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Ultrastructural visualization of the transmembranous and cytomatrix-related part of nicotinic acetylcholine receptor of frog motor endplate by means of an immunochemical avidity of IgG for d-tubocurarine

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Abstract: In the present study, a fine ultrastructural localization of nicotinic acetylcholine receptor (nAChR) was attempted, using d-tubocurarine (d-TC), a quaternary ammonium compound binding to nAChR. The localization was based on the binding avidity of immunoglobulin G (IgG) for acetylcholine (ACh) and other quaternary ammonium compounds, such as d-TC. d-TC was applied to the frog neuromuscular preparation and caused a blockade of neuromuscular transmission. Then, d-TC was rendered insoluble *in situ* by silicotungstic acid (STA), a precipitating agent of soluble proteins and quaternary ammonium compounds. After tissue fixation, a normal rabbit serum was applied to the fine precipitate of the insoluble salt of d-TC silicotungstate (quaternary ammonium radical of d-TC) to form the immunochemical complex d-TC- rabbit IgG at ACh binding sites. The IgG of the complex was revealed by means of the conventional immunoperoxidase procedure used for ultrastructural localization. Under the electron microscope, fine diaminobenzidine (DAB) precipitates appeared as regular rod-like structures oriented to cytoplasmic side of the horizontal part (crest) of the postsynaptic membrane (between the junctional folds) which is known to be endowed with nAChR. The rod-like precipitates were not observed in the postsynaptic junctional folds which are devoid of nAChR. The distance separating the rods each other was rather constant (12 - 15 nm), while the length of the rods was variable and exceeded the usual length of nAChR. The present work indicates that the rod-like structures, already observed in association with sarcoplasmic side of the postsynaptic membrane, did correspond to the intramembranous and intracytoplasmic part of nAChR and related proteins. These cytochemical results confirm that d-TC binds to ACh binding sites in the pore of nAChR, and raise the question of DAB staining of cytoskeletal proteins related to the nAChR complex.

Keywords: Immunoglobulin - Quaternary ammonium compound - d-tubocurarine - Nicotinic acetylcholine receptor - Silicotungstic acid - Immunoperoxidase

Introduction

Bernard [1] was the first physiologist who observed that curare (d-tubocurarine: d-TC) was a blocking agent of the neuromuscular transmission. Langley [2] deduced from experimental data that curare reacted with "a receptive substance" (nowadays identified as nicotinic acetylcholine receptor, nAChR) on the muscle side. It was demonstrated that d-TC binds, competitively with ACh, to nAChR of the postsynaptic membrane [3-5].

The nAChR protein was characterized, for the first time, by Changeux *et al.* [6] after its isolation from the electric organ of the electric eel (a homologous organ of skeletal muscle) by means of α-bungarotoxin, a specific snake neurotoxin. Today, nAChR is one of the most studied ligand-gated channels of fast chemical synapses [7].

In the electric organ, nAChR was localized at optical microscopic level on the postsynaptic membranous area by autoradioautography with radioactive curare

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compounds [8] and by immunofluorescence with snake naja α-toxine [9] or with fluorescent dye conjugated α-bungarotoxin [10]. At the electron microscopic level, nAChR was localized by autoradiography using radioactive α-bungarotoxin [11-17] and by immunoperoxidase visualization of α-bungarotoxin bound to nAChR [18]. However, limited resolving power of autoradiography and diffusion of diaminobenzidine (DAB) precipitates in the synaptic cleft did not provide localization of nAChR at molecular level. By means of conventional electron microscopic technique [19,20] and cryoultramicrotomy [21], comb-like structures were observed protruding in the synaptic cleft. These structures were interpreted as visualization of arrays of the external parts of nAChR molecules. Freeze etching technique applied on the Torpedo electroplaque provided clusters of crystal-like nAChR molecules [22-24] and negative staining gave an oligomeric molecular form with central pore [25-27]. Outline of nAChR molecules attached to postsynaptic membrane fragments of Torpedo electroplaque was visualized by high resolution cryo-electron microscopy [28]. These studies, and the following ones, revealed that nAChR was composed of an extracellular pentameric subunit with a central pore toward the synaptic cleft, of an intramembranous part with the gate at open and closed state, of a cytoplasmic part endowed with openings in the channel wall in the subsynaptic sarcoplasm, and of proteins associated to the cytoplasmic side of nAChR [29-33].

