

## SELECTIVE LABELLING BY [<sup>3</sup>H]TRIMETHISOQUIN AZIDE OF POLYPEPTIDE CHAINS PRESENT IN ACETYLCHOLINE RECEPTOR-RICH MEMBRANES FROM *TORPEDO MARMORATA*

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### 1. Introduction

Membrane fragments in which the acetylcholine (ACh) receptor may represent up to forty percent of the proteins can be purified in large quantities (decigram levels) from *Torpedo* electric organ [1–3]. These membrane fragments, which most likely derive from subsynaptic areas of the electroplaque plasma membrane, appear particularly convenient to study the structural and functional properties of the ACh receptor and of the associated ionophore (reviewed [4]).

SDS–polyacrylamide gel electrophoresis in one dimension of the purified membranes yields several polypeptide chains [3,5,6]. The predominant subunit has an app. mol. wt 40 000 (40 k) and is affinity labelled by reagents (TDF [7], MPTA [3], MBTA [8], BrACh [9,10], DAPA [11] known to bind covalently to, or in the close vicinity of, the ACh-receptor site. The 40 k subunit therefore carries the ACh-receptor site.

Subunits of app. mol. wt 43 k, 50 k and 66 k have been found in most preparations of receptor-rich membranes with, depending on the authors, fixed [12] or variable [3,13–15] stoichiometries. In addition,

several laboratories have reported the presence of a band of app. mol. wt 60 k [5,12] (but [3,13–15]). The 43 k polypeptide can be labelled by iodo [<sup>14</sup>C]acetate [13] or by a covalent derivative of the local anesthetic procaine [16] but does not seem to contain the binding site for non-competitive blockers of the response to acetylcholine [17]. The suggestion has been made that the 43 k peptide represents actin [18]; but it does not comigrate with *Torpedo* actin (J. Hofler, unpublished) and has a different amino acid composition than skeletal muscle actin [13]. Photoaffinity derivatives of the snake  $\alpha$ -toxins label the 66 k chain in addition to the 40 k one [12,19,34] and labelling of the 50 k and 66 k chains has also been reported with bis-azido- [<sup>3</sup>H]ethidium bromide in the presence of carbamylcholine [12]. The functional significance of these transmembrane [20] polypeptides is still a matter of controversy.

These receptor-rich membranes contain a category of sites, distinct from the ACh-receptor site, to which bind non-competitive blockers of the response to ACh such as the aminated local anesthetics and the frog toxin histrionicotoxin. In order to identify which of the polypeptide chains present in the receptor-rich membranes carry this site(s), we have employed a radiolabelled photoaffinity derivative of the non-competitive blocker trimethisoquin, 5-azido [<sup>3</sup>H]trimethisoquin whose synthesis and pharmacology is presented in [21].

Specific labelling of the site for the pharmacological action of the non-competitive blockers should fulfil criteria defined by studies of the non-covalent binding of radioactive local anesthetics to *Torpedo* membranes

*Abbreviations:* ACh, acetylcholine; 5-A [<sup>3</sup>H]T, 5-azido- [<sup>3</sup>H]trimethisoquin; SDS, sodium dodecyl sulfate; MPTA, 4-(*N*-maleimido)phenyltrimethylammonium; MBTA, 4-(*N*-maleimide)benzyltrimethylammonium; TDF, trimethylammonium diazonium fluoroborate; DAPA, bis-(3-azidopyridinium)-1,10-decane perchlorate; BrACh, bromoacetylcholine

[15,22]: the labelling should be inhibited by histri-  
nicotoin and unlabelled local anesthetics, enhanced  
by carbamylcholine, and the effect of carbamylcholine  
blocked by  $\alpha$ -bungarotoxin.

Finally, the labelling should disappear after dissolu-  
tion of the ACh-receptor protein by high concentra-  
tion of Na-cholate under conditions where the  
allosteric interaction between the ACh-receptor site  
and the site for non-competitive blockers is abolished  
[23,24]. We report here the labelling of two peptides  
of mol. wt 50 k and 66 k that fulfill the requirements  
for specific binding to the site for non-competitive  
blockers.

