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Affinity Labeling of the Subunits of the Membrane Associated Cholinergic Receptor

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SUMMARY

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Membranes enriched in acetylcholine receptor have been purified from *Torpedo californica* electric organs by gradient sedimentation and affinity partitioning. The preparations contain 40,000, 49,000, 60,000, 67,000 and 105,000 dalton peptides; the latter peptide can be virtually removed by the partitioning step. Two-dimensional electrophoresis reveals that the 67,000 and to a lesser extent the 49,000 dalton peptides exist as disulfide linked dimers. *p*-(Trimethylammonium) benzenediazonium fluoroborate (TDF), a structural analogue of phenyltrimethylammonium, binds covalently to the 40,000 and 105,000 dalton peptides present in the membrane fragments but only the labeling of the 40,000 dalton subunit is blocked by prior association of cholinergic agonists, antagonists and α-toxin. The protectable labeling is saturable and stoichiometric with irreversibly blocked toxin sites; one molecule of [^{3}H]TDF binds per toxin site. TDF thus appears to label specifically the ligand/toxin binding site of the receptor. Procedures which convert the receptor to its high affinity state for agonists such as prolonged incubation with agonist or short term incubation with agonist and local anesthetic enhance the extent of protection against irreversible labeling by TDF. Hence, covalent labeling by TDF appears to distinguish the two interconvertible receptor states identified previously in the membrane preparation by their different affinities for agonists.

INTRODUCTION

The availability of membrane fragments of high specific activity in α-toxin sites has enabled investigators to characterize the composition and properties of the *Torpedo* cholinergic receptor while residing in its native membrane environment (1-5). To purify membranes enriched in receptor, we have relied on a combination procedure of gradient sedimentation and affinity partitioning (2). This results in obtaining membranes having a high degree of morphologic uniformity and specific activities which approach those of the solubilized receptor. Using these membranes we have analyzed further the subunit composition of the receptor and have attempted to characterize the ligand binding site with the use of the irreversible label, TDF[3] (6).

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[3] The abbreviations used are: TDF, *p*-(trimethylammonium)benzenediazonium fluoroborate; MBTA, 4-(N-maleimido)-benzyltrimethylammonium iodide; SDS, sodium dodecyl sulfate; DTT, dithiothreitol.

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Prolonged application of agonists to the cholinergic receptor results in a progressive loss of the response achieved initially. As originally shown by Katz and Thesleff (7), the kinetics of this desensitization process appear to be consistent with the following cyclic scheme (cf. 8):

$$\begin{array}{ccc} L + R & \rightarrow & LR \\ \text{slow}\downarrow\uparrow & & \uparrow\downarrow\text{slow} \\ L + R' & \rightarrow & LR' \end{array}$$

The receptor, in absence of ligand, L, prevails in R, a low affinity state for agonists and certain antagonists. The conversion from the active complex, LR, to the desensitized form, LR′, is driven by the higher affinity of the ligand for the R′ state. Removal of ligand results in a slow reversion to activatable receptor (R′ → R). The isolated membrane-associated receptor *in vitro* can also be shown to exist in two discrete states which differ in their affinity for agonists (9–11). The kinetics of ligand-induced conversion to the high affinity state and its reversion following removal of ligand indicate that the R′ state reflects the desensitized form of the receptor *in situ* (9, 10).

An additional technique that could be used to demonstrate two receptor states *in vitro* is affinity labeling. The specific covalent binding of an affinity label is dependent on the ligand's affinity in a reversible complex with the binding site and on the apposition while in the complex of reactive groups on the ligand and the macromolecule (12). An increase in the affinity of the binding site for the ligand is likely to be reflected in an increase in specific labeling. The alkylating reagent, MBTA, a cholinergic antagonist, has been shown to affinity label after disulfide reduction the purified nicotinic receptor from *Torpedo* and *Electrophorus* electric organs (13, 14) and rat diaphragm (15). Photoaffinity labeling reagents have been employed recently to label the membrane-associated receptor from *Torpedo* (16, 17); however, the specificity of these compounds appears to be lower.

A candidate for a rapidly reacting ligand which can discriminate between the R and R′ states of the receptor is the cholinergic affinity agent TDF. This ligand with its reactive diazonium group can form covalent bonds with tyrosine, histidine, and lysine side chains of proteins. Prior reduction of the receptor is not essential for achieving labeling. *In vivo* TDF irreversibly blocks the nicotinic receptor in the *Electrophorus* electroplaque cell (18, 19).

MATERIALS AND METHODS

Materials. The cholinergic ligands and cobra α-toxin were obtained as reported (10, 20). All other chemicals were of the highest purity available.

Receptor-rich membrane fragments from *Torpedo californica* were purified and concentrated as described previously (2, 20, 21). Specific activity of the various preparations ranged between 0.8–1.6 nmoles of α-toxin sites/mg of protein following gradient centrifugation and 2.9–4.5 nmoles/mg following recovery from the affinity partitioning step (2, 10, 20). Proteins were measured by the Lowry assay using bovine serum albumin as a standard.

Two dimensional gel electrophoresis. This procedure essentially followed the techniques of Laemmli (22) and Cleveland *et al.* (23) with the following specifications. Receptor-rich membranes collected from the affinity partitioning step were dissolved in 1% SDS, placed in a boiling water bath for one minute and subjected to SDS gel electrophoresis on 8.0 cm tubes using 5% acrylamide. A 3 mm thick slab gel of 5% acrylamide was prepared on the same day containing a 1% agarose stacking gel. The tube gel immediately following electrophoresis was placed in the stacking gel and covered with an additional 0.5 cm of agarose gel. After 20 min electrophoresis was run in the second dimension and the slab gel subsequently stained with Coomassie blue. To achieve reduction in the second dimension 150 mM DTT was added to the stacking gel.