In this work, an ultrastructural localization of d-TC bound to nAChR was performed by means of conventional immunoperoxidase procedure. The present method is based on the recently discovered avidity of IgG for quaternary ammonium compounds, such as ACh and d-TC [34]. The immunoperoxidase staining showed regularly disposed rod-like structures, probably corresponding to the pores of nAChR, oriented not to the synaptic cleft but to the cytoplasmic side of the postsynaptic membrane. Since the rod-like structure penetrates deeply in the subneural sarcoplasm and exceeds largely the length of nAChR, its relationship with fibrous structure in the subsynaptic sarcoplasm [35-38] was discussed.

Materials and methods

Frog cutaneous pectoris muscles were taken out with the motor nerve bundles and pinned on the bottom of an experimental chamber containing 5 ml of Ringer solution (112 mM NaCl, 2.0 mM KCl and 1.8 mM $CaCl_2$). Ringer solution was buffered with 25 mM Tris(hydroxymethyl)aminomethane - HCl (Tris-HCl) buffer solution at pH=7.8.

The bundle of motor nerves was stimulated electrically with a pair of silver chloride electrodes (supramaximal voltage: 3-7 V, during 0.3 ms) at 0.3 Hz and the muscle contractions were monitored on a chart recorder (San-ei, Tokyo, Japan; Recti-Horiz) by isometric mechano-electric transducer (Nihon Koden, Tokyo, Japan; TB-611T). Peak tension of control muscular twitch due to nerve stimulation was approximately 3g.

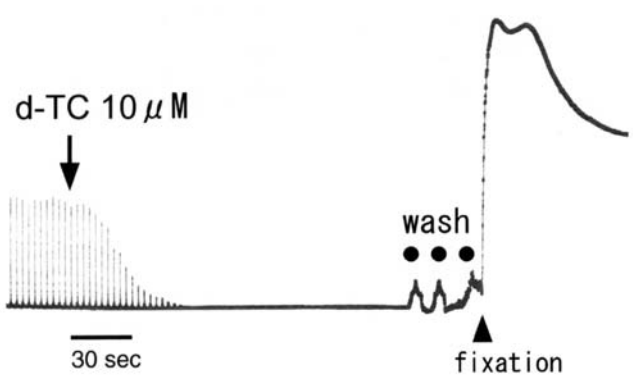

Fig. 1. Blockade of the neuromuscular transmission with 10 μM d-TC. The neuromuscular preparation (frog cutaneous pectoralis muscle) under submaximal electrical stimulation of the motor nerve. Synaptic transmission was blocked within 1 min, and the preparation remained in contact with d-TC for two additional min. After three rapid washes in single Ringer solution, the preparation was fixed with 5% aqueous solution of silicotungstic acid (STA).

10^{-5} M d-tubocurarine (d-TC) was applied to the preparation. The subsequent blockade of neuromuscular transmission was monitored and confirmed by submaximal electrical stimulation of the motor nerve after 3 min (Fig. 1). After a rapid wash (30 s) of the preparation with Ringer solution without buffer (simple Ringer), the preparation was fixed in 5% aqueous solution of silicotungstic acid (STA) for 15 min. The tissue was washed three times in the simple Ringer solution for 3 min, and fixed for 12 h with 3.7% formalin dissolved in the simple Ringer. Then, the preparation was washed in the simple Ringer for 3 min, and in two bathes of a phosphate buffered saline (PBS: 120 mM NaCl with 0.01 M sodium phosphate buffer at pH=7.4) for 2 × 3 min. The binding of IgG to d-TC silicotungstate precipitate, based on the avidity of IgG for ammonium quaternary compounds, was performed as presently described: The preparation was treated with the following immunochemical agents dissolved in PBS: 1% bovine serum albumin (Biosis) (30 min), 1% rabbit normal serum (3h), washing in PBS (3 × 5 min), 1/500 diluted Anti-Rabbit IgG (whole molecule)-peroxidase (Sigma, USA) (2h), washing in PBS (3 x 5 min), 0.02% diaminobenzidine tetrahydrochloride (DAB) and 0.01% H_2O_2 (20 min). After washing in PBS, the preparation was postfixed in 2% aqueous solution of OsO_4 (30 min), washed in water, dehydrated in graded series of ethanol solutions and embedded in Araldite. Ultrathin sections were observed without counterstaining under JEOL 100 CX electron microscope at 80 kV. As control experiment of d-TC reaction, the experimental procedure described above was performed without d-TC blockade of the neuromuscular junction.

