Ligand Binding Sites and Subunit Interactions of *Torpedo californica* Acetylcholine Receptor†

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ABSTRACT: A [^{3}H]bisazido derivative of ethidium bromide was synthesized to identify sites of interaction of ethidium with the acetylcholine receptor from *Torpedo californica* and to aid in localization of ligand binding sites. For purified solubilized acetylcholine receptor it was shown (a) that the photolabel was competitive with ethidium bromide, (b) dodecyl sulfate–polyacrylamide gel electrophoresis revealed that all four polypeptide components were labeled with [^{3}H]ethidium azide, and (c) α-bungarotoxin inhibited the labeling of the 40 000-dalton subunit. Photolabeling of acetylcholine–receptor enriched membrane fragments led to the following conclusions: (a) the photochemical reaction was more selective than for purified acetylcholine receptor, since the 40 000-dalton subunit was preferentially labeled; this result demonstrated differences in the topography of receptor subunits depending on whether the molecule was in detergent solution or in a membrane-bound state, (b) α-bungarotoxin inhibited labeling of the 40 000-dalton subunit, (c) ligand-induced conformational changes resulted in different subunit labeling patterns. The results imply that conformational changes generated at the 40 000 molecular weight subunit upon cholinergic ligand interaction cause further intermolecular structural changes that involve subunits of higher molecular weight. These higher molecular weight subunits therefore belong to a supramolecular complex of polypeptides associated with the postsynaptic membrane.

The use of snake venom α-toxins (Lee, 1972) and curare-like agents has made possible the characterization and purification of a receptor for the neurotransmitter AcCh,[1] namely, the AcChR from the electric tissues of *Narcine entemedor* (Schmidt and Raftery, 1972) and *Torpedo* species (Karlsson et al., 1972; Schmidt and Raftery, 1973a; Eldefrawi and Eldefrawi, 1973; Raftery et al., 1974; Weill et al., 1974; Gordon et al., 1974; Ong and Brady, 1974) and from *Electrophorus electricus* (Olson et al., 1972; Biesecker, 1973; Karlin and Cowburn, 1973; Klett et al., 1973; Lindstrom et al., 1974; Chang, 1974). The electroplax responds in vivo to AcCh and other agonists in that the postsynaptic receptors mediate an increase in the permeability of the postsynaptic membrane to sodium and potassium ions and this effect is inhibited by *d*-tubocurarine (an antagonist), α-neurotoxins, or the neurotoxin histrionicotoxin (Albuquerque et al., 1973; Kato and Changeux, 1976). In addition, ligand interactions have been studied in vitro with membrane vesicles highly enriched in receptor protein or with solubilized, purified AcChR. Understanding of the mechanism(s) by which ion translocation through the excitable membrane is achieved is at the initial stages; little is known even regarding the detailed structure of the receptor and the topography of its subunits in the membrane. The protein isolated and purified from *Torpedo californica* contains four types of polypeptide chains with apparent molecular weights of 40 000, 50 000, 60 000, and 65 000 (Vandlen et al., 1976 and references therein). A specific function has been assigned only to the 40 000-dalton subunit, which apparently bears the binding site(s) for ligands such as the quaternary ammonium alkylating agent MBTA (Karlin et al., 1975), DAP (Witzemann and Raftery, 1977), and for α-neurotoxins (Hucho et al., 1976) all of which are cholinergic antagonists. We have recently shown that this same subunit also binds a cholinergic agonist, BrAcCh (Hsu and Raftery, to be published). No specific role(s) has been assigned to the other three subunit types, and the functional aspects of receptor quaternary structure have yet to be ascertained for the various polypeptide chains.

Recently, we have shown that the fluorescent membrane probe ethidium bromide (Gitler et al., 1969) binds to the membrane-bound AcChR from *Torpedo californica* with a resultant increase in fluorescence, and that this fluorescent enhancement is subject to the effects of a variety of cholinergic ligands as well as to α-neurotoxins and local anaesthetics (Schimerlik and Raftery, 1976). In this communication, we describe the synthesis of a photolabel, bis(azido)ethidium chloride, which we have used to identify the ethidium binding sites and to aid in localization of other ligand binding sites.