Formation and maintenance of disulfide bonds. To minimize disulfide bond formation or interchange following denaturation, the membranes were reacted with 10 mM N-ethylmaleimide prior to addition of SDS. Membranes were treated with 0.1 mM Cu^{++} $(phenanthroline)_2$ for 30 min at 22° to maximize formation of disulfide bonds (24, 25). The reaction was quenched with 2.0 mM

EDTA and 10 mM N-ethylmaleimide prior to addition of SDS.

Preparation of non-radioactive and [^{3}H]TDF. TDF was synthesized from dimethyl(p-aminophenyl)ammonium chloride as described by Pressman *et al.* (26) and Traylor and Singer (27). The product was recrystallized in a minimal volume of 25% fluoroboric acid by addition of methanol, to yield fluffy white needles which degassed and melted at 150°. The initial product degassed and melted at 146°. The elemental analysis (Galbraith Laboratories, Knoxville, Tenn.) was consistent with one mole of water of hydration. Found (recrystallized product): C 29.90, H 3.55, N 11.71, F 43.17%, C:N 2.57; calculated ($C_8H_{13}N_3F_8B_2 \cdot H_2O$): C 30.46, H 4.27, N 11.92, F 42.83, C:N 2.55. Therefore a molecular weight of 354.9 was assumed. The λ_{max} and ϵ^M_{max} at pH 6.0 for the product of the reaction of the recrystallized TDF with a 100-fold molar excess of N-chloroacetyl-L-tyrosine (Sigma) were 322.2 nm and 18,200, respectively [lit. (27): λ_{max} = 322.5 nm; $\epsilon^M_{322.5}$ = 19,900 at pH 5.6–6.2].

Radiolabeled [^{3}H]-TDF was synthesized as above except that [^{3}H]CH_3I (New England Nuclear) was used in the methylation step and the final product was not recrystallized but immediately dissolved in 0.01 M HCl at a concentration of 1–2 × 10^{-3} M and stored at −65°. The molarity of the solution was determined from the $OD_{322.2}$ after reaction with excess N-chloroacetyl-L-tyrosine. The specific activity determined from the molarity of the N-chloroacetyl-L-tyrosine adduct was 2.22 × 10^{13} cpm/mole or approximately 30 Ci/mole (~30% efficiency). The reagent was radiochemically pure as shown spectroscopically and by thin-layer chromatography of N-chloroacetyl-L-tyrosine coupled [^{3}H]-TDF on Eastman silica gel sheets using (66)n-butanol-(17)H_2O-(17)glacial acetic acid (R_f ~0.47) and Eastman cellulose gel sheets using (8)n-butanol-(2)ethanol-(1) glacial acetic acid-(3)H_2O (R_f ~0.79) as the solvent systems. Only a single peak of radioactivity was observed which contained all the radioactivity applied to the plates.

Covalent binding of TDF to the membrane fragments. Determination of covalent binding of TDF to the membrane-bound receptor was done by two methods: indirectly by measurement of the loss of α-toxin binding sites and directly by measurement of [^{3}H]TDF labeling of the membrane proteins. Typically, to 20 μl of a membrane suspension 0.5 to 15 μM in α-toxin binding sites in 0.05 M NaCl, 0.03 M sodium phosphate, pH 7.4, at 22° was added 1 μl of buffer or buffer containing 0.01 M carbamylcholine, followed by 6 μl of the desired concentration of TDF in 0.01 M HCl. Thirty seconds after the TDF addition, 1 μl of 0.1 M tyramine hydrochloride (Sigma) was added to quench the unreacted TDF. Twenty minutes later an aliquot of the reacted membranes was diluted 300- to 1000-fold into 0.1 M NaCl, 0.01 Na phosphate, pH 7.4, and the concentration of receptor-bound [^{125}I]α-toxin at equilibrium determined with α-toxin in large molar excess, using the DEAE filter disc assay described previously (10). The effect of the products of the decomposition of TDF in solution and of the reaction of TDF with tyramine on toxin bound at equilibrium was examined and after dilution no inhibition was observed. To determine irreversibly blocked α-toxin sites after TDF labeling, bound toxin at equilibrium was compared with binding to a control aliquot of membranes which had not had TDF added.

For direct measurement of [^{3}H]TDF labeling, 6 to 10 μl of the tyramine-quenched reaction solution was made 1% in SDS and β-mercaptoethanol and placed in boiling water for two minutes. The samples were then electrophoresed in the presence of 0.1% SDS on 5% acrylamide disc gels according to the method of Weber *et al.* (28). Gels were stained in a solution of 0.15% Coomassie blue, 50% methanol, and 7% acetic acid, destained in 50% methanol, 7% acetic acid, and rehydrated in 7% acetic acid. After rehydration, the gels were cut into 2 mm slices and each slice placed in a scintillation vial with 300 μl of 30% hydrogen peroxide. The vials were tightly capped and heated to 120° for 20 min or until the gel slice was dissolved. After the vials had cooled, 200 μl of 4 M urea, 1% SDS was added to each vial. Five milliliters of scintillation fluid, made up of 4 gr of Omnifluor (New England Nuclear) per liter of 33% Triton X-100, 67% toluene, was added and

the ^3H activity counted on a Packard Tricarb scintillation spectrometer. Specific [^3H]TDF labeling was defined as the difference between the ^3H counts covalently bound in the absence and in the presence of 0.4 mM carbamylcholine. Standard aliquots of [^3H]TDF added to control gels prior to digestion were used to correct for variations in counting efficiency. TDF concentrations in the gel slices could then be ascertained from the recovered CPM and the previously determined specific activity and concentration of the [^3H]TDF standard.

