissue and about 85 % of the toxin binding activity.

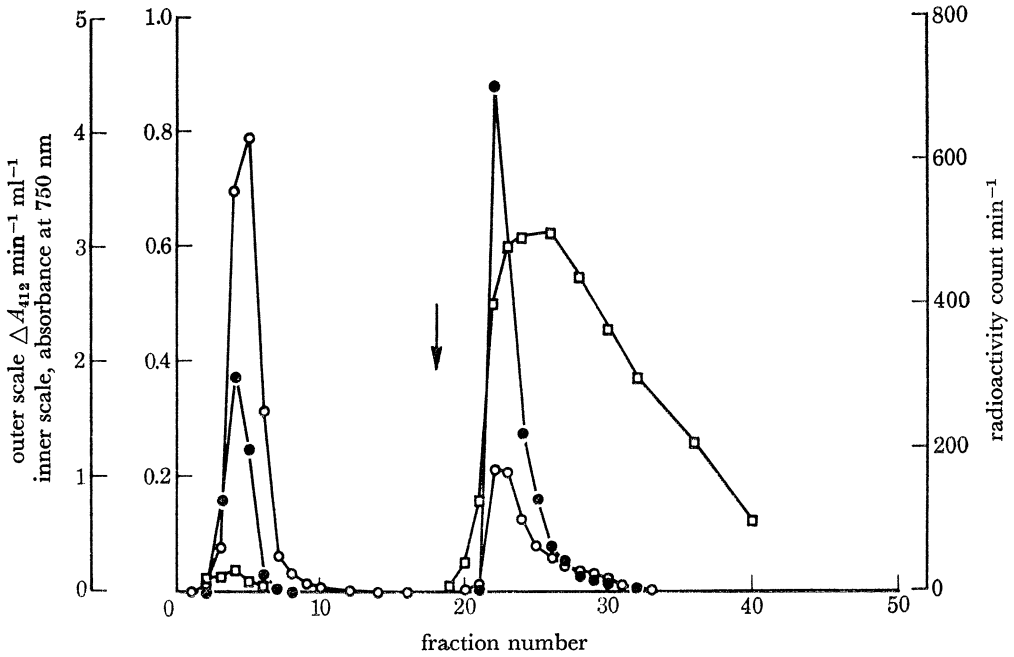

FIGURE 2. The behaviour of a crude Triton X-100 extract of *Torpedo marmorata* membranes on a Concanavalin A–Sepharose 4B column. The extract (as in figure 1) applied to a 10 cm × 0.9 cm column and eluted, after washing with starting buffer, with a 2 % solution of α-methyl-D-mannoside in the same buffer, starting at the arrow. ●, Esterase; ○, protein; □, α-bungarotoxin binding activity.

The final activity of receptor preparations is usually expressed in terms of moles of toxin binding activity per gram of protein, standardized against bovine serum albumin using the Lowry method. While not an absolute standard, it provides a convenient method for the comparison of specific activities. The highest obtained from *Torpedo*, *Narcine* and *Electrophorus* is generally in the region of 6.5–7.5 μmol/g, and this corresponds to an uncorrected molecular mass for the protein of about 130 000–150 000 per toxin binding site (Olsen *et al.* 1972; Eldefrawi & Eldefrawi 1973; Klett *et al.* 1973; Patrick, Lindstrom, Culp & McMillan 1973; Schmidt & Raftery 1973; Green *et al.*, unpublished). If this specific activity is taken as a working base, then each gram of tissue contains about 0.14 mg of receptor protein and a purification of 20-fold is required from the solubilized extract. In practice, the yields are always considerably less than 100 % at this last stage and final yields in the best preparations are about 0.05–0.06 mg per gram of tissue. *Electrophorus* contains 20 to 30-fold fewer receptors per gram of tissue than *Torpedo* or *Narcine*, and the purification needed at this last stage is correspondingly greater.

The toxin binding material is a glycoprotein and the result of an experiment demonstrating this is shown in figure 2. A crude extract of *Torpedo* has been passed over concanavalin A

covalently attached to an insoluble support. About 25 % of the protein, 75 % of the esterase and essentially all the toxin binding activity is retained initially and eluted subsequently with a 2 % solution of α-methyl-D-mannoside. The receptor constitutes approximately 20 % of the recoverable glycoprotein retained by the concanavalin A.