Experimental Procedures

Materials. Electric organs of *Torpedo californica* were used for the preparation of solubilized, purified AcChR (Vandlen et al., 1976) and of AcChR-enriched membrane fragments (Duguid and Raftery, 1973). Organs were used either immediately after sacrificing the animals or were stored at −90 °C. Crude venom from *Bungarus multicinctus* (Sigma Chemical Co.) was used for purification of α-Butx and this was labeled with ^{125}I as described by Clark et al. (1972).

Synthesis of ETA. The synthesis of ETA has been described by De Nobrega Bastos (1975), where the bisazido derivative was obtained, and by Hixon et al. (1975), whose methods resulted in the monoazido compound. We used a slightly different procedure to obtain bisazidoethidium. ETBr (0.5 g) was dissolved in 10 mL of 2 N HCl and cooled in an ice/salt mix-

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[1] Abbreviations used are: AcCh, acetylcholine; AcChR, acetylcholine receptor; α-Butx, α-bungarotoxin; BrAcCh, bromoacetylcholine; Carb, carbamoylcholine; DAP, bis(3-aminopyridinium)-1,10-decane diiodide; ETA, bis(azido)ethidium chloride; ETBr, ethidium bromide; H_{12}-HTX, perhydrohistrionicotoxin; MBTA, 4-(*N*-maleimido)-α-benzyltrimethylammonium iodide.

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ture. Dropwise addition of about 3 mL of a 2.9 M $NaNO_2$ solution was carried out over a period of 45 min, while the temperature was kept below 5 °C. Addition of 3 mL of NaN_3 (3.1 M) was followed again with stirring over a period of 30 min and the resulting precipitate was filtered off. The dried powder was dissolved in chloroform, applied to an aluminum oxide (Aluminum Oxide Woelm, neutral, ICN Co.) column (1 × 20 cm), and eluted with 1-propanol. The eluate was dried, dissolved in dilute acetic acid, precipitated with NH_4OH, and again dried.

IR spectroscopy of the product in chloroform revealed the appearance of a strong band at 2110 cm^{-1}, characteristic of azide groups, whereas NH_2 signals around 3400 cm^{-1} were completely absent. The absorption spectrum, using 10% acetic acid as solvent, is shown in Figure 1. Instead of the visible ETBr adsorption at 480 nm, one band appeared at 432 nm (ϵ 6.5 × 10^3 M^{-1} cm^{-1}). Changes also occurred in the UV region, where the original ETBr band at 286 nm was replaced by two absorption maxima at 283 and at 295 nm (ϵ 5.2 × 10^4 M^{-1} cm^{-1}). The azide was highly sensitive to light and decomposition occurred even in daylight. Upon irradiation with UV light (Mineral light UVSL 25 from Ultra-Violet Products Inc., San Gabriel, Calif.), using the short-wavelength range, a rapid decrease of the azide absorptions at 283 and 295 nm was observed, whereas the absorption band at 432 nm was shifted to longer wavelengths, around 500 nm (Figure 1).

An elemental analysis was conducted on the synthetic material. Anal. Calcd for $C_{21}H_{19}N_2O$: C, 65.43, H, 4.96; N, 25.44. Found: C, 65.69; H, 4.61; N, 24.93.

ETA was tritiated by the Wilzbach method (by ICN Corp.) and the radioproduct was chromatographed on an aluminum oxide column to spectroscopic purity. Radiochemical purity was demonstrated by thin layer chromatography on alumina (neutral) using 1-propanol as solvent. The R_f value (0.6) of the singly migrating band was identical with the value for unlabeled ETA and contained all the applied radioactivity. Specific activity was determined by counting aliquots of a stock solution and was 90 mCi/mmol.

Methods. The concentration of α-Butx binding sites as well as the time course of α-[^{125}I]Butx binding were measured by DEAE filter-disk assay methods (Schmidt and Raftery, 1973b). The amount of bound [^{3}H]ETA was assayed using the same DEAE filter-disk procedure. Aliquots of AcChR-[^{3}H]ETA solutions were pipetted onto the disks before and after photolysis, and free azide and decomposition products were removed by extensive washing in the dark in 10 mM sodium phosphate buffer containing 50 mM NaCl and 0.1% Triton X-100. The difference in radioactivity bound before and after irradiation was used as an indication of the amount of receptor labeled with [^{3}H]ETA.

Photolabeling was carried out at room temperature using all-quartz cells with a path length of 1 cm. The distance from the UV-light source was 1 cm and the time of exposure was 1 min. Irradiation for 1 