RESULTS

Gel electrophoresis of the peptides in the membrane fragments. Following solubilization of the membranes purified by affinity partitioning, electrophoresis in the presence of DTT reveals predominate peptides of 40,000 (α), 49,000 (β), 60,000 (γ), 67,000 (δ) and 105,000 daltons (Fig. 1). The latter peptide, an abundant entity in the crude fractions following the centrifugation step, is removed to a large extent by the final affinity partitioning step. Thus, as suggested previously (2), this peptide likely reflects the extent of contamination of the preparation.[4] Integration of Coomassie blue staining intensities from five separate preparations reveals molar ratios of: $\alpha = 1.0$, $\beta = 0.32 \pm 0.02$, $\gamma = 0.20 \pm 0.02$, $\delta = 0.29 \pm 0.02$ (means $\pm$ standard errors—12 scans from six different preparations).

Electrophoresis in the absence of DTT yields a different pattern and the origin of the peptides can be best discerned by two-dimensional gel electrophoresis. When electrophoresis is conducted in the absence of DTT and the gel is subsequently reduced and electrophoresed at right angles, peptides appearing off the diagonal should have contained disulfide bonds susceptible to reduction. Two such peptides are evident. The dominant one appears at 135,000 daltons and should be a dimer of the 67,000 or δ subunit (Fig. 2a). A second peptide, although it is less intense, appears at 102,000 daltons in the non-reduced dimension. Its position and molecular weight would indicate it to be a dimer of the β subunit. Hence two peptides of 49,000 and 67,000 molecular weight appear to exist at least in part as dimeric species containing interchain disulfides.

The Cu^{++}-(phenanthroline)$_2$ complex is known to catalyze the oxidation of sulfhydryl groups and maintain disulfide bonds in their oxidized state (24). When applied to the membrane fragments, it appears to increase slightly the density of the dimeric spots relative to the monomeric peptide species (Fig. 2b). The influence of Cu^{++}-(phenanthroline)$_2$ should only serve to promote dimerization while the receptor is in the membrane since the metal is scavenged by EDTA prior to SDS solubilization. If EDTA was added prior to Cu^{++}-(phenanthroline)$_2$, no enhancement of dimerization was evident. Moreover, for the electrophoretic patterns obtained in Figs. 2a and b, the membranes are treated with N-ethylmaleimide prior to solubilization. This should minimize disulfide bond formation or interchange that might occur upon addition of detergent. These experiments do not exclude the possibility that air oxidation of free thiols occurs during preparation of the membranes. This would also require that the receptor subunits have adjacent free thiols at a common interface.

Covalent binding of TDF to the α-toxin binding site. Equilibrium binding of α-toxin to the receptor-containing membranes is blocked irreversibly by prior TDF reaction with the receptor, and the extent of blockade is dependent on the concentrations of TDF and of toxin binding sites (Fig. 3). The reaction appears to be quenched endogenously within a few seconds, since addition of 4 mM tyramine 30 sec or more after initiation of the reaction has no effect on the number of toxin sites blocked. Endogenous quenching is also apparent from the decrease in the extent of toxin site blockade at a single concentration of TDF as the

[4] Following affinity partitioning, occasionally we have observed only modest increases in specific activity of α-toxin sites but a substantial enhancement of receptor purity as judged from electrophoretic profiles. Since the affinity partitioning procedure fractionates entire membrane patches, it is entirely possible to recover receptor with diminished toxin binding capacity but containing normal subunit profiles following SDS denaturation.

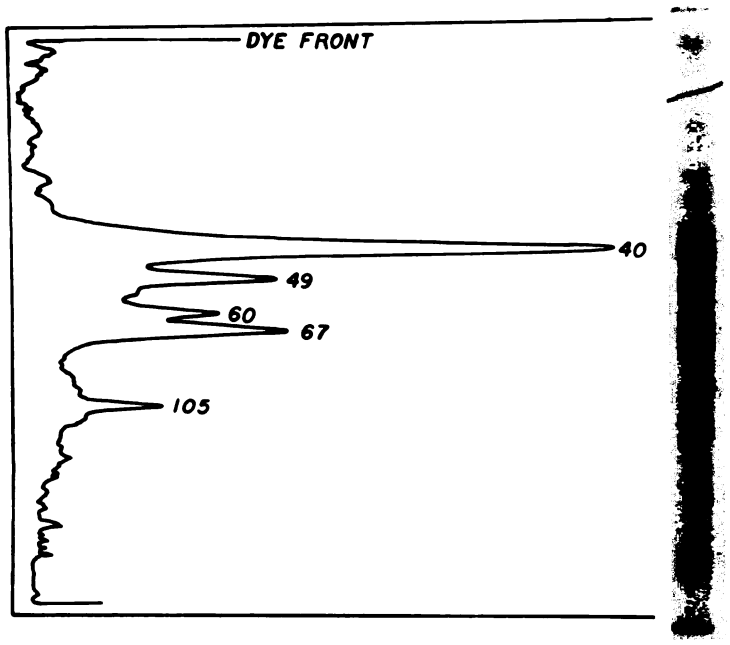

FIG. 1. *Polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate for the membrane associated acetylcholine receptor*

Receptor-containing membranes were purified by the sedimentation and affinity partitioning methods previously described (2) to specific activity of 3.5 μmol of α-toxin sites/mg of protein. DTT (5 mM) was added, and the membranes were then solubilized with 1% SDS and subjected to gel electrophoresis. Proteins were stained with Coomassie blue and scanned at 660 nm on a Gilford linear transport scanner.

membrane concentration is increased (Fig. 3). In addition to a specific reaction with the receptor, TDF must also be reacting rapidly with other components of the membrane.