Isolation of receptors from skeletal muscle

In a muscle fibre whose length may be 20–30 mm, the end-plate, being of the order of 50 μm in diameter in the rat, occupies a very small part of the total surface. In normally innervated mammalian muscle fibres, acetylcholine receptors are almost wholly restricted to this end-plate region (Miledi 1960*b*). However, it is known that after denervation acetylcholine receptors appear along the entire length of the muscle fibres (Axelsson & Thesleff 1959; Miledi 1960*a*) and over a period of 2–3 weeks their total number increases about 20-fold (Miledi & Potter 1971). This increase can be utilized in the purification of receptors from skeletal muscle.

Table 2.

rat skeletal muscle	protein yield/mg / mass of tissue/g	toxin binding activity/pmol / protein/g	recovery of binding activity (%)
intact tissue	123.5	24	–
membrane fragments	62.1	41	85
detergent extract	15.7	109	57
purified receptor	—	not less than 3×10^5	10–30

The recovery of binding activity is related to that in intact muscle.

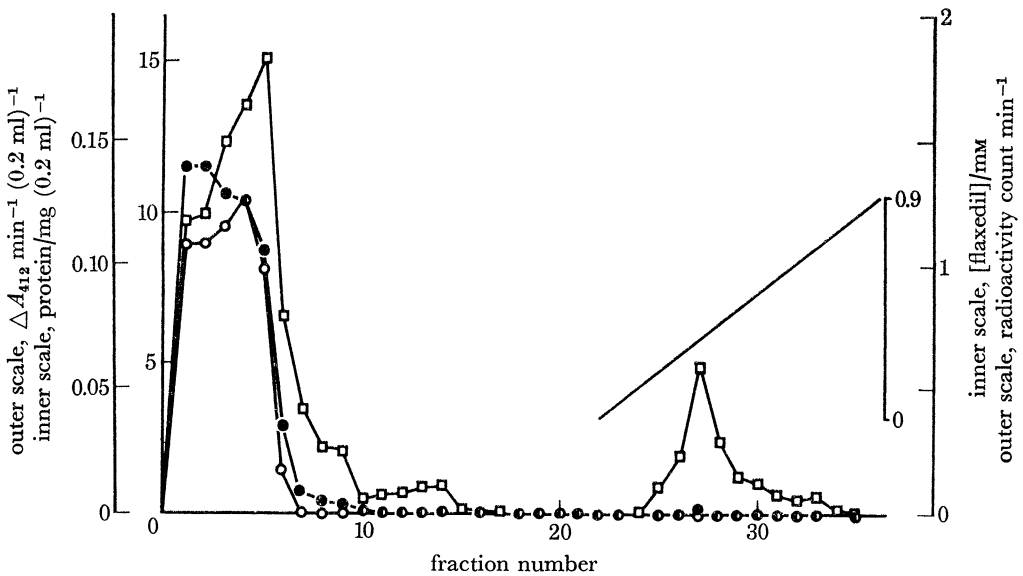

Figure 3. The purification of acetylcholine receptors from denervated skeletal muscle by affinity chromatography on a curare-Sepharose 4B column (0.7 μmol/ml packed gel). The crude Triton X-100 extract of rat skeletal muscle membranes (protein concentration about 16 mg/ml) in 0.1 mol/l tris HCl and 0.1 mol/l sodium chloride, containing 1 % Triton X-100 (by vol.), pH 8.0, applied to a 20 cm × 0.3 cm column and the effluent recycled once. After washing with starting buffer, the column eluted with a linear gradient of 0–0.9 mmol/l flaxedil in the same buffer solution. The yield of receptor from the gradient was about 13 %. ●, Esterase; ○, protein; □, α-bungarotoxin binding activity.

In a typical experiment a crude membrane fraction is prepared from the denervated diaphragms of 5–10 rats. This contains almost all the toxin binding activity but also a substantial quantity of the original protein. This crude preparation is solubilized with Triton X-100 and purified using a curare affinity column. The purification achieved at each stage is shown in table 2. The number of receptors per gram of whole tissue is difficult to state, but the best yield obtained so far is about 30–50 pmol; that is, 20 to 30-fold less than *Torpedo* and of the same order as in *Electrophorus*. In figure 3 is shown the affinity chromatography of these receptors on a curare affinity column. In contrast to *Torpedo*, a large proportion of the toxin binding activity is not retained by such a column, for reasons which are not yet clear, but which may be due to a heterogeneity of the receptor. Even so, there is some material retained which is eluted by the cholinergic antagonist flaxedil. Acetylcholine has also been used as an eluting agent. The specific activity of this material has been estimated at not less than 300 nmol of toxin binding activity per gram of protein, but this is a lower limit and it could be considerably greater.

