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CONDITIONS FOR THE SELECTIVE LABELLING OF THE 66 000 DALTON CHAIN OF THE ACETYLCHOLINE RECEPTOR BY THE COVALENT NON-COMPETITIVE BLOCKER 5-AZIDO-[^{3}H]TRIMETHISOQUIN

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1. Introduction

The subsynaptic membrane fragments prepared from *Torpedo* electric organ are composed of a few discrete polypeptide entities (reviewed [1–3]). The predominant chain of app. mol. wt 40 000 (40 k) is covalently labelled by affinity reagents specific for the acetylcholine receptor site and therefore carries this site [4–10]. The NH_2 terminal sequence of its first 20 amino acids has been established in the case of *Torpedo marmorata* receptor [11] and confirmed with *Torpedo californica* [12]. The 43 k polypeptide is a peripheral protein which is released from the subsynaptic membrane by treatment at pH 11. Its alkaline extraction does not significantly change the known regulatory properties of the ACh receptor protein [13–16] but alters its thermal stability [17] and its rotational [18–21] and translational [22] diffusion. Most likely, the 43 k protein plays a 'structural' role in maintaining the ACh receptor protein in a highly immobilized and stable aggregated form in the subsynaptic membrane.

Minor polypeptide components of app. mol. wt 50 k, 57 k, 66 k [23–25] are also encountered in the ACh-receptor rich membranes and copurify with the α-bungarotoxin labelled ACh-receptor solubilized by non-denaturing detergents. A photoaffinity derivative of the local anesthetic trimethisoquin, 5-azido-[^{3}H]-trimethisoquin (5-A[^{3}H]T) [26] labelled bands of app. mol. wt 50 k and 66 k [27]. This labelling is inhibited by histrionicotoxin or unlabelled local anesthetics; it is enhanced by carbamylcholine and the effect of carbamylcholine is blocked by α-bungarotoxin [27]. The criteria for a selective attachment of 5-A[^{3}H]T to the site for the non-competitive blockers of the permeability response thus seem fulfilled.

To explain the rather paradoxical labelling by 5-A[^{3}H]T of two polypeptide chains instead of one, three possibilities were considered:

(i) The site for non-competitive blockers is not carried by the 50 k or 66 k chain; however, these chains lie in the vicinity of the binding site and become preferentially labelled since they present chemical groups with which the nitrene group of 5-A[^{3}H]T reacts.

(ii) The 50 k and 66 k chains are different but carry binding sites for non-competitive blockers with similar reactivities.

(iii) The 50 k chain labelled by 5-A[^{3}H]T derives from the 66 k chain by proteolysis.

These results show that the third alternative is the correct one. Under conditions which limit proteolysis, 5-A[^{3}H]T selectively labels the 66 k polypeptide chain.

2. Materials and methods

2.1. *Preparation of ACh-receptor rich membranes from* Torpedo marmorata *electric organ*

ACh-receptor rich membranes were purified from freshly dissected electric organ of *T. marmorata*, except when otherwise noted. Membranes were

Abbreviations: ACh, acetylcholine; 5-A[^{3}H]T, 5-azido-[^{3}H]-trimethisoquin; SDS, sodium dodecyl sulfate; MPTA, 4-(*N*-maleimido)phenyltrimethylammonium; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis-(β-aminoethyl ether) *N,N'*-tetraacetic acid

purified as in [28]. To investigate the effect of protease inhibitors the following buffer (PI) was used: 50 mM Tris-HCl (pH 7.5 at 20°C), 3 mM EDTA, 1 mM EGTA, 0.1 mM phenyl-methyl-sulfonyl fluoride (PMSF), 5 units/ml aprotinine, and 0.5 μg/ml pepstatin.

2.2. *Affinity labelling of the binding site for non-competitive blockers by tritiated 5-azido-trimethisoquin*

200 μl of ACh-receptor rich membranes (15–20 μM ^{125}I-labelled α-bungarotoxin sites) were extracted at pH 11 according to [13] and centrifuged for 30 min at 27 000 rev./min in a Beckman Ti 30 Rotor. The pellet was resuspended in 200 μl *Torpedo* saline solution (250 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$ and 5 mM Na-phosphate, pH 7.5) and recentrifuged at full speed in a Beckman airfuge for 1 min. The final pellet was resuspended to 100 μl with *Torpedo* saline solution. Carbamylcholine was added to 10^{-4} M (final) followed by the addition of 5-A[^{3}H]T (5×10^{-6} M, final) in the dark. The reaction chamber was flushed with N_2; and the sample equilibrated for 15 min in the dark. The mixture was then irradiated for 5 min with vigorous stirring under N_2 with a Mineralight shortwave ultraviolet lamp (Ultraviolet Products). Following irradiation, the membranes were washed twice by centrifugation in a Beckman airfuge and resuspended in PI buffer.