Results

After blockade of the neuromuscular junction with d-tubocurarine (d-TC), precipitation of d-TC with silicotungstic acid (STA), and postfixation of the tissue with formalin, the conventional indirect immunoperoxidase reaction revealed the presence of rabbit IgG that bound to d-TC precipitated in the vicinity of its binding site with nAChR. At the electron microscopic level, diaminobenzidine (DAB) staining was observed at the

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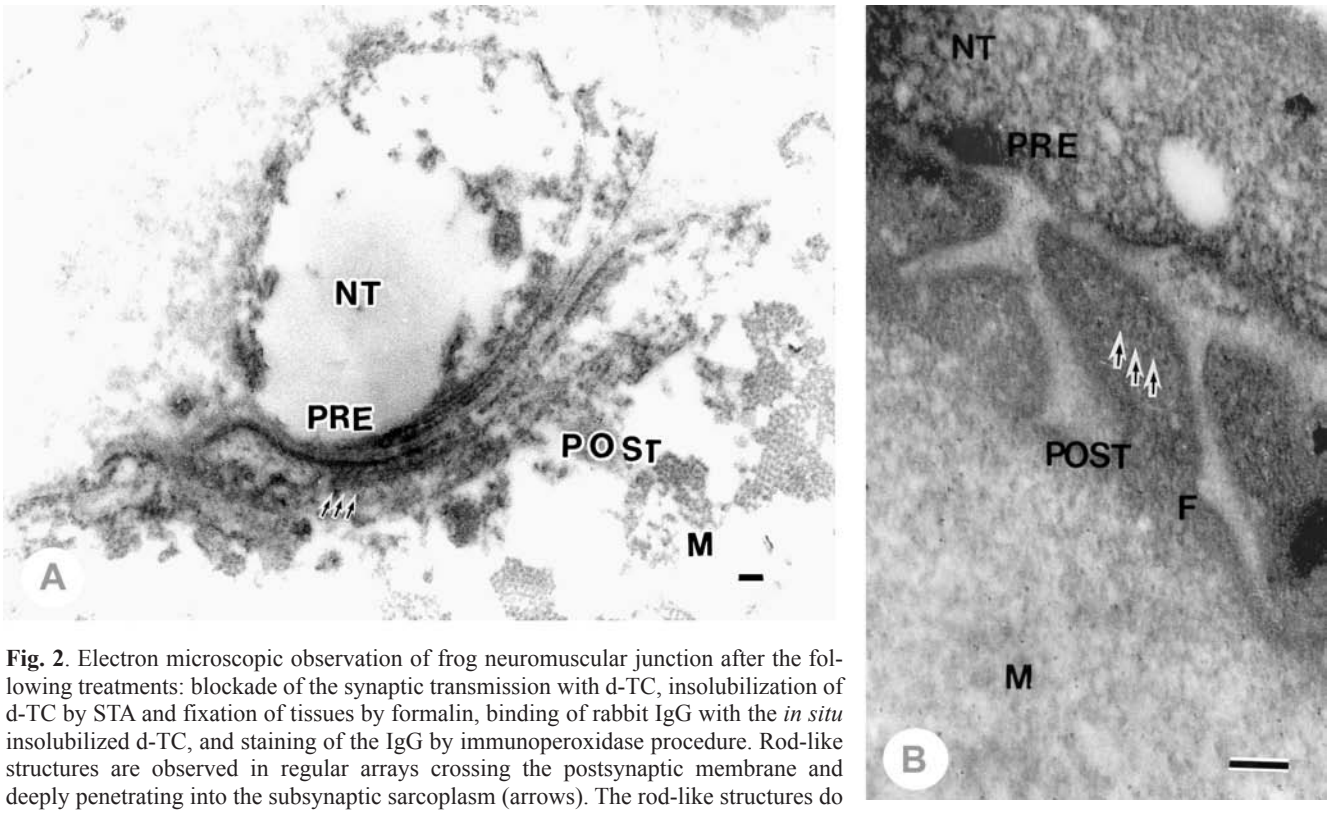