Estimate of acetylcholine receptors in muscle fibres

The ability of radioactively labelled α-bungarotoxin to combine, for practical purposes, irreversibly and specifically with acetylcholine receptors gives one an opportunity to study their number, distribution and behaviour in intact tissues (Miledi & Potter 1971). In early experiments, a muscle, previously incubated with ^{131}I-α-bungarotoxin, was solubilized and radioactivity estimated by liquid scintillation spectrometry. Subsequent non-destructive estimates were undertaken with ^{125}I-α-bungarotoxin, and radioactivity was now measured by γ-ray spectrometry.

We have studied the binding of ^{125}I-α-bungarotoxin to several types of frog, rat and rabbit muscle, and over the last year or so have undertaken similar studies on human muscle with Dr J. Newsom-Davis of the Royal Free Hospital. This is of interest because it was shown by Elmqvist, Hofmann, Kugelberg & Quastel (1964) that in myasthenia gravis, a disease that is accompanied by muscular weakness, the amplitude of the miniature end-plate potentials was reduced. It was thought that this reduction in size was due to a lower content of acetylcholine in the package released from the nerve terminals. However, the possibility that a post-synaptic change in acetylcholine sensitivity is involved has not been fully excluded. In human intercostal muscle from non-myasthenic patients, there are about 1.8×10^7 sites per end-plate. By contrast, in myasthenic patients the number is down by between a half and a third. Similar results have been obtained recently by Fambrough, Drachman & Satyamurti (1973).

In all muscles examined, α-bungarotoxin was bound mainly at the end-plate region. For example, figure 4*a* shows the distribution of binding along a bundle of muscle fibres from the rat diaphragm. In this case, non-end-plate segments had, on average, about 1% of the peak segment and the total amount of toxin bound to the extra-junctional regions was about 14%. This extra-junctional binding together with that to tendons, was rather variable within a given species, and was generally greater in human muscle. The nature of this 'non-specific' irreversible binding is unknown and under investigation at the present time. However, it is not antagonized by D-tubocurarine or acetylcholine. In figure 4*b* is shown the distribution of α-bungarotoxin binding along a bundle of fibres from a frog sartorius muscle. The fibres in this muscle have two

or more end-plates (Katz & Kuffler 1941; Miledi 1960*a*) and this is reflected in the multiple peaks.

If we disregard the mass of individual animals, then each rat diaphragm end-plate has an average value of about 3.5×10^7 toxin binding sites. This compares with 3.4×10^7 for rabbit diaphragm, 1.8×10^7 for human intercostal and 1.3×10^8 for frog sartorius end-plates. Similar values have been obtained by others (Miledi & Potter 1971; Porter, Barnard & Chiu 1973; Porter, Chiu, Wieckowski & Barnard 1973; Hartzell & Fambrough 1972; Fambrough *et al.* 1973). From these numbers, and estimates of the synaptic membrane area, it appears that acetylcholine receptors in the various end-plates examined occur at an average density of the order of $10^4/\mu m^2$ (cf. Salpeter & Edelfrawi 1973). In considering the location and density of acetylcholine receptors in the muscle membrane, some preliminary electron microscopic observations may be of interest. Several years ago, it was noticed (Miledi, unpublished) that in normal or denervated frog and mammalian muscles, fixed in either osmium tetroxide or glutaraldehyde, the external part of the synaptic membrane possessed a palisade structure,