2.3. *Sucrose gradient purification of the ACh-receptor protein*

The 5-A[^{3}H]T-labelled membranes were diluted to 450 μl with PI buffer. β-mercaptoethanol was added to 0.6% (v/v) final conc., followed by 40% (w/v) Na-cholate to 3%. The sample was then loaded on an 11.5 ml 5–20% (w/v) sucrose gradient containing 1% Na-cholate and 0.6% β-mercaptoethanol in PI buffer. The gradients were centrifuged for 16 h at 33 000 rev./min in a Beckman SW 41 rotor at 4°C. Seven-drop fractions were collected from the bottom of the tube. Each fraction was assayed for α-toxin binding activity with ^{125}I-labelled α-bungarotoxin and for 5-A[^{3}H]T by counting a 25 μl aliquot in 4 ml of Bray's solution. Under these conditions, all ACh-receptor was present as a 9 S monomer. In all cases, the profiles of ^{125}I-labelled α-bungarotoxin binding activity and 5-A[^{3}H]T labelling were superimposable along the sucrose gradient.

2.4. *Affinity chromatography purification of the ACh-receptor protein*

ACh-receptor protein was purified using a 2 ml erabutoxin B–Sepharose 4B affinity column equilibrated in PI buffer supplemented with 0.1% Triton X-100 and 0.6% β-mercaptoethanol. Membranes solubilized in 3% Triton X-100 were cycled through the column for 3 h. The column was then washed with 100 ml equilibration buffer containing 1 M NaCl. Following the wash, the column was equilibrated with the equilibration buffer supplemented with 1 M carbamylcholine and incubated overnight to elute the ACh-receptor. All purification steps were performed at 4°C.

2.5. *Polyacrylamide gel electrophoresis*

Two polyacrylamide gel systems, that are modifications of the system in [29], were used here. Both systems contained 10% acrylamide; however, the highly crosslinked system 1 contained 0.26% bisacrylamide and system 2 contained 0.13% bisacrylamide. The standard system 2 was described in [5].

Sample preparation, fluorography and gel scanning were done as in [27] except that films for fluorography were preflashed to an absorbance of 0.2 [30]. For quantitation of the radioactivity in each band, appropriate sections of the stained and dried gel were cut out and counted in a scintillation medium containing 900 ml toluene/PPO/POPOP, 100 ml tissue solubilizer and 20 ml NH_4OH (4 M) after a 2 day incubation at room temperature. Except where otherwise noted, samples were not heated prior to loading on the gel.

2.6. *Chemicals*

5-Azido-dimethisoquin was a gift of Gilles Waksman and Bernard Roques and was prepared as in [26]. 5-A[^{3}H]T was synthesized from 5-azido-dimethisoquin using [^{3}H]methyliodide at the CEA, Saclay, Service des Molécules Marquées. ^{125}I-labelled α-bungarotoxin was purchased from NEN. Aprotinine, PMSF, and pepstatin were obtained from Sigma; acrylamide, bisacrylamide and TEMED were products of Kodak.

3. Results and discussion

3.1. *Effect of protease inhibitors on the pattern of polypeptide chains labelled by 5-azido-[^{3}H]trimethisoquin*

The contribution of proteolysis to the pattern of chain(s) labelled by 5-A[^{3}H]T was tested in the following manner. ACh-receptor rich membranes were prepared from fresh electric organ in the presence or absence of a cocktail of protease inhibitors and chelating agents (section 2), labelled by 5-A[^{3}H]T and solubilized by Na-cholate or Triton X-100. The receptor protein was then purified in the presence of PI buffer.