## 2. Methods

### 2.1. Preparation of membranes

ACh-receptor rich membranes were purified from  
the electric organ of *Torpedo marmorata* as in [3] and  
stored in liquid nitrogen until use. Non-receptor  
peptides, including the 43 k protein [13], were  
removed from the membranes by pH 11 treatment as  
in [17] modified [14,15] yielding 'alkaline-treated  
membranes.' Alkaline-treated membranes were sub-  
jected to the 'reconstitution cycle' [14] which consists  
of dispersing the membranes in 3% (w/w) Na-cholate,  
dilution to 1% (w/w) Na-cholate, and centrifugation  
yielding the 'soluble extract', followed by reconstitu-  
tion of the extract by filtration on a Sephadex G-50  
column. The fractions excluded from the column  
which did not contain Na-cholate were pooled as  
'reconstituted AChR'. ACh-receptor sites were  
quantitated as in [25] using  $^{125}\text{I}$ -labelled  $\alpha$ -bungaro-  
toxin.

### 2.2. Affinity labelling

Samples of ACh-receptor rich membranes were  
diluted in *Torpedo* physiological solution (250 mM  
NaCl, 5 mM KCl, 4 mM  $\text{CaCl}_2$ , 2 mM  $\text{MgCl}_2$ , and  
5 mM Na-phosphate (pH 7.0)), and 5-A $^{[3}\text{H}]\text{T}$  and  
other test compounds were added to 100  $\mu\text{l}$  final vol.  
The final concentration of  $\alpha$ -bungarotoxin binding  
sites was 4–5  $\mu\text{M}$ , and that of 5-A $^{[3}\text{H}]\text{T}$  was 5  $\mu\text{M}$ .  
Incubations were performed in the dark for 1 h,  
after which the tubes were cooled on ice and irradiated  
for 5 min with a Mineralight short wave ultraviolet  
lamp (Ultraviolet Products, Inc.). Following irradiation,  
the samples were diluted with 100  $\mu\text{l}$  sodium  
dodecylsulphate (SDS) sample buffer (5% SDS, 4%

$\beta$ -mercaptoethanol, 13% glycerol, 0.002% bromphenol  
blue, 0.2 M Tris-HCl (pH 6.8)), and 10  $\mu\text{l}$  were  
layered directly onto SDS-polyacrylamide gels.

Membranes were labelled with  $^{[3}\text{H}]\text{M PTA}$  as in  
[3].

### 2.3. Polyacrylamide gel electrophoresis

SDS-polyacrylamide gels (10% acrylamide) were  
prepared as in [3] with 1.1 mm thick slabs. Gels were  
stained in 0.05% Coomassie brilliant blue R-250,  
destained, and scanned with a Vernon gel scanner  
using a yellow filter.

Fluorography was performed as in [26] using PPO  
and dimethylsulfoxide. Gels were dried and exposed  
to Kodak X-Omat R film for 48 h at  $-70^\circ\text{C}$ . The  
developed fluorograms were scanned on a Vernon gel  
scanner.

### 2.4. Chemicals

5-Azido-trimethisoquin was prepared as in [21]  
and tritiated using  $^{[3}\text{H}]\text{methyl iodide}$  at the CEA,  
Saclay, Service des Molécules Marquées;  $^{125}\text{I}$ -labelled  
 $\alpha$ -bungarotoxin was purchased from NEN, trimethiso-  
quin was a gift from Lab. Roger Bellon; and histrio-  
nicotoin was a gift of Dr G. Kato. Native  $\alpha$ -bungaro-  
toxin was a gift of Dr Cassian Bon. Live *Torpedo*  
*marmorata* were provided by the Biological Station  
of Arcachon.

## 3. Results and discussion

When native ACh-receptor-rich membranes from  
*Torpedo marmorata* were labelled with 5-A $^{[3}\text{H}]\text{T}$   
[21] in the absence of any effector ('resting' condi-  
tions) following the procedure in section 2, the major  
labelled product was the 40 k polypeptide (fig.1B,  
2B). The presence of the label in this band was con-  
firmed by the comigration of the 5-A $^{[3}\text{H}]\text{T}$  labelled  
polypeptide with the  $^{[3}\text{H}]\text{M PTA}$ -labelled 40 k  
protein (fig.1J) and by two dimensional gel electro-  
phoresis (Dr T. Saitoh, personal communication) using  
the O'Farrell method [27]. In addition to the 40 k  
band, a few minor bands of app. mol. wt 50 k, 66 k and  
95 k became labelled but to a smaller extent (<10%)  
than the 40 k polypeptide. When the labelling of the  
membranes was carried out in the presence of an  
excess of unlabelled non-azido trimethisoquin ( $10^{-4}$  M;  
fig.1C) or of histrionicotoin ( $10^{-4}$  M; fig.1D,2C) the  
incorporation of radioactivity into the 50 k and 66 k

