CROSSLINKING OF α-BUNGAROTOXIN TO THE ACETYLCHOLINE RECEPTOR FROM *TORPEDO MARMORATA* BY ULTRAVIOLET LIGHT IRRADIATION

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1. Introduction

The acetylcholine (ACh) receptor purified from the electric organ of *Torpedo* (sp) is an oligomer composed of 4 different subunits having app. M_r-values on SDS–polyacrylamide gels of 40 000 (α), 50 000 (β), 60 000 (γ), and 66 000 (δ) and a stoichiometry of $\alpha_2\beta\gamma\delta$ [1–3]. The α subunit is labeled by affinity reagents (TDF [4,5]; MBTA [6,7]; MPTA [8]; BrACh [9,10]; DAPA [11]) known to bind to, or in the close vicinity of, the ACh binding site. However, the δ chain reacts covalently with 5A[^{3}H]T [12–14], a photoaffinity derivative of the non-competitive blocker trimethisoquin [15], and with other reversible noncompetitive blockers (e.g., phencyclidine, histrionicotoxin) by simple UV irradiation [16]. Other non-competitive blockers label subunits in addition to the δ chain. Following UV irradiation, chlorpromazine labels all of the subunits [16]. Thus, the ACh binding site is carried, mainly, by the α chain and the site for non-competitive blockers, mainly, by the δ chain, but other subunits may also contribute to one or both of these sites[17].

The α-toxins from snake venoms behave as competitive antagonists of ACh for its site [18,19] but differ from the classical curare-like agents by the fact that they are polypeptides in nature and of high M_r (7000–8000). Because of their size, they may be useful in exploring the vicinity of the ACh binding site. Studies with photolabile derivatives of α-bungarotoxin [20] or with added crosslinking agents and radioactive α-bungarotoxin [21,22] gave crosslinking products of 7000–8000 M_r (on SDS–polyacrylamide gels) greater than some of the ACh receptor subunits. The major crosslinked products were the α and δ chains, but the β and γ chains also could be labeled slightly under certain conditions [22]. The conclusions were that the binding site for snake α-toxins resides on the α chain; whereas, the other chains, in particular the δ subunit, were 'within crosslinking distance of the bound toxin' *or* that 'some if not all of the crosslinking was due to diffusion and collision' [22].

We have found, using the same method as that used with some non-competitive blockers [16], that α-[^{125}I]-bungarotoxin (Bgt) can be crosslinked covalently to ACh receptor subunits by simple UV irradiation. This allows the analysis of toxin–receptor crosslinked products without the complication of an intervening 'crosslinking arm'.

Abbreviations: ACh, acetylcholine; α-Bgt, α-bungarotoxin; BrACh, bromoacetylcholine; DAPA, bis(3-azidopyridinium)-1,10-decane perchlorate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; MBTA, 4-(*N*-maleimino)benzyltrimethylammonium; MPTA, 4-(*N*-maleimido)phenyltrimethylammonium; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; TDF, *p*-trimethylammonium benzene diazonium fluoroborate

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2. Methods

2.1. *Preparation of membranes*

ACh receptor-rich membranes were purified from freshly dissected *Torpedo marmorata* membranes as in [13,23] using a buffer designed to inhibit endogenous proteolytic activity (PI buffer: 50 mM Tris–HCl, pH 7.5 (20°C), 3 mM EDTA, 1 mM EGTA, 0.1 mM PMSF, 5 units aprotinin/ml and 5 μg pepstatin/ml). Membranes were stored in liquid nitrogen at 20 to 25 μM in α-Bgt sites until use.

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2.2. *Ultraviolet light-induced crosslinking*

Samples of ACh receptor rich membranes were diluted to 10 μM in α-Bgt sites and incubated with varying [α-^{125}I-Bgt] in PI buffer. After 1 h incubation, the mixture was centrifuged for 2 min at top speed in a Beckman airfuge. The pellet was washed 3 times with 170 μl PI buffer by centrifugation and resuspension as above. The pellet was resuspended to the original volume, and 20 μl aliquots were placed in a chamber for irradiation. The chamber was flushed with nitrogen for 15 min and the membranes were irradiated with a Mineralight short-wave UV lamp (Ultra-Violet Products, San Gabriel CA) placed outside the chamber at a distance of 15 cm (254 nm, 1200 μW/cm^2) from the samples. The solutions were separated from the light source by a thin sheet of cellophane that absorbed <5% of the light. The time of irradiation varied from 1–30 min, with the value for each experiment given either in the text or in a figure legend. Irradiation times were limited by crosslinking of receptor subunits, which results in products that do not enter the gels, and by the loss of radioactive iodine from α-^{125}I-Bgt after prolonged irradiation. The iodinated species liberated after irradiation did not label the receptor peptides as no label comigrated with native receptor subunits. The labeled bands migrated on denaturing gels with app. M_r 7000–8000 greater than those of receptor subunits.