First, the distribution of 5-A[^{3}H]T and of ^{125}I-labelled α-bungarotoxin labelling were compared during the purification on sucrose gradient after dissolution by Na-cholate. Fig.1 shows that the profile

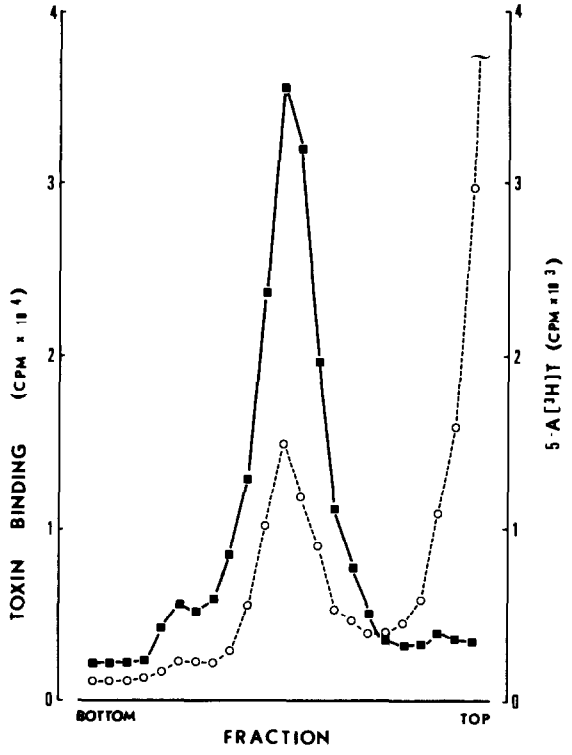


Fig.1. Distribution of 5-A[^{3}H]T and α-bungarotoxin binding activity on a sucrose density gradient. Membranes were labelled with 5-A[^{3}H]T in the presence of carbamylcholine, solubilized in Na-cholate, and centrifuged for 16 h in a 5–20% sucrose gradient containing 0.6% β-mercaptoethanol and 1% Na-cholate (see section 2). Fractions were counted for ^{3}H to reveal the 5-A[^{3}H]T label (o---o) and assayed for toxin binding activity with ^{125}I-labelled α-bungarotoxin (▪——▪).

of ^{3}H and ^{125}I labelling superimposed almost exactly. In other words, the component labelled by 5-A[^{3}H]T is strongly bound, or belongs to, the detergent-extracted receptor protein labelled by the α-toxin.

Then, the patterns of polypeptide chains given by the preparations of receptor prepared in the presence and in the absence of protease inhibitors were investigated by electrophoresis in SDS in a highly crosslinked (fig.2A–D) and standard (fig.2E–H) systems of polyacrylamide gels (see section 2).

The receptor purified in the presence of protease inhibitors yielded, in the two gel systems, the four characteristic bands stained by Coomassie blue of app. mol. wt 40 k, 50 k, 57 k and 66 k (fig. 2A, E). In both gel systems, the 66 k band was, of these four, the only one labelled by 5-A[^{3}H]T in the presence of carbamylcholine (fig.2B, F).

On the other hand, the pattern of bands given by the receptor protein prepared in the absence of protease inhibitors was always more complex. In the highly crosslinked gel system, a band positioned slightly below the 50 k band, that will be referred to as *bis* 50 k, and a distinct smear above the 40 k band were observed in addition to the already mentioned four bands (fig.2C). The corresponding fluorograms (fig.2D) indicated that both the 66 k and the *bis* 50 k bands were labelled by 5-A[^{3}H]T. In addition, the Coomassie blue staining intensities of the 66 k, 57 k and 50 k bands and the 5-A[^{3}H]T labelling of the 66 k band appeared fainter than what was observed with the receptor protein purified in the presence of protease inhibitors. The simplest interpretation of these data is that the *bis* 50 k band results from the proteolytic degradation of the 66 k chain.

In the standard gel system 2 (fig.2E–H), the receptor protein prepared in the absence of protease inhibitors gave the standard four bands found in the presence of protease inhibitors but, in addition, a new band of app. mol. wt 45 k; moreover, the 66 k, 57 k and 50 k appeared again fainter. In this slot, and in agreement with [27], the 5-A[^{3}H]T labelling coincided with the Coomassie blue stained band of app. mol. wt 50 k. Taking into account the information given by the highly crosslinked gels, we conclude that, in the standard gel system, the 50 k band is composed of the native 50 k chain superimposed on the *bis* 50 k chain which appears to be a degradation product of the native 66 k chain.