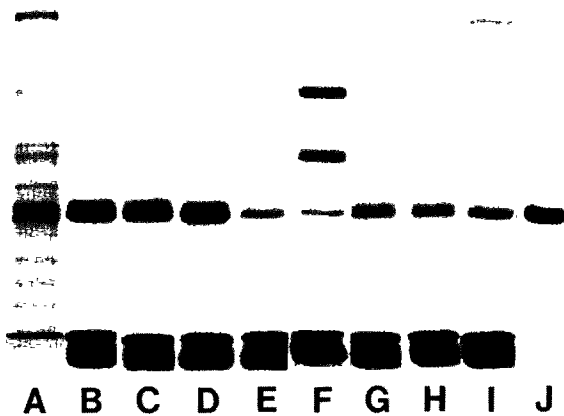


Fig.1. Effect of various competitive and non-competitive blocking agents on the labelling of the ACh-receptor rich membranes from *Torpedo marmorata* by 5-A[<sup>3</sup>H]T. The membranes were incubated, irradiated, solubilized in SDS sample buffer, and peptide chains separated by electrophoresis as in section 2. Illustrated are the Coomassie blue stain (A) of the protein components and fluorograms of 5-A[<sup>3</sup>H]T (B-I) and [<sup>3</sup>H]M PTA (J) labelling. 5-A[<sup>3</sup>H]T-labelling is shown under the following conditions: (B) resting; (C) unlabelled trimethisoquin ( $10^{-4}$  M); (D) HTX ( $10^{-4}$  M); (E)  $\alpha$ -bungarotoxin ( $10^{-5}$  M); (F) carbamylcholine; (G) unlabelled trimethisoquin ( $10^{-4}$  M) and carbamylcholine; (H) HTX ( $10^{-4}$  M) and carbamylcholine; (I)  $\alpha$ -bungarotoxin ( $10^{-5}$  M) and carbamylcholine. Carbamylcholine was present at  $10^{-4}$  M in F-I. All competitive and non-competitive agents were added prior to the addition of 5-A[<sup>3</sup>H]T.

polypeptides decreased similarly, but under the same conditions no effect was observed on the labelling of the 40 k polypeptide. On the other hand, when the membrane fragments were pre-incubated with  $\alpha$ -bungarotoxin (fig.1E), the labelling of the 40 k chain decreased markedly (but not completely) indicating that, a significant fraction of 5-A[<sup>3</sup>H]T incorporated into the 40 k band results from its covalent bonding to the ACh-receptor site. This finding is consistent with the fact that the 5-AT molecule contains a quaternary ammonium group [21] and therefore might exhibit a significant affinity for the ACh-binding site. Moreover it is known that local anesthetics may bind to the ACh-receptor site in addition to their specific binding site [22,28,29].