Following irradiation, the samples were diluted with 20 μl SDS sample buffer (5% SDS, 4% β-mercaptoethanol, 13% glycerol, 0.002% bromphenol blue, 0.2 M Tris–HCl, pH 6.8 at 20°C), and 35 μl were layered directly onto SDS–polyacrylamide gels.

2.3. *Polyacrylamide gel electrophoresis*

SDS–polyacrylamide gels (10% acrylamide) were prepared as in [8] using 1.1 mm thick slabs. Gels were stained in 0.5% Coomassie brilliant blue R-250, destained, and scanned with a Vernon gel scanner using a yellow filter. Gels were then dried and exposed to Kodak X-Omat AR film preflashed to an absorbance of 0.2. Alternatively, dried gels were cut into 1 mm slices and counted in a Beckman multiwell γ-counter.

2.4. *Chemicals*

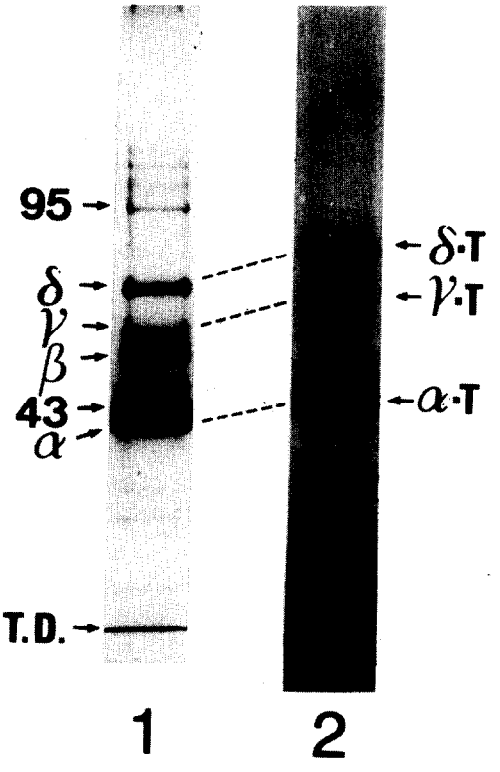
Purified α-Bgt was a gift of Drs B. Holton and T. Saitoh, and α-^{125}I-Bgt was purchased from NEN. Acrylamide, bisacrylamide, and TEMED were products of Kodak. Live *Torpedo marmorata* were provided by the Biological Station of Arcachon.

3. Results and discussion

Fig.1 shows that UV irradiation of α-^{125}I-Bgt bound to native ACh receptor-rich membranes from *Torpedo marmorata* results in the covalent crosslinking of α-^{125}I-Bgt to several ACh receptor subunits. After SDS–polyacrylamide gel electrophoresis and β-mercaptoethanol (section 2), 3 sharp radioactive bands were detected by autoradiography. Their app. M_r was 48 000, 66 000 and 74 000 respectively, which coincided with the app. M_r of the α,γ and δ chains increased by 7000–8000; i.e., the M_r of α-Bgt.

The observed labeling and change in migration of the polypeptide chains resisted heating at 100°C for 5 min and treatment with 5% β-mercaptoethanol which both destroy all α-Bgt binding activity in non-irradiated samples. Thus, the labeling did not correspond to a low affinity reversible association between α-Bgt and receptor polypeptides [24,25] but to the covalent attachment of the α-toxin.

In general, ≤5% of bound α-^{125}I-Bgt was recovered as a crosslinked product after SDS gel electrophoresis. Under these conditions, as shown in fig.1b and 2, the