Examination of the same gel slot (fig.2G) also showed that the appearance of the 45 k band coin-

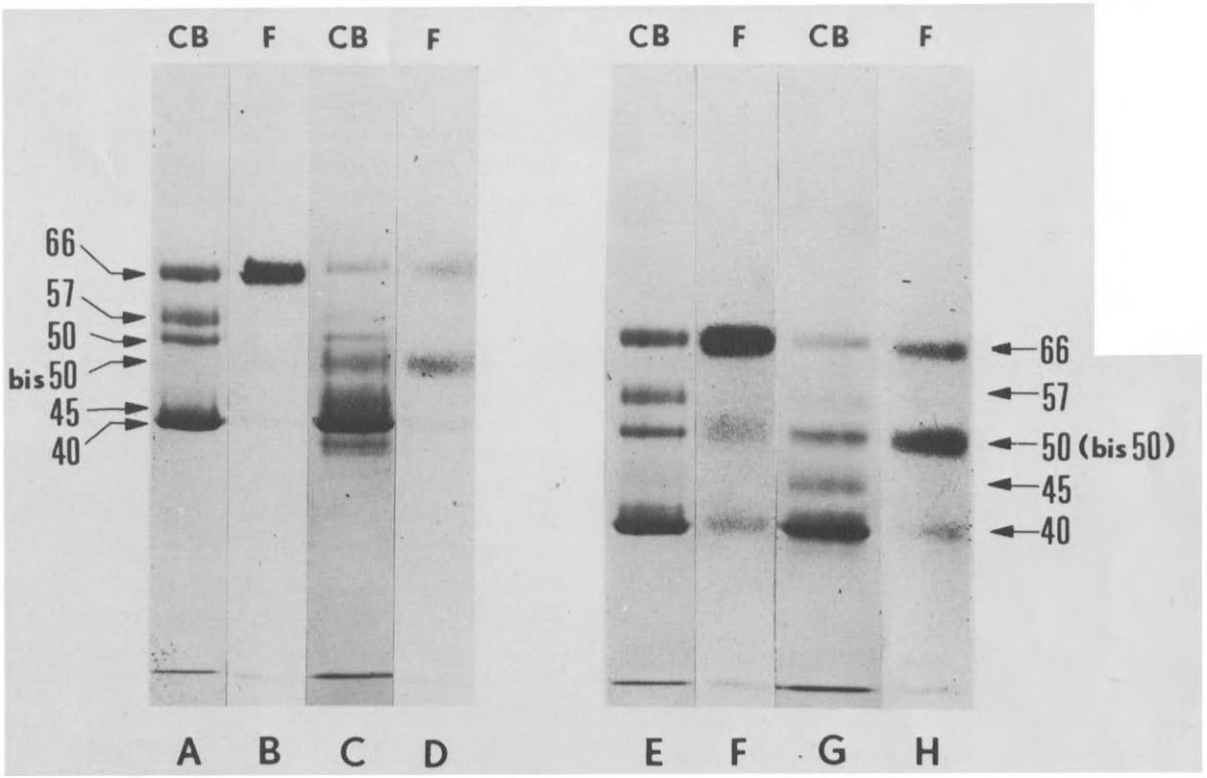


Fig.2. Gel patterns given by the ACh-receptor protein purified from membranes prepared in the presence and in the absence of protease inhibitors. A–D are highly crosslinked system 1 polyacrylamide gels in SDS; E–H are standard system 2 gels (see section 2). The Coomassie blue stain of ACh-receptor purified with protease inhibitors is shown in A, E, and the corresponding fluorograms of 5-A[^{3}H]T labelling in B, F. The Coomassie blue staining of ACh-receptor purified without protease inhibitors is shown in C, H, and the corresponding fluorograms in D, H. Because of the anomalous behaviour of some of the chains on the two gel systems, nomenclature derived from the comparison of the chains with protein of known mol. wt in the standard gel system 2 will be used exclusively. The four major bands in E will be referred to as 40 k, 50 k, 57 k and 66 k dalton peptides. The band between the 40 k and 50 k peptides in G will be referred to as the 45 k peptide; and the band below the 50 k chain in C, which comigrates with the 50 k chain in G, will be referred to as the *bis* 50 k peptide. The minor peptides below the 40 k chain in C and G are degradation products of the 40 k chain as they can be labelled with [^{3}H]MPTA, an affinity reagent for the ACh binding site [5].