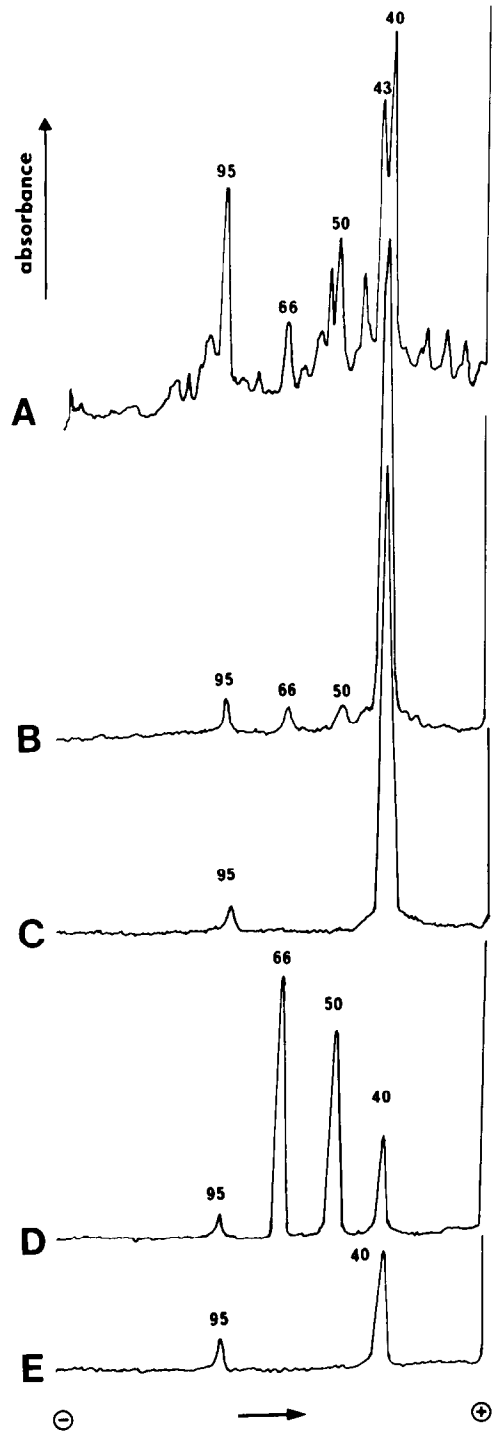


Fig.2. Scans of the Coomassie blue stained gel (A) and fluorograms of 5-A[<sup>3</sup>H]T labelling (B-E) from fig.1. The following conditions are illustrated: (B) resting; (C) HTX; (D) carbamylcholine; (E) HTX and carbamylcholine. Concentrations are given in the legend to fig.1.

Equilibration of the receptor-rich membranes with carbamylcholine, under conditions where the ACh-receptor is stabilized into its high affinity state [29,30], resulted in a marked change of the labelling pattern by 5-A[<sup>3</sup>H]T (fig.1F,2D). In the presence of carbamylcholine, the incorporation of radioactivity in the 50 k and 66 k protein increased markedly while the labelling of the 40 k became similar to that found with the resting membranes in the presence of  $\alpha$ -bungarotoxin (fig.1E). As found with the resting membranes, the labelling of the 50 k and 66 k bands (but not that of 40 k) was blocked by unlabelled trimethisoquin (fig.1G) and by histrionicotoxin (fig.1H,2E). Moreover, preincubation of the membranes with  $\alpha$ -bungarotoxin abolished the effect of carbamylcholine on the labelling of the 50 k and 66 k polypeptides (fig.1I). These data

are consistent with the results of binding studies carried out with reversible fluorescent [31] and radioactive [15,22] local anesthetics. In the presence of cholinergic agonists the high affinity binding of local anesthetics increases and this binding is blocked by histrionicotoxin, a characteristic ligand of the binding site for non-competitive blockers [32,33]. Occupation of the ACh-receptor site by  $\alpha$ -bungarotoxin both prevents the accessory binding of local anesthetics to the ACh-receptor site and the allosteric effect of carbamylcholine on the high affinity binding of local anesthetics to their 'specific' site [15].

The selectivity of 5-A[<sup>3</sup>H]T binding in the presence of carbamylcholine is evident from the rather impure membrane preparation used for the experiments illustrated in fig.1,2 (see Coomassie blue stain in