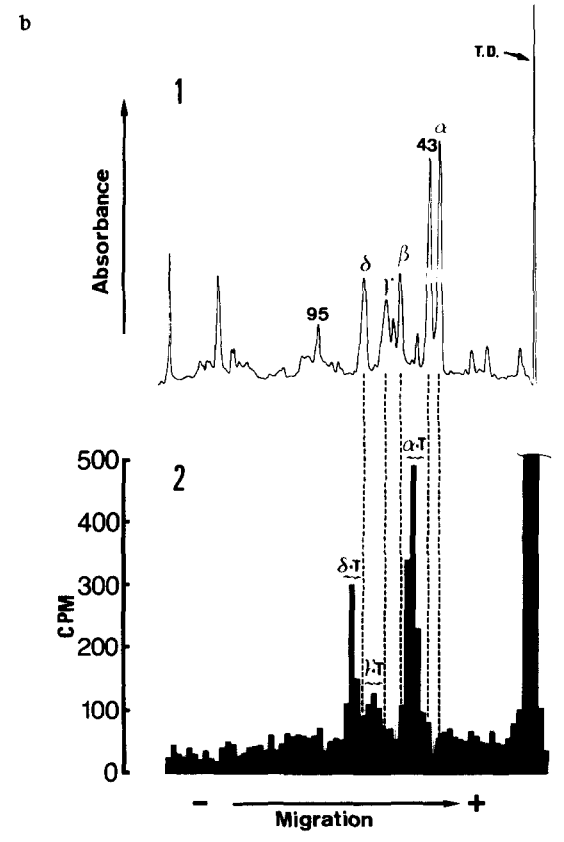


Fig.1. Ultraviolet light-induced crosslinking of α-^{125}I-Bgt to the membrane-bound ACh receptor from *Torpedo marmorata*. About 3% of the α-toxin sites were occupied by α-^{125}I-Bgt, and the irradiation time at 254 nm was 20 min. (a) (1) Coomassie blue stain of a 10% polyacrylamide SDS gel showing the membrane preparation crosslinked with α-^{125}I-Bgt; (2) autoradiogram of the dried gel showing the positions of the labeled crosslinked products.
(b) (1) Scan of the Coomassie blue-stained gel shown in (a,1); (2) quantitation of the α-^{125}I-Bgt incorporated into receptor peptides by counting 1 mm strips of the dried gel.

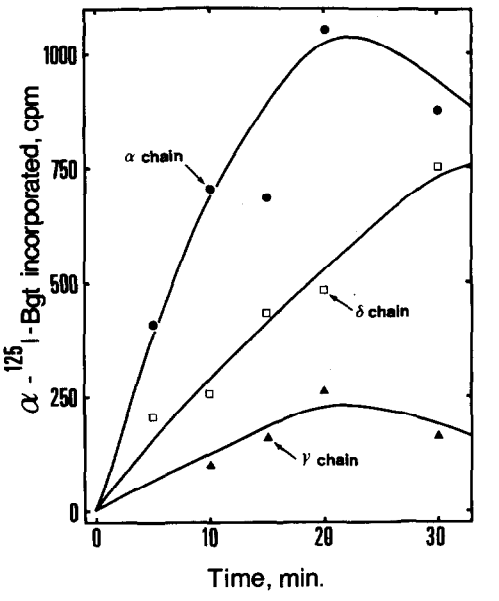


Fig.2. Time course of the incorporation of α-^{125}I-Bgt into ACh receptor subunits. α-^{125}I-Bgt was bound to the ACh receptor at 3% saturation, free toxin was removed, and toxin–receptor complexes were irradiated at 254 nm for the times indicated. Quantitation was performed as in fig.1, and 1000 cpm represented ~2.5% of the total α-^{125}I-Bgt bound.

stoichiometry of the labeling of the α,β,γ and δ chains was 4:0:1:2. The same stoichiometry was found up to irradiation times of ~20 min. After this time crosslinking between receptor subunits (or possibly other extrinsic proteins) began to alter their electrophoretic mobility, thus making impossible an accurate measurement of the crosslinked products.

The selectivity of the crosslinking process is illustrated in fig.3. Identical crosslinked products were observed with the same stoichiometry both when only 5% or when 100% of the available sites were occupied by α-^{125}I-Bgt (fig.3A,C). In both cases, preincubation with 20 μM unlabeled α-Bgt (fig.3B,D) completely abolished the crosslinking of α-^{125}I-Bgt. MPTA, the affinity reagent known to label the agonist (ACh) binding site on the α chain, inhibited the binding of α-^{125}I-Bgt and, thus, its covalent crosslinking (fig.3E). The non-competitive blocker phencyclidine [14,26] at a concentration 10-fold higher (10 μM) than the K_d for its high affinity site had no effect on the crosslinking process even when maintained at 10 μM throughout the washing and irradiation steps.