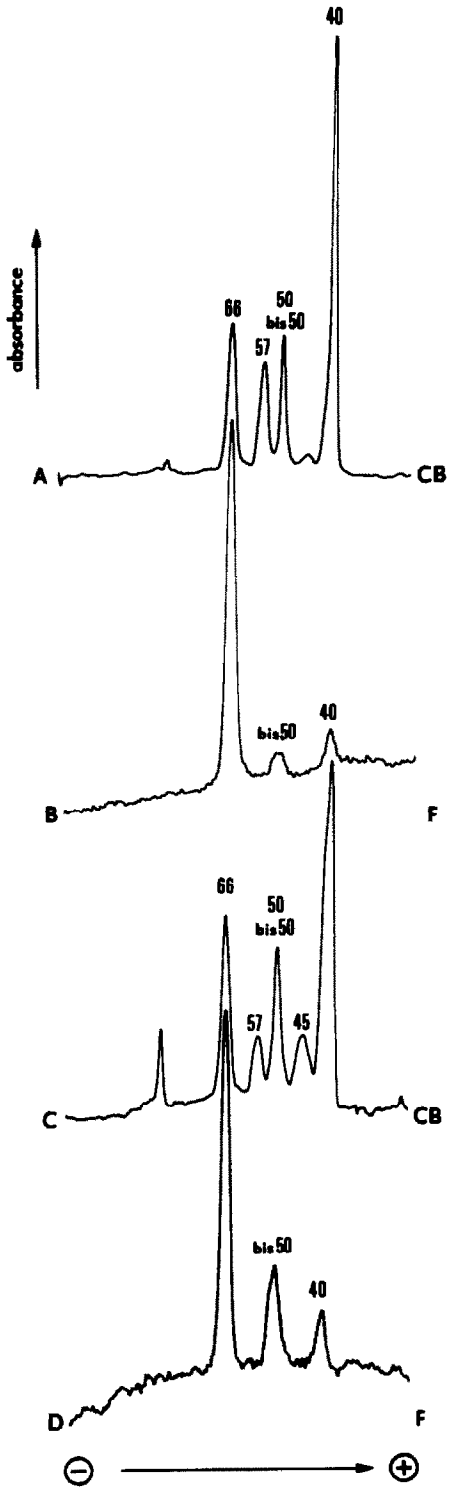
cided with a quantitative decrease of intensity of the 57 k band. Most likely, the 45 k band, which, in the highly crosslinked gels, gave only a smear above the 40 k band resulted also from the proteolysis of the 57 k polypeptide.

3.2. *Causes for irreproducibility of the pattern of chains labelled by 5-azido-[^{3}H]trimethisoquin*

3.2.1. Freezing the electric organ

Frozen electric organ has been used commonly as biological material to purify the ACh-receptor protein [8,31,32]. However, freezing and thawing of the tissue might release intracellular proteases which would attack the receptor protein in situ before blocking of the proteases.

To test this point, a freshly excised electric organ was frozen in liquid nitrogen, thawed to room temperature and homogenized in the presence of protease inhibitors to purify the ACh-receptor-rich membranes. In fig.3B, is presented a scan given by a preparation of receptor protein purified from these membranes after electrophoresis in the standard gel system 2. The height of the peak given by the 57 k band appeared smaller and that given by the 45 k band higher than those of the corresponding chain in the control preparation of receptor protein purified from fresh electric tissue. In addition, a larger quantity of 5-A[^{3}H]T was found in the *bis* 50 k band than in the corresponding



region of the control (fig.3C, D). Thus, the preparation of ACh-receptor from frozen electric tissue results in the proteolytic degradation of the 57 k and 66 k chains even though all the subsequent steps, starting from the homogenization, had been carried out in the presence of protease inhibitors.

3.2.2. Presence of Ca^{2+}

The chelating agent EDTA has been used in [8,32–34] to prevent proteolysis during the preparation and purification of the receptor protein. To test for the presence of a Ca^{2+}-sensitive protease, ACh-receptor-rich membranes, purified in the presence of protease inhibitors and chelators, were solubilized in the presence of Ca^{2+} and the receptor protein purified from these membranes in the presence of the PI buffer (with PMSF, aprotinin and pepstatin) except that EDTA and EGTA were replaced by 4 mM $CaCl_2$. Fig. 4A shows that, under these conditions, the 57 k and 66 k were degraded. On the other hand, when an excess of Ca^{2+} was added to the receptor protein after purification under the standard conditions (see section 2), no degradation was observed after 16 h incubation at 4°C. A Ca^{2+}-dependent protease, resistant to PMSF, aprotinin and pepstatin is therefore present in the homogenate of electric organ and copurifies with the ACh-receptor-rich membranes; however, this protease is removed by the purification of the detergent-extracted receptor.