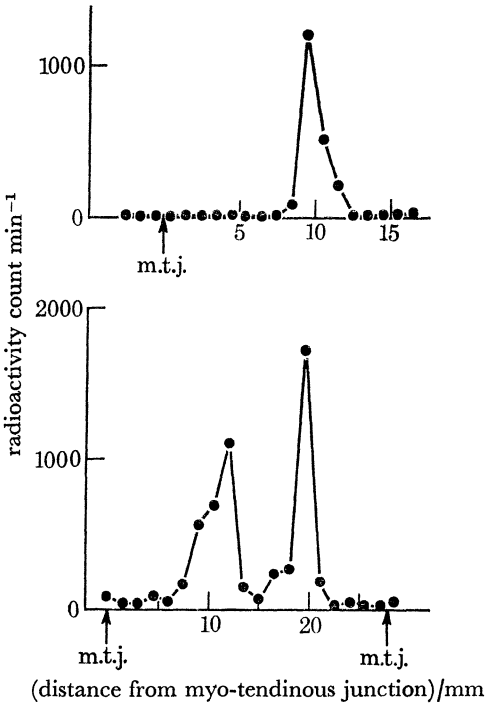

FIGURE 4. The distribution of [^{125}I]bungarotoxin along (*a*) a strip of rat hemi-diaphragm and (*b*) a frog sartorius muscle.

the result of small projections rising from the muscle membrane and extending towards the basement membrane. Similar, but somewhat larger, projections have been seen in earthworm muscle (Rosenbluth 1972). The projections are approximately 7 nm long and consist of a small rodlet surmounted by a globular head. The spacing between rodlets is 4–7 nm, but a more precise determination must await a detailed examination of frontal views of the membrane. The palisade is seen on the muscle membrane immediately below the axon but may sometimes extend beyond this region and that covered by the Schwann cell. It extends some way into the synaptic folds but not into their deeper portions. In general, the projections appear to coincide

with those specialized regions of the muscle membrane which are more densely stained. These specialized regions are underlined by a network of fine filaments which may play a role in anchoring the projections. The possibility that these projections represent acetylcholine receptors or cholinesterase molecules raises questions which could be resolved by either removing the cholinesterase (Betz & Sakmann 1971; Hall & Kelly 1971) or identifying acetylcholine receptors using markers attached to α-bungarotoxin.

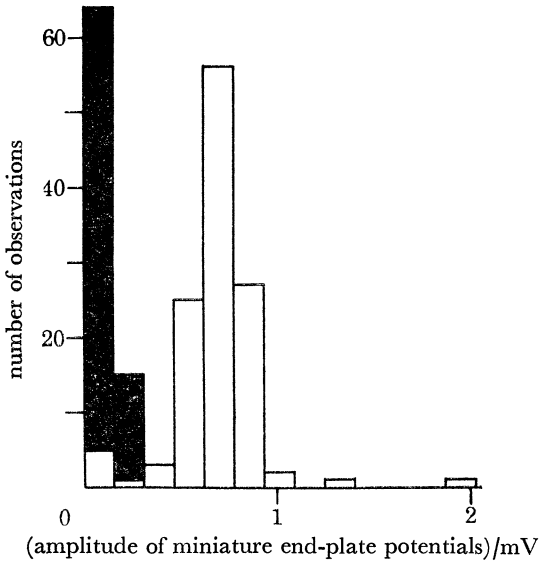

FIGURE 5. Amplitude of miniature end-plate potentials in a frog end-plate before (clear columns) and 1 h after applying anti-acetylcholine receptor serum (dark columns).

Finally, in common with others (Patrick & Lindstrom 1973; Sugiyama, Benda, Meunier & Changeux 1973) we have injected purified receptor preparations obtained from *Torpedo* electric organ into rabbits. After some time these animals develop a fatal muscular paralysis which can be temporarily relieved by injection of cholinesterase inhibitors. The resting potential and the ability of the muscle fibres to generate action potentials and contractions was not obviously altered. On the arrival of a nervous impulse, the motor nerve terminals still released a large number of acetylcholine packages. However, the amplitude of the miniature end-plate potentials was reduced to 0.2 or less of their normal amplitude, indicating that the effect produced by a single quantum of acetylcholine on the muscle membrane was greatly reduced. This decrease in sensitivity of the muscle membrane is probably due to blockage of receptors by circulating antibodies, which can be detected in the serum of immunized animals. Furthermore, as shown in figure 5, this serum is also capable of reducing the amplitude of miniature end-plate potentials in frog muscle. Subsequent binding studies on the end-plates of immunized animals showed that the number of α-bungarotoxin binding sites was reduced to between 12 and 50 % of their control value. We have also obtained similar results in rats. Immunization of animals with the Triton X-100 extract from which acetylcholine receptor had been removed by affinity chromatography does not produce these effects. There is thus an interesting parallel between myasthenic end-plates and those of rabbits and rats which have been immunized against purified acetylcholine receptor from *Torpedo*.

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