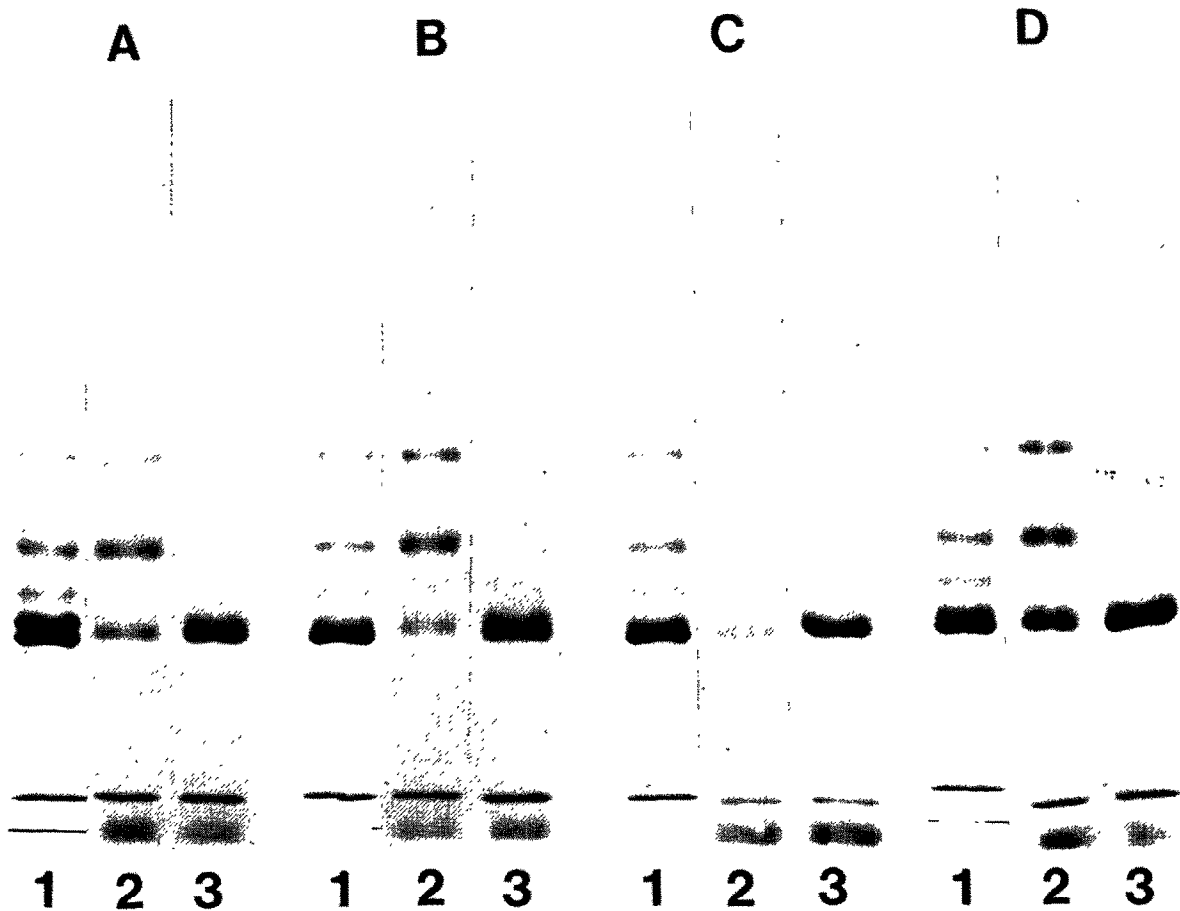


Fig.3. 5-A[<sup>3</sup>H]T labelling of several fractions from the reconstitution cycle. Coomassie blue staining (1) and fluorograms of 5-A[<sup>3</sup>H]T labelling in the presence (2) and absence (3) of  $10^{-4}$  M carbamylcholine are shown for native (A), alkaline-treated (B), Na-cholate solubilized (C) and reconstituted (D) membranes.

fig.1A and the scan in fig.2A). In this preparation, a number of polypeptide chains with app. mol. wt  $\geq 95$  k, 50–66 k and  $<40$  k were present. The only non-receptor peptide labelled by 5-A[ $^3$ H]T was the 95 k band, but the labelling of this chain was unaffected by competitive and non-competitive blocking agents and therefore must be considered as non-specific. In addition, no small molecular weight degradation products were observed when the sample in fig.1F was run on a 20% acrylamide–SDS gel.

Extraction of the 43 k polypeptide by pH 11 treatment did not change the labelling pattern of the receptor-rich membranes by 5-A[ $^3$ H]T (fig.3B<sub>2</sub>,3B<sub>3</sub>). As found with the native membranes the labelling of the 50 k and 66 k bands was still enhanced by carbamylcholine and this labelling disappeared in the presence of unlabelled trimethisoquin, histrionicotoxin and  $\alpha$ -bungarotoxin. This confirms that the 43 k protein is not involved in the binding of the non-competitive blockers to their specific site and in the reciprocal allosteric interaction between this site and the ACh-receptor site [14,17].

When the alkaline-treated membranes were dispersed into solution by Na-cholate under conditions where the allosteric interaction between cholinergic agonists and non-competitive blockers was abolished [14,23], the enhanced incorporation of 5-A[ $^3$ H]T into the 50 k and 66 k bands caused by carbamylcholine was no longer observed. On the other hand, removal of Na-cholate from the soluble receptor extract under conditions which preserve the allosteric properties of the ACh receptor protein [14] yielded a 'reconstituted' receptor which presented a labelling pattern by 5-A[ $^3$ H]T similar to that found with the native membranes.

Conclusively, the covalent labelling of the 50 k and 66 k polypeptides by 5-A[ $^3$ H]T follows the same regulation as the reversible binding of radioactive local anesthetics to their high affinity sites. Most likely, both series of compounds label the same class of sites.

The question remains, however, as to why 5-A[ $^3$ H]T labels two different polypeptide chains rather than a single one (e.g., [ $^3$ H]M PTA and the 40 k polypeptide). Several explanations may account for this rather paradoxical observation:

- (i) The 50 k and 66 k chains would not carry the binding site for non-competitive blockers but lie in its close vicinity and possess chemical residues with which the azide group of 5-A[ $^3$ H]T reacts when this compound is bound to its specific

site (for instance on the 40 k chain).

- (ii) The 50 k and 66 k polypeptides would differ from each other but nevertheless possess sites for non-competitive blockers with similar binding properties.
- (iii) The 50 k chain would carry the same site as the 66 k chain and derive from this polypeptide by proteolysis.

Future studies on the relative stoichiometries of the 50 k and 66 k polypeptides and on their quantitative labelling by 5-A[ $^3$ H]T may distinguish between these various alternatives.

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