3.2.3. Heating the sample before gel electrophoresis in SDS

When the sample of purified receptor was heated for 3 min at 100°C in a medium containing 2% SDS and 0.1% Triton X-100, the pattern of bands stained with Coomassie blue appeared strikingly different (fig.4B). As described in [16], the height of the 40 k band decreased and gave a smear below the 40 k band which was still labelled by the affinity reagent of

Fig.3. Effect of freezing and thawing of the electric organ on the Coomassie blue staining of ACh-receptor purified by affinity chromatography in the presence of protease inhibitors. The gel scans correspond to the following samples: (A) control, ACh-receptor labelled with 5-A[^{3}H]T and purified in the presence of protease inhibitors; (B) fluorogram of (A); (C) same as (A) except that the ACh-receptor was prepared from electric tissue pre-frozen and thawed; (D) fluorogram of (C).

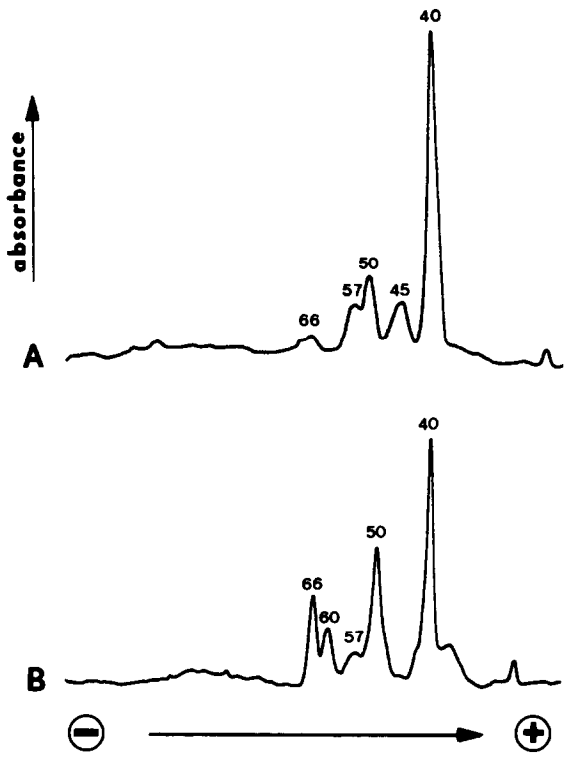


Fig.4. Consequences on the polypeptide pattern of the ACh-receptor protein of the presence of Ca^{2+} during purification and of sample heating before loading on gels. (A) ACh-receptor purified in the presence of Ca^{2+} + protease inhibitors; and (B) same as fig. 3(A) but the ACh-receptor sample was heated in the presence of SDS for 3 min at 100°C. All samples were run on standard gel system 2.

the ACh-receptor site [^{3}H]MPTA [5]. A band at app. mol. wt 60 k was observed in addition to the 57 k band which decreased in height. In the preparation of receptor purified in the absence of protease inhibitors, the 45 k chain was no longer present (not shown).

4. Conclusion

When the ACh-receptor protein is purified from fresh electric tissue, in the presence of protease inhibitors (including chelating agents), and analyzed by SDS gel electrophoresis without heating the sample, a reproducible pattern of polypeptide bands is obtained. Those bands include:

(1) The 40 k chain which is labelled by [^{3}H]MPTA and therefore carries the ACh-receptor site [1,2];

(2) The 66 k which chain is labelled by the covalent local anesthetic 5-A[^{3}H]T under conditions where it attaches selectively to the site for non-competitive blockers (in the presence of cholinergic agonist).

These results indicate that the 66 k chain, like the 40 k chain, is functionally and/or topographically related to the molecular device involved in the regulation of ion translocation by ACh. The 66 k chain might carry the site for non-competitive blockers and therefore be an integral part of the ACh-regulator. However, it may be present in the close vicinity of this site (which would then be carried by another chain, e.g., the 40 k polypeptide) and possess amino acid side group(s) with which the nitrene residue of 5-A[^{3}H]T would preferentially react. Experiments are now designed to distinguish between these two alternatives.

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