Fig. 2. Electron microscopic observation of frog neuromuscular junction after the following treatments: blockade of the synaptic transmission with d-TC, insolubilization of d-TC by STA and fixation of tissues by formalin, binding of rabbit IgG with the *in situ* insolubilized d-TC, and staining of the IgG by immunoperoxidase procedure. Rod-like structures are observed in regular arrays crossing the postsynaptic membrane and deeply penetrating into the subsynaptic sarcoplasm (arrows). The rod-like structures do not protrude in the synaptic cleft. Transversal (A) and longitudinal section (B) of the muscle fibers. NT: Nerve terminal, PRE: presynaptic membrane, POST: postsynaptic membrane, M: muscle cell, F: folding of the postsynaptic membrane. Bar 100 nm.

horizontal part (crest) of the postsynaptic membrane (Fig. 2A). The perpendicular junctional folds including the bottom of the postsynaptic membrane (trough) were devoid of staining (Fig. 2B). At closer observation, the postsynaptic membrane stained with DAB reveals transmembranous rod-like structures separated each other by a distance of 12-15 nm. These transmembranous rod-like structures were not oriented to the synaptic cleft but crossed the membrane and penetrated deeply into the cytoplasm (Fig. 2A and B: arrows). The length of the rods was variable (25-100 nm) and largely overpassed the length of nicotinic acetylcholine receptor (nAChR).

Control neuromuscular preparation, without d-TC, revealed a moderate staining, weaker than in the experiment including d-TC. (Fig. 3: arrowhead).

Discussion

The rod-like structures stained by diaminobenzidine (DAB) in the postsynaptic membrane area of the neuromuscular junction probably correspond to arrays of nicotinic acetylcholine receptor (nAChR). Indeed, d-tubocurarine (d-TC) dropped on a nitrocellulose membrane has been shown to be a good target of the avidity of IgG molecules after its precipitation by silicotungstic acid (STA) [34]. In this experimental model, an intense staining was obtained after immunoperoxidase reaction performed on d-TC-STA complex. Therefore, in the present study, the staining associated with the rod-like structures is presumably due to IgG bound to d-TC-STA complex and visualized by immunoperoxidase procedure in the pore of nAChR. Thus, the present work confirms pharmacological binding of d-TC to the nAChR of frog endplate. Furthermore, it provides complementary data to previous ultrastructural localizations of nAChR. Indeed, immunoperoxidase reaction for bungarotoxine gave diffuse DAB precipitates in the synaptic cleft [18] and autoradiography with radioactive neurotoxine provided intense and quantifiable signal, nevertheless not appropriate to a fine localization of neurotoxine bound structures [17].

The weak persistence of the reaction in the control experiment without d-TC was not expected. It might be due to the endogenous acetylcholine (ACh) fixed into the nAChR by STA and bound to IgG. Indeed, it was shown that IgG could bind ACh precipitated with STA on a nitrocellulose membrane [34].

The stained rod-like structures were oriented into the cytoplasmic side of the postsynaptic membrane, and did not extend in the synaptic cleft. These rods were separated each other by a rather regular space of about 12-15 nm, which corresponds to the conventional electron microscopic observations of the part of nAChR oriented to the synaptic cleft [19-21].

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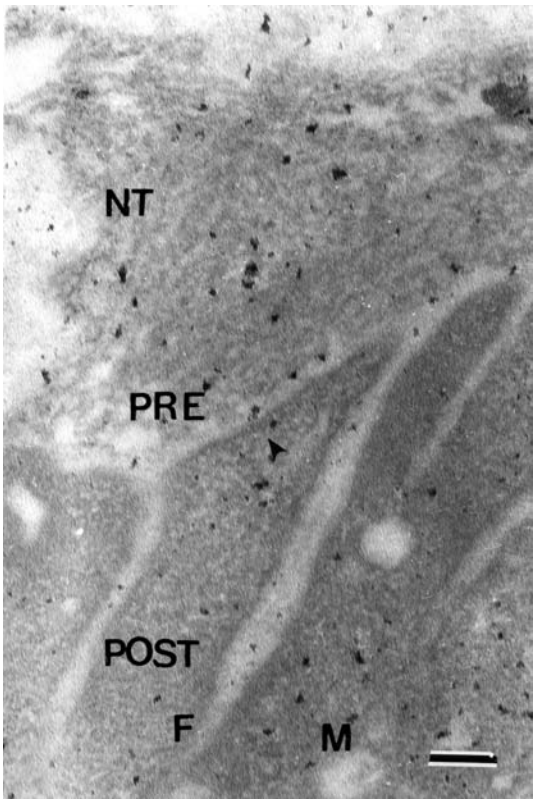