Covalent binding of [^{3}H]TDF to the membrane fragment proteins. Receptor-rich membrane fragments were reacted with [^{3}H]TDF in the presence and absence of carbamylcholine and the covalent labeling analyzed by polyacrylamide gel electrophoresis in SDS. Of the five polypeptides identified in the gels (Fig. 1), only the 40,000 and 105,000 dalton species were significantly labeled. In the absence of carbamylcholine the major portion of the protein associated counts coincided with the former even when the two bands by protein staining were of equal intensity (Fig. 4). The 67,000, 60,000, and 49,000 dalton species were not found to be labeled above gel background. The labeling of only the 40,000 dalton polypeptide was markedly reduced by the presence of carbamylcholine during covalent binding. Reaction of the membranes with [^{3}H]TDF in the presence of 1 mM gallamine, 20 μM α-toxin, or 30 μM acetylcholine protected only the labeling of the 40,000 dalton species and each compound produced essentially the same extent of protection. Therefore the specific labeling site of [^{3}H]TDF as defined by the covalent binding that is protectable by cholinergic ligands is on the 40,000 dalton subunit and appears to be the ligand/toxin binding site of the receptor. The amount of specific and non-specific labeling was affected by the concentration of receptor and the specific activity of the receptor preparation as well as the concentration of [^{3}H]TDF. For the 40,000 dalton band, the ratio of specific to non-specific radioactivity incorporated above gel background ranged from 6.7 to 1.0.

The effect of TDF concentration on specific covalent binding of [^{3}H]TDF for two concentrations of receptor is shown in Fig. 5. The specific labeling measured directly is

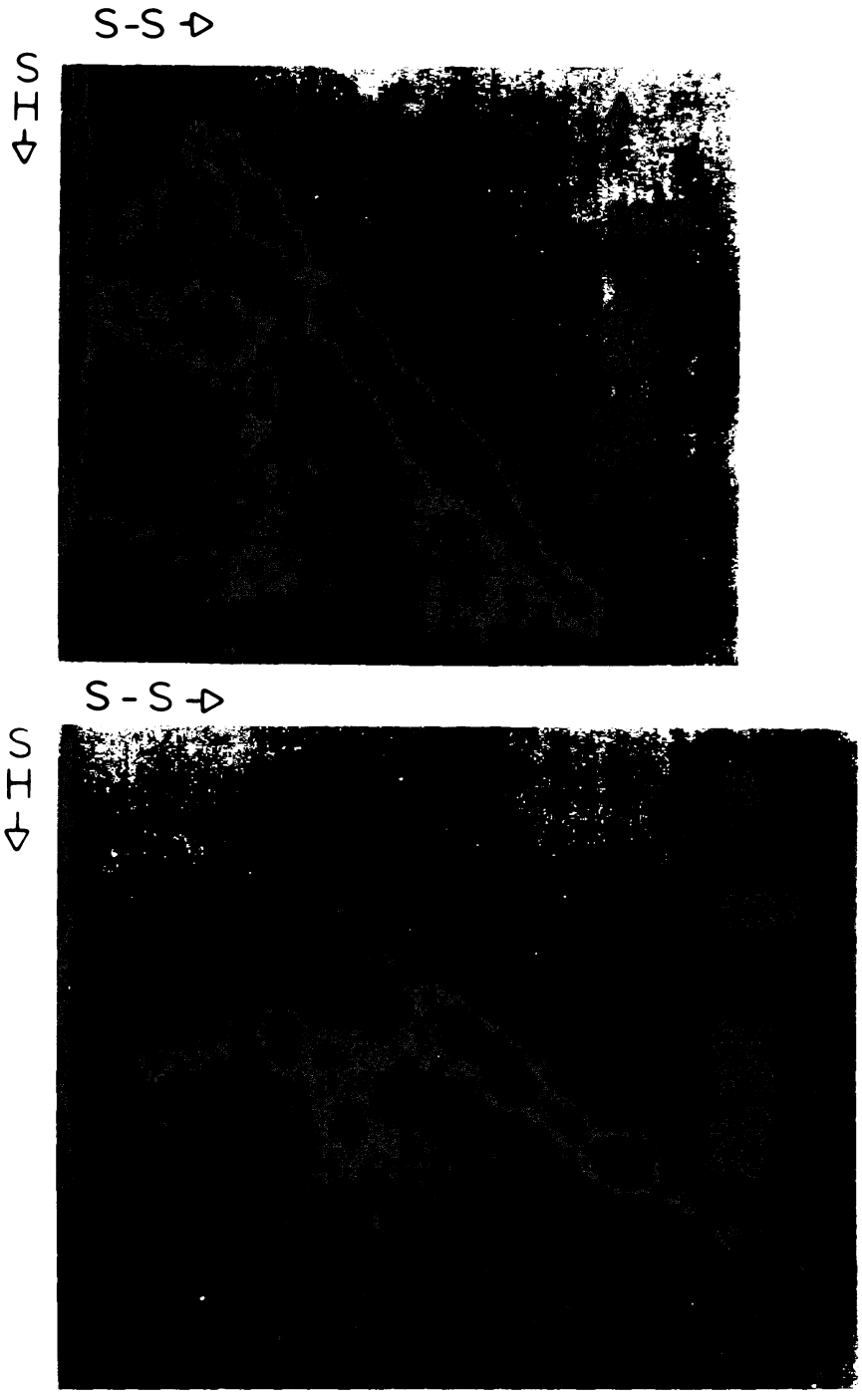

FIG. 2. *Two dimensional slab gel electrophoresis in the presence of sodium dodecyl sulfate for the membrane associated acetylcholine receptor*

Receptor-containing membranes were purified as described in Fig. 1 and subjected to gel electrophoresis. For electrophoresis in the second dimension the samples were reduced by the addition of 150 mM DTT to the agarose stacking gel.