The question of whether the portion of the δ subunit labeled by the photoaffinity reagent for the site for non-competitive blockers [12–14], 5-azido-[^{3}H]-trimethisoquin (5A[^{3}H]T, [15]), overlaps the portion of the δ subunit labeled by α-^{125}I-Bgt was investigated by irradiating complexes of unlabeled α-Bgt and ACh receptor prelabeled with 5A[^{3}H]T. A faint band labeled with ^{3}H was observed at the 72 000 M_r position on an SDS gel. This band was not present under the following conditions: (i) in non-irradiated samples; (ii) in samples irradiated in the absence of unlabeled α-Bgt; (iii) in samples in which excess phencyclidine (a specific inhibitor of the labeling of the site for non-competitive blockers by 5A[^{3}H]T) was included in the initial incu-

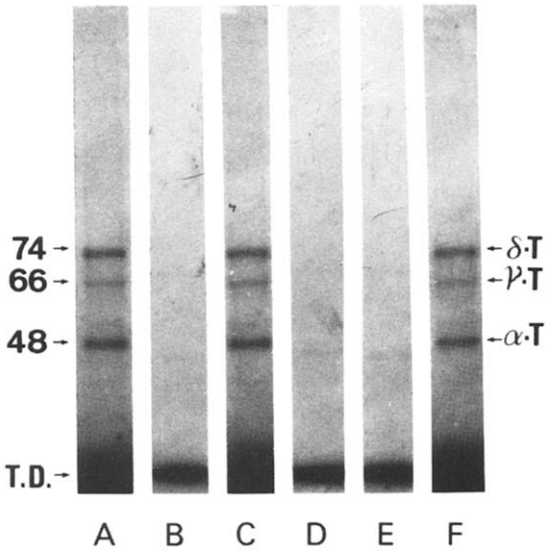


Fig.3. Autoradiograms of the incorporation of α-^{125}I-Bgt into ACh receptor peptides under the following conditions: (A) 3% of the α-toxin sites occupied by α-^{125}I-Bgt; (B) pretreatment with unlabeled α-Bgt, same concentration of α-^{125}I-Bgt as in (A); (C) 100% saturation with α-^{125}I-Bgt; (D) pretreatment with unlabeled α-Bgt, same concentration of α-^{125}I-Bgt as in (C); (E) pretreatment of DTT-reduced receptor with MPTA, same concentration of α-^{125}I-Bgt as in (A). (F) inclusion of unlabeled phencyclidine (10 μM) in all solutions, same concentration of α-^{125}I-Bgt as in (A). The large amount of radioactivity at the tracking dye in conditions A, C and F reflects α-^{125}I-Bgt bound to the receptor but not covalently crosslinked. In conditions B, D and E, a much lower quantity of α-^{125}I-Bgt was bound due to the presence of inhibitors and that which was not bound was washed out prior to irradiation.

bation with 5A[^{3}H]T. The most plausible explanation for this result is that α-Bgt interacts with a portion of the α chain that is distinct from the site for non-competitive blockers. In addition, this confirms the assumption that the 72 000 M_r complex is composed of α-Bgt and the δ subunit.

These results are consistent with those in [20–22] where α-Bgt was bound covalently to *Torpedo californica* ACh receptor using crosslinking agents or derivatives of α-Bgt with photolabile arylazide sidechains. In all cases the major crosslinking occurred with the α and δ chains and to a lesser extent with the γ chain (review [27]). The major contribution of this study is that no 'crosslinking arm' was employed so that the labeling of the subunits can be assigned to a direct interaction of α-Bgt with these chains. The slight labeling of the γ chain is consistent with the finding that, under conditions in which 5A[^{3}H]T labels the ACh site, the γ chain is also slightly labeled [14].

The observed stoichiometry of labeling cannot be readily interpreted in terms of site stoichiometry since only a fraction of the bound toxin molecules are covalently attached to the receptor chains. Nevertheless, the fact that the ratio of incorporation of α-^{125}I-Bgt into the α and δ chains (2:1) is the same as the stoichiometry of these subunits in the 250 000 M_r oligomer of the ACh receptor (light form) suggests the possibility that the 'reactivity' of α-Bgt bound to the ACh receptor is the same for the α and δ chains. However, this 'reactivity' may be smaller with the γ chain. Since a maximum of 2 α-Bgt molecules bind/receptor oligomer (review [27,28]), a possible interpretation of these data is that each α-Bgt site is built from 1 α chain + 1 of the neighboring subunits. Accordingly, 1 of the α-Bgt sites would be made up of 1 of the α chains + the δ chain and the other by the second α chain + the γ chain. This interpretation is consistent with the observation that the 2 ACh α-toxin sites exhibit different binding properties ([29], review [28]).

In conclusion, the UV irradiation method for crosslinking appears to reflect accurately the interactions of a polypeptide ligand with its receptor and, thus, could possibly be extended to the study of other receptors for polypeptide hormones and neurotransmitters.

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