Fig. 3. A control tissue without d-TC treatment is not completely immuno-negative and reveals moderately stained transmembranous rod-like structures (arrowhead). The persistence of the reaction is presumed to be derived from the endogenous ACh rendered insoluble by STA inside the nAChR and bound to IgG. Longitudinal section of the muscle fiber. NT: Nerve terminal, PRE: presynaptic membrane, POST: postsynaptic membrane, M: muscle cell, F: folding of postsynaptic membrane. Bar 100 nm.

Precedent authors studied the ultrastructure of the neuromuscular junction with conventional cytological methods. They noticed a particular density of the postsynaptic membrane on the subneural sarcoplasm [39]. The exact thickness of this dense part of the postsynaptic membrane was hindered by "fringes" attached to the sarcoplasmic side of the postsynaptic membrane [35,36]. A close observation of the fringes reveals, though shorter in length, nothing but the rod-like structures stained by d-TC in the present work. In a similar study on subsynaptic sarcoplasm of mammalian neuromuscular junction, a cortical network associated by a lattice to the postsynaptic membrane was shown [37]. This lattice partially corresponds to the rod-like structures evidenced in the present study. In an ultrastructural image of frog neuromuscular junction obtained after cryosubstitution [40], a similar rod-like structure oriented to cytoplasmic side of the postsynaptic membrane was seen. At the end, after chemical washing, a submembranous meshwork was observed just beneath the postsynaptic membrane of the neuromuscular junction [41]. Thus, these authors have provided figures corresponding to the rod-like structure that was identified, in the present work, as cytoplasmic part of nAChR.

The rod-like structures penetrated through postsynaptic membrane and entered into the cytoplasm at variable depth (25-100 nm), which overpassed the length of nAChR (16 nm) [33]. This pattern of staining may correspond to the filamentous structures bound to the cytoplasmic side of nAChR [37,38,42]. Indeed, a 43-kD protein is supposed to be connected to the cytoplasmic side of nAChR molecule, [29,43,44]. This protein was identified as an acetylcholine receptor-associated-protein (RAPsyn) [30] which has a role of clustering nAChR [45]. More recently, cryoelectron microscopic technique, applied on reconstituted postsynaptic membrane of Torpedo electric organ, has shown the cytoplasmic side of nAChR, including cytoplasmic mass and attached protein [31-33]. It is probable that RAPsyn and a part of cytoskeleton connected to the nAChR [46,47] have been stained by DAB in the present work.

Why the rod-like structures stained by DAB deeply extended in the sarcoplasm remains an unsolved question. d-TC is assumed to be bound to ACh binding sites [5] which are situated inside nAChR about halfway between the extracellular ends of the α-subunits and the membrane [31,33]. Since IgG is supposed to be bound to d-TC, DAB, the reaction product of immunoperoxidase, can diffuse in the pore of the channel both toward the synaptic cleft and the sarcoplasmic side. The staining of the cytoplasmic part of nAChR and related proteins suggests that the channel pore remained open in the present experimental conditions. The synaptic cleft, which is an extracellular space, is voluminous and rich in free water [48]. Thus, it does not provide a good condition for the precipitation of DAB. On the contrary, the pore, though also being an extracellular space, is incomparably less voluminous than the synaptic space and, so, much more favourable for precipitation of DAB. After staining inside the pore of the transmembranous nAChR, DAB might come out the pore through the openings in the channel wall, enter into the cytoplasm [31,33] and stain the fibrous structure connected to nAChR.

An other question is why a so small quantity of DAB has not been diluted in the cytosol after its penetration through the pore? To answer this enigma it is of interest to recall that the cytosol is considered rather as a cytogel, a structured cytomatrix [49], where free water is much less abundant than in the extracellular space [48]. In that case, even after cytological fixation, the cytomatrix might hinder diffusion of DAB and favor its precipitation on the surface of the fibrous structures. Further studies are necessary to verify these interpretations concerning the DAB staining of different subsynaptic fibrous structures.

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