A. Samples were treated with 10 mM N-ethylmaleimide prior to solubilization in SDS.

B. Samples were treated with 0.1 mM Cu^{++} (phenanthroline)$_2$ for 30 min, scavenged with 2.0 mM EDTA and 10 mM N-ethylmaleimide prior to solubilization in SDS.

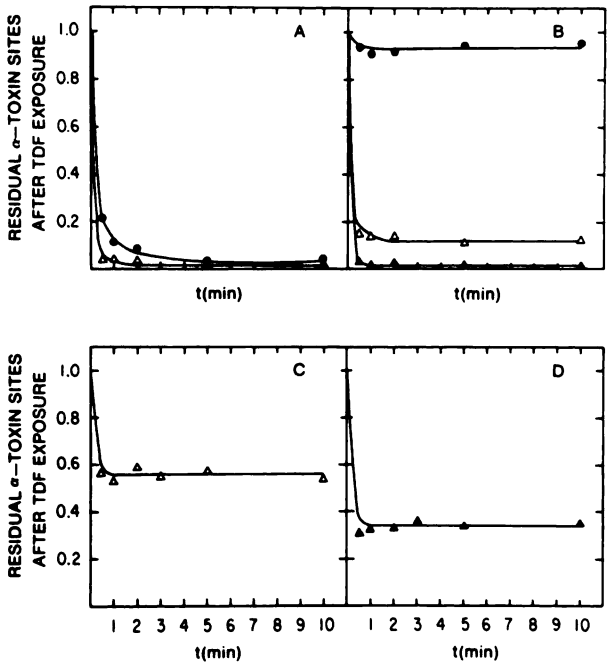

FIG. 3. *Time course of irreversible blockade of α-toxin binding sites by TDF*

Membrane fragments were reacted with unlabeled TDF for the time specified on the abscissa prior to the addition of 4 mM tyramine. α-Toxin bound at equilibrium was determined after 300- to 1000-fold dilution of the reaction mixture as described in the METHODS. The concentration of α-toxin sites during the reaction was A, 0.1 μM; B, 1.0 μM; C, 3.0 μM; D, 8.1 μM. Concentration of TDF during the reaction was: ●, 10 μM; △, 30 μM; ▲, 100 μM.

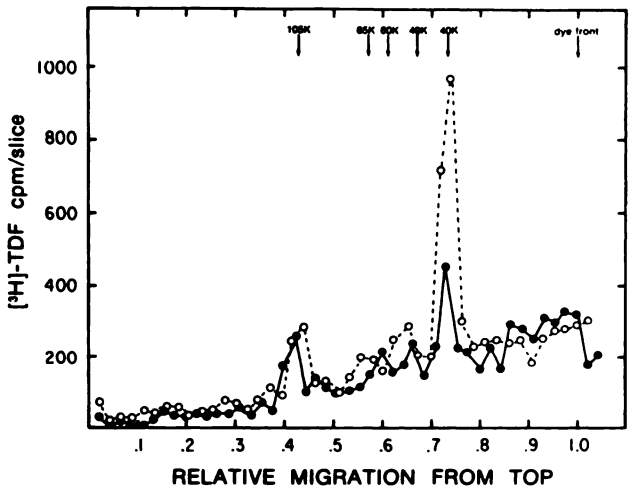

FIG. 4. *SDS-acrylamide gel electrophoresis profile of ^3H activity for [^3H]TDF labeled membrane fragments*

Receptor-rich membranes (13 μM in toxin sites) were reacted with 250 μM [^3H]TDF in the presence (●) or absence (○) of 0.1 mM carbamylcholine. The samples were electrophoresed and the gels prepared for counting as described in the METHODS. ^3H activity per slice is plotted as a function of migration distance relative to the dye front. The arrows designate the positions of the major polypeptide species and their apparent molecular weights.

compared to the inhibition of α-toxin binding at equilibrium after sufficient dilution of the membranes. The amount of specific labeling in both cases is concentration dependent and proportional to the number of toxin sites irreversibly blocked. At the lower concentration of receptor up to 95% of the toxin sites are blocked and specific labeling levels off at the higher concentrations of [^{3}H]TDF (Fig. 5b). Under these conditions non-specific labeling becomes substantial and measurement of the specific component is difficult to quantitate accurately. The specific covalent binding of [^{3}H]TDF does, however, appear to be saturable. The 50% saturation concentration for specific [^{3}H]TDF labeling of the receptor is approximately 0.1 mM when reacted at the higher receptor concentration (Fig. 5a), but it decreases with lower receptor concentrations (Fig. 5b) or higher specific activity of receptor. Therefore if TDF is behaving as an affinity agent, 0.1 mM is only a lower limit of the equilibrium dissociation constant for its reversible binding.

By comparing specifically bound [^{3}H]-TDF to the decrease in α-toxin sites, the stoichiometry of TDF to toxin sites can be determined. In Fig. 6 the concentration of specific TDF sites labeled is plotted versus the concentration of toxin sites irreversibly blocked by TDF labeling for several receptor preparations obtained over a period of eight months. Each measurement of binding, [^{3}H]TDF and α-toxin, was made on the same sample. The number of α-toxin sites irreversibly blocked is directly proportional to the number of TDF sites labeled specifically, with a proportionality constant of 0.98. Hence we identify one specific TDF site per toxin site.

