PREFERENTIAL CHEMICAL MODIFICATION OF A BINDING SUBSITE ON THE ACETYLCHOLINE RECEPTOR*

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Summary

Following modification with trimethyloxonium tetrafluoroborate, the binding parameters of the acetylcholine receptor from Torpedo californica in the solubilized, purified state and in its native membrane environment have been studied. α-Bungarotoxin binding was not affected, but cholinergic ligand binding was. Bis-quaternary cholinergic ligands were especially sensitive to the modification. Protection studies have shown that the site of specific modification is adjacent to the acetylcholine binding site, most likely at the carboxyl group which interact with the second quaternary nitrogen group of bifunctional ligands.

Introduction

Trialkyloxonium salts have been used to modify biological macromolecules because they combine high reactivity with the capability of alkylating weakly nucleophilic groups under mild aqueous conditions (1). In cases where the reagent has been used to modify proteins, as in lysozyme (2) and trypsin (3), only a limited number of carboxyl groups were found to react, indicating a certain degree of specificity by the reagent. Recently, Rawn and Lienhard (4) have used the trimethyloxonium ion (TMO†), $(CH_3)_3O^+$, as an affinity ligand for acetylcholinesterase, reasoning that the similarity in size and charge between TMO and the functional group $(CH_3)_3N^+$- should direct the reagent towards the quaternary ammonium binding site of the enzyme.

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† Abbreviations used: TMO, trimethyloxonium ion; AcChE, acetylcholinesterase; AcChR, acetylcholine receptor; α-BuTx, α-bungarotoxin; [^{125}I]-α-BuTx, [^{125}I]-iodinated α-bungarotoxin; DAP, bis-(3-amino pyridinium)1,10-decane.

The isolation and preliminary characterization of the acetylcholine receptor (AcChR) has recently been described (5-9,13). With the availability of substantial quantities of purified AcChR in solution and in the membrane state, the chemical and physical environment around the cholinergic ligand binding site can be probed. The presence of at least one quaternary nitrogen in all cholinergic ligands strongly suggests that the receptor contains one or more anionic binding sites. In this paper we report the modification of one anionic binding site by the active-site directed reagent TMO to AcChR isolated from the Pacific electric ray, Torpedo californica.

Materials and Methods

TMO tetrafluoroborate was synthesized by the procedure of Curphy (10) and stored at -20°C under N_2 until used. [Methyl-^{3}H]carbamylcholine was prepared from $(CH_3)_2NCH_2CH_2O_2CNH_2$ and [^{3}H]-CH_3I (11). [Methyl-^{3}H]-decamethonium, [methyl-^{3}H]acetylcholine and [methyl-^{14}C]-d-tubocurarine were purchased from New England Nuclear Corp.

Acetylcholine receptor-enriched membrane fragments were prepared on a large scale by sucrose gradient-zonal centrifugation (11, 12). Solubilized, purified AcChR was prepared by affinity chromatography techniques (13, 14). Binding of small ligands to the solubilized receptor was measured by equilibrium dialysis (15) and binding to membrane fragments was measured by equilibrium dialysis or by a centrifugation assay (12). The binding of [^{125}I]-α-BuTx was measured by DEAE filter disk assay (16). AcChE was assayed with acetylthiocholine as substrate (17).

Typically, modification of the receptor by TMO was performed in the following manner: To 5 ml of purified AcChR or purified membrane fragments containing about 1 x 10^{-8} moles of α-BuTx binding sites in 10 mM Na phosphate buffer, pH 7.4, 25 mg of TMO·BF_4 was added as the solid in 5 mg portions over a 3 minute time period. The reaction mixture was maintained at 4°C in an ice bath and stirred with a magnetic stirrer throughout the addition and for 30 minutes thereafter. The pH of the reaction mixture was maintained between 6.5 and 7.0 throughout

Table I

Effect of TMO Treatment on Ligand Binding Parameters to AcChR

a. Purified AcChR

	K_D[M] Before	K_D[M] After	% Sites Remaining After TMO Treatment
α-BuTx[1]	-	-	100
Acetylcholine	1.8×10^{-6}	6.7×10^{-6}	100
Decamethonium[2]	4.0×10^{-5}	-	0
d-Tubocurarine	7.7×10^{-6}	20×10^{-6}	100

b. Membrane-Bound AcChR

α-BuTx	-	-	90
Acetylcholine	1.3×10^{-8}	1.0×10^{-8}	47
Decamethonium	1.7×10^{-7}	1.4×10^{-7}	24
Carbamylcholine	5.3×10^{-8}	5.7×10^{-8}	60

[1] α-BuTx-AcChR complex is essentially irreversible under these conditions.

[2] No decamethonium binding was observed after modification at concentrations of decamethonium used in experiments.

the reaction by the addition of solid K_2CO_3 as needed. Although control experiments showed that the TMO·BF_4 hydrolysis products did not interfere with the binding studies, the reaction mixture was routinely dialyzed before use. In experiments where cholinergic ligands had been added to protect the AcChR from TMO modification, four changes of dialysis buffer over 24 hours were made.

Results and Discussion

Small Ligand Binding to TMO-Treated AcChR:

a. Membrane fragments: Samples of purified AcChR-enriched membrane

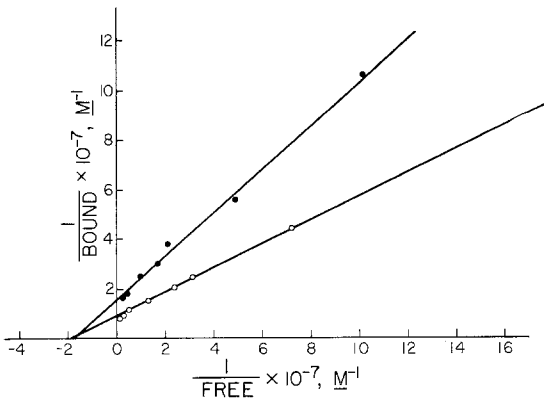

Figure 1: Double reciprocal plot of [^{3}H]-carbamylcholine binding to native and TMO modified AcChR membrane fragments. Binding data were obtained by centrifuge binding assay. Lines drawn through experimental data points obtained from least squares analysis. O - Control fragments, K_D = 5.3 x 10^{-8}M, number of sites = 1.1 x 10^{-7}M; ● - TMO treated fragments, K_D = 5.7 x 10^{-8}M, number of sites = 6.5 x 10^{-8}M. A reduction of 40% in number of sites for carbamylcholine was observed after TMO modification. Concentration of α-BuTx sites for untreated AcChR is 2.3 x 10^{-7}M.

fragments were modified by TMO and the binding of small ligands measured by centrifugation assay. For all ligands tested, the observed decreased binding was manifested as a decrease in the number of sites, with the dissociation constant for the remaining sites essentially unaltered (Table I); the extent of the decrease in sites was roughly proportional to the extent of TMO treatment. Figure 1 shows a typical double reciprocal plot obtained with carbamylcholine as the ligand. For a given TMO concentration during modification, some ligands are affected much more than others. For example, decamethonium, a bis-quaternary ligand, suffers a four-fold decrease in binding sites. Fluorescence studies with the decamethonium analog, DAP, show that its binding is totally eliminated in the TMO-treated receptor (18). On the other hand, mono-quaternary ligands such as acetylcholine or carbamylcholine are affected to a much lesser extent.

b. Purified, solubilized AcChR: The effect of TMO-treatment on purified AcChR is somewhat different than that observed for membrane fragments. In this case, the decreased binding after TMO treatment is manifested as a decrease in the affinity for a ligand with little change in the number of sites. In the case of acetylcholine and d-tubocurarine, the decrease in affinity was comparable; whether the abolition of decamethonium binding is due to a drastic decrease in

affinity or due to the elimination of sites is unknown.

The different effects on the binding of cholinergic ligands observed in the membrane-bound state and in the solubilized state are probably due to the molecular changes which occur upon detergent extraction and solubilization of the AcChR from membranes. Ligand affinities for all ligands decrease upon solubilization; the affinities of the agonists acetylcholine and carbamylcholine decrease by at least two orders of magnitude, while the antagonists of the AcChR are minimally affected (12, 19).

Protection Studies: Sodium chloride was used to protect against modification with TMO·BF_4 since it is known that the concentration of NaCl greatly affects the affinities of some ligands like decamethonium and d-tubocurarine but has little effect on other ligands such as acetylcholine or carbamylcholine (12). Our results (Table II) show that the decrease in the number of binding sites for carbamylcholine is approximately the same regardless of whether a "protecting" ligand was present or not during the TMO treatment. When decamethonium binding

Table II

Protection Against TMO Inactivation of Membrane Bound AcChR

a. Carbamylcholine Binding

Protecting Ligand, Concentration	% Sites Remaining After TMO Treatment
None	60
Carbamylcholine, 10^{-4}M	74
NaCl, 2 x 10^{-1}M	60

b. Decamethonium Binding

None	24
NaCl, 2 x 10^{-1}M	80

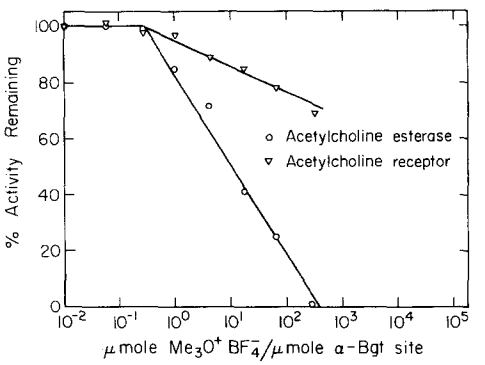

Figure 2: Inhibition of AcChR and AcChE by trimethyloxonium ion. Decamethonium binding to the AcChR (▽ — ▽) was followed by equilibrium dialysis procedures at fixed ^{3}H-decamethonium and AcChR concentrations as a function of TMO concentration. AcChE activity (o — o) as a function of TMO concentration was followed by the hydrolysis of acetylthiocholine (17).

to TMO-treated AcChR was investigated, it was found that NaCl (200 mM) afforded substantial protection against the inactivation of decamethonium binding. While TMO-treated membrane fragments lose about 80% of their decamethonium binding ability, those treated with TMO in the presence of NaCl lose only 20%. Similar results were obtained when decamethonium was used as the protecting ligand. In contrast, carbamylcholine did not protect against the loss of decamethonium binding

In order to account for the relatively high affinity of the AcChR for decamethonium-like bis-quaternary ligands, it is assumed that both ends of the bifunctional ligand bind to distinct sites on the receptor molecule. One site corresponds to the acetylcholine binding site and the second site lies within a 10-12Å radius of the former. These studies suggest that it is the second site which is esterified by TMO. This would account for the extreme sensitivity of decamethonium and DAP binding to TMO modification of the AcChR and to the relative insensitivity of acetylcholine or carbamylcholine. The protection studies suggest that cations bind to the second site and account for the cation sensitivity of bis-quaternary ligands as compared to mono-quaternary ligands which bind at the first site only. The small effect that TMO has on mono-quaternary ligands may be due to a slight local conformational change which affects the first site indirectly after modification of the second site.

Inactivation of Acethycholinesterase: Inactivation of AcChE activity was observed at much lower TMO concentrations than was the inactivation of decamethonium binding to the AcChR (Figure II). These results on the Torpedo AcChE agree with those of Rawn and Lienhard (4) who showed that Electrophorus electricus AcChE is effectively inactivated by TMO at low concentrations.

Effect on [^{125}I]-α-Bungarotoxin Binding: Treatment of purified AcChR with TMO at concentrations normally used produced no measurable effect on receptor toxin complex formation (Table I), although the binding of small cholinergic ligands is affected. Treatment with substantially greater amounts of TMO resulted in partial abolition of toxin binding. The decrease in binding is probably due to non-specific alteration of the protein structure, as it is accompanied by some precipitation. Treatment of membrane fragments at low concentrations of TMO produced an aggregation of a fraction of the membrane fragments and a reproducible decrease in the number of toxin sites (~ 10%).

The substantial effect of TMO modification on the binding of cholinergic ligands and the comparatively small effect on toxin binding could be explained by a) toxin and small ligand binding sites do not overlap, or b) binding of toxin to the cholinergic site involves multisite interactions and the presumed carboxyl group on the AcChR responsible for cholinergic ligand binding is relatively unimportant for toxin binding. While it has not been definitively shown that toxin and ligand sites overlap, it is generally regarded that they may, since treatment of solubilized AcChR with α-BuTx abolishes all small ligand binding (15) and, conversely, small ligands slow the rate of toxin binding (20). Also, the quaternary nitrogen binding sites, the target of our modification efforts, is present in both AcChR and in AcChE and is essential for the binding of small ligands. However, α-BuTx binds only to the AcChR and not to the AcChE. This supports our hypothesis that this site is not the major determinant in the formation of the toxin-receptor complex.

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