[^{3}H]TDF labeling and the agonist-induced receptor state transition. Measurements of the specific labeling of the receptor by [^{3}H]TDF in the presence of carba-

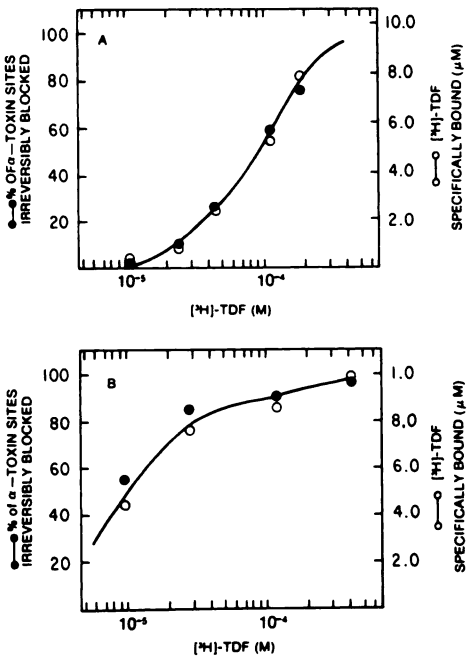

FIG. 5. *Concentration dependence of specific [^{3}H]TDF labeling of the receptor*

Membranes 9.6 μM (A) or 1.0 μM (B) in toxin binding sites were reacted with the specified concentration of [^{3}H]TDF for 30 sec prior to the addition of 1 mM tyramine. Specific labeling of the 40,000 dalton subunit (○) and irreversible blockade of α-toxin bound at equilibrium after 300- to 1000-fold dilution of the samples (●) were determined in parallel as described in the METHODS.

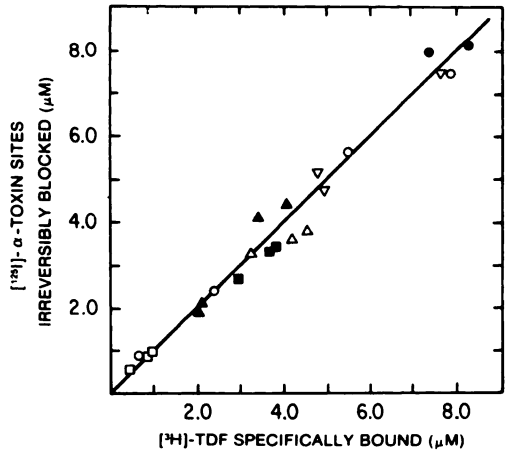

FIG. 6. *Relationship between [^{3}H]TDF specifically bound and α-toxin sites irreversibly blocked*

Membranes were reacted with [^{3}H]TDF and the concentrations of specifically bound [^{3}H]TDF and irreversibly blocked toxin sites determined as described in the METHODS. Determinations over a period of eight months using various membrane preparations and concentrations of [^{3}H]TDF are plotted. The concentrations of membrane toxin binding sites were: ○, ●, 14.2 μM; △, 11.4 μM; ▽, 9.9 μM; ■, 4.6 μM; □, 1.4 μM. Linear regression of these data gives a slope of 0.98 with a correlation coefficient (r^2) of 0.99.

mylcholine at agonist concentrations near its apparent dissociation constant were made to determine if the duration of exposure of the receptor to carbamylcholine would affect its inhibition of [^{3}H]TDF binding. Prolonged exposure (10 min) of the membranes to 3 or 10 μM carbamylcholine enhanced the inhibition of specific [^{3}H]-TDF labeling when compared with 10 sec of prior carbamylcholine exposure (Table 1). This influence of duration of prior ligand exposure is qualitatively similar to what has been observed from the inhibition of the initial rate of α-toxin binding in the presence of agonists (20). As also demonstrated by carbamylcholine competition with the α-toxin binding kinetics (20), the local anesthetic, prilocaine, increases the rate of the carbamylcholine-induced transition in receptor state (Table 1). Prilocaine alone at this concentration had no effect on [^{3}H]TDF labeling (Table 2). The apparent affinity of carbamylcholine increases less as measured by its effect on [^{3}H]TDF labeling than on α-toxin binding which may reflect a concomitant but smaller increase in TDF affinity associated with the receptor transitions. Hence an agonist-induced change in receptor binding properties where the affinity for agonists is enhanced preferentially can be demonstrated indirectly with [^{3}H]TDF labeling.

Because local anesthetics increase the rate of receptor conversion induced by agonists (20), the effect of a local anesthetic on [^{3}H]TDF labeling was examined. In the presence of a local anesthetic, TDF, which could be regarded as an analogue of the agonist phenyltrimethylammonium, may induce a sufficiently rapid transition in receptor state to be detected by an increase in specific labeling. For three concentrations of [^{3}H]TDF, prior incubation of the membranes with 0.2 mM prilocaine had no effect on the amount of specific labeling (Table 2). It would appear either that TDF does not have a higher affinity for R′ or that the reaction of TDF with the membranes is complete before any receptor conversion can take place, even under conditions where the rate of conversion is greatly increased.

TABLE 1

Effect of the duration of prior exposure of the membranes to carbamylcholine on specific [^{3}H]TDF labeling

Membranes (7 μM in toxin sites) were exposed to carbamylcholine in the presence or absence of 0.2 mM prilocaine for 10 sec or 10 min prior to the addition of 160 μM [^{3}H]TDF. Specific labeling of the receptor was determined as described in the METHODS. Labeling relative to control is in parentheses.

[Carbamylcholine] (M)	Specifically bound [^{3}H]TDF (μM)		
	10 sec prior exposure	10 min prior exposure	10 sec prior exposure in the presence of prilocaine (2×10^{-4} M)
0	3.54 (100%)	—	—
3×10^{-6}	3.26 (92%)	2.36 (64%)	1.71 (48%)
1×10^{-5}	1.33 (38%)	0.24 (7%)	0.52 (15%)

TABLE 2

Effect of prilocaine on specific [^{3}H]TDF labeling

Membranes 7 μM in toxin sites were reacted with the specified concentration of [^{3}H]TDF in the presence or absence of 0.2 mM prilocaine and specific labeling determined as described in the METHODS. Labeling relative to control is in parentheses.

[TDF] (M)	Specifically bound [^{3}H]TDF (μM)	
	Control	2×10^{-4} M prilocaine
5×10^{-5}	0.25	0.24 (96%)
1×10^{-4}	1.41	1.36 (96%)
2×10^{-4}	2.72	2.39 (88%)
		(93 ± 3%)[a]

[a] Mean ± SEM.

DISCUSSION

Subunit structure of the membrane associated receptor. The combined procedures of gradient sedimentation and affinity partitioning enable one to obtain membrane fractions of sufficient purity to characterize the polypeptide subunits by electrophoretic profiles and affinity labeling. Based on staining intensity, ratios of 1:0.32:0.20:0.29 were found for α, β, γ and δ subunits, respectively. Other workers using solubilized (4, 13, 29) or membrane-associated receptor (1, 3) have found the stoichiometries of the β, γ and δ subunits to be nearly equivalent with the α subunit being present in a 2- or 4-fold molar excess. It is noteworthy that affinity partitioning which employs

a different methodology for membrane fractionation yields such similar subunit stoichiometries. However, using a purification procedure that employs sonication, Sobel *et al.* (5) have virtually eliminated the higher molecular weight subunits from their preparations. Sufficiently harsh procedures could result in detachment of functional subunits from the receptor. Furthermore, Coomassie blue staining intensities will vary with the amino acid composition of the subunits. Thus, at present it becomes difficult to attach much significance to the subunit stoichiometries obtained. Moreover, a simple summation of subunit molecular weights in their apparent stoichiometric ratios to achieve a receptor molecular weight of 330,000 (29, 30) or 250,000 (31) daltons may prove difficult to reconcile with the constraints of symmetry necessary to achieve cooperativity in receptor activation.

The presence of the δ subunit as a dimeric species has been observed previously in the membrane-associated receptor (3, 32, 33) and is retained following solubilization (33). The 8S and 13S receptor species obtained following Triton solubilization contain 67,000 and 135,000 dalton chains and the δ subunit is responsible for the monomeric and dimeric forms of soluble receptor (33, 34).

Electrophoresis in two dimensions allows for a clearer definition of the origin of dimeric species, and as has been observed with the soluble receptor (33), the dominant dimer migrates as a 67,000 molecular weight entity. We also observe partial dimerization in the region of the 49,000 dalton peptide which may well be obscured in one dimensional electrophoresis. For the latter peptide dimerization is not complete, and it is increased by Cu^{++}-(phenanthroline)$_2$ treatment. These findings suggest alternative subunits could be responsible for dimerization of the receptor.

Affinity-labeling of the α-subunit by TDF. The carbamylcholine-protectable covalent binding of [^{3}H]TDF satisfies the basic requirements for specific affinity labeling of the binding site of the receptor. This labeling is concentration dependent, saturable, stoichiometric with α-toxin sites, and blocked by agonists and antagonists, as well as α-toxin. The reversible component of the binding appears to have a relatively high dissociation constant (~0.1 mM); however, with high concentrations of receptor and the apparent efficiency of endogenous quenching, non-specific labeling can be minimized. The apparent binding constant for the reversible component is within a factor of 3 of the apparent K_D for phenyltrimethylammonium (21).

The kinetics of the labeling could not be determined because the reaction appeared to be complete in less than thirty seconds. Since the protectable binding in many cases amounts to less than 10% of the total [^{3}H]-TDF added, TDF must be reacting non-specifically with other components of the membrane, causing rapid endogenous quenching of the reagent. This is not unexpected since the diazonium ion is a highly reactive electrophile. However, these non-specific reactions are not reflected in the labeling of membrane proteins, but probably membrane lipids, and the major portion of the [^{3}H]TDF present during the reaction does not appear on the gel. This endogenous quenching is actually fortuitous since it probably raises the ratio of specific to non-specific polypeptide labeling significantly. Moreover, endogenous quenching decreases the duration of exposure of ligand to the preparation.

Specific labeling by [^{3}H]TDF occurs only on the 40,000 dalton polypeptide. This binding has been shown to be blocked by both agonists and antagonists and to irreversibly inhibit α-toxin binding stoichiometrically. From these results it appears that the 40,000 dalton subunit of the receptor contains the ligand and toxin binding site(s) and that there are an equal number of TDF sites to α-toxin sites. The non-protectable labeling by TDF which becomes more dominant at high TDF concentrations appears only on the α-subunit. This may in part be a consequence of accessibility of the α relative to other subunits on the membrane surface (cf. 35). Although there is some labeling of the 105,000 dalton polypeptide, it is not protected by agonists or antagonists. This polypeptide does not appear to be associated with the receptor but

is likely a contaminant of the membrane fragment preparation. Further purification of the membranes (2, 3, 5) or solubilization and purification of the receptor (13, 29, 33) leads to the loss of this component.

The 40,000 dalton polypeptide has been implicated as the binding site subunit of the nicotinic receptor in many studies. Crosslinking of [^{125}I]α-toxin to the *Torpedo* receptor with suberimidate produces only one radioactive subunit of molecular weight approximately 40,000 (36).

The quaternary ammonium alkylating agent, MBTA, has been utilized with a number of nicotinic receptor preparations to affinity label the binding site of the receptor. After reduction of the receptor with dithiothreitol, MBTA alkylates specifically only the 40,000 dalton subunit of the receptor from *Electrophorus* (14) and *Torpedo* (5, 13). This labeling is blocked by cholinergic agonists and antagonists and MBTA reacts with one-half of the number of toxin sites (37). Recently MBTA has been shown to affinity label the nicotinic receptor purified from denervated rat skeletal muscle (15) where two subunits with molecular weights of 45,000 and 49,000 were labeled.

Recent results of photoaffinity labeling of the receptor from *Torpedo californica* have been less clear and unfortunately no information on stoichiometry is available. All four components of the receptor react covalently with [^{3}H]4-azido-2-nitrobenzyltrimethylammonium fluoroborate and cholinergic ligands appear to protect all the subunits against labeling (16). Only the 40,000 dalton subunit, however, was partially protected by toxin from covalent reaction. It would appear that small quaternary ligands can bind non-specifically to many sites on the receptor while only the 40,000 dalton subunit contains the specific α-toxin site that is related to the functional properties of the receptor. Photoaffinity labeling of more than one subunit of the *Torpedo* receptor has also been reported for the covalent binding of [^{3}H]bis(3-aminopyridinium)-1,10-decane azide (DAPA) (17). Although specific labeling appeared to occur on two of the four subunits, the authors argued that only the covalent binding to the 40,000 dalton subunit reflected reaction with the functional binding site. The labeling of the other subunit was thought to be non-specific and due, perhaps, to its proximity to the ligand binding site. The covalent binding of DAPA only decreased the initial rate of toxin binding and had no effect on toxin bound at equilibrium, which clearly differs from the finding for TDF. Ternary complexes of receptor, α-toxin, and photolyzed DAPA may have formed and the DAPA sites and toxin sites may only partially overlap.

Since toxin binding, being reversible, must be measured prior to dissociation of the subunits and TDF binding is determined in the dissociated α peptide following electrophoresis, the estimate of one TDF to one α-toxin site assumes that all of the α-subunit migrates into the gel. This assumption appears valid since under reducing conditions we do not find appreciable counts remaining at the origin of the SDS gels.

Reports on the ratios of α-toxin to ligand sites divide into two groups: those which demonstrate 1:1 stoichiometry and those which demonstrate 2:1 stoichiometry. An equal number of α-toxin sites and ligand sites has been found for detergent-solubilized (3) and membrane-associated *Torpedo* receptor (39–41) determined by equilibrium dialysis of radiolabeled ligands. A ratio of toxin sites to ligand sites of 1:1 has also been determined for *Torpedo* membrane-bound receptor by electron spin resonance measurements of the binding of decamethonium mononitroxide (10). A ratio of 2:1, however, has been reported by some investigators for purified *Torpedo* receptor using equilibrium dialysis of [^{3}H]acetylcholine (42–44). MBTA (see above) appears to bind to only half the toxin sites (13, 37); however, the receptor has been perturbed by reductive cleavage of a disulfide bridge within the binding site prior to labeling. The reason for the discrepancy in stoichiometry is still unclear. It has been suggested that during some purification procedures, ligand binding can be reduced without an effect on α-toxin binding which would lead to high ratios of toxin to ligand sites (40).

Protection of specific [^{3}H]TDF labeling by carbamylcholine is increased by prolonged exposure of the receptor to the ag-

onist or by the presence of a local anesthetic during a short agonist exposure. The effect of the local anesthetic is expressed only in the presence of agonist and there is no change in the extent of labeling in its absence. These results may be reflecting a carbamylcholine-induced increase in agonist affinity or decrease in TDF affinity and/or reactivity. The former interpretation agrees with the results of the α-toxin binding competition experiments and indeed local anesthetics appear to enhance the rate of the conversion to the high affinity state (20). The magnitudes of the apparent increases in carbamylcholine affinity determined by the two methods, however, differ slightly; the increase is less as measured by TDF binding. In the case of the α-toxin binding experiments it could be shown that the intrinsic rate of toxin binding is unchanged with receptor conversion. The effect of conversion on TDF affinity and reactivity and thus on the extent of labeling one would observe in the absence of agonist has not been determined. A concomitant, but smaller, increase in TDF affinity with carbamylcholine conversion of the receptor would decrease the apparent change in carbamylcholine protection. Therefore, although the results from studies on agonist protection of α-toxin and TDF association cannot be compared quantitatively, there is good qualitative agreement in showing a slow transition to a state where the affinity for agonists is enhanced.

Based on structural analogy, it is to be expected that the difference in affinity of TDF for R and R′ states of the receptor would be far smaller than the 300-fold difference found for carbamylcholine (20). For example, in the bisquaternary methonium series, agents with intramolecular cationic site distances similar to TDF showed affinity differences of 1/10 to 1/20 of carbamylcholine (21). Hence, it might be anticipated that based on affinity considerations alone, TDF could discriminate between the R and R′ receptor states but not with the large differences seen for full agonists. However, such considerations cannot discern whether different intrinsic rates of reactivity with TDF of the two receptor states prevail following formation of a reversible TDF-receptor complex.

TDF appears to have sufficient selectivity to be employed as an affinity label to identify the active site sequence of the α-subunit. Irreversible labeling can be protected by competing agonist or antagonist. It is saturable and occurs with a precise stoichiometry. Differences in selectivity of TDF and the photoaffinity label, 4 azido-2-nitrobenzyltrimethylammonium which is a fairly close congener, are striking (16) and it appears that the selectivity of TDF for the α-subunit approaches that of MBTA (13). TDF and a monocatonic congener, p-nitrobenzene diazonium, irreversibly antagonize the depolarization induced by agonists in the intact electroplaque (19). However, following reduction with DTT, TDF becomes a reversible activator (18, 19).

TDF may also prove useful in the differential labeling of the R and R′ states of the receptor. The ligand carries the inherent advantage that prior covalent modification of the receptor is unnecessary and the intrinsic scavenging behavior allows the time of TDF reactivity to be shorter than the lifetime of the R′ state. Acetylcholine could be used to convert the receptor to the high affinity, R′-Ach complex. Rapid removal of acetylcholine by exogenous acetylcholinesterase would leave the receptor in the uncomplexed R′ state. Since the reversion of R′ back to its original state R has a half time of 190 sec (20), labeling of R′ could be affected within this time frame and compared with that of the receptor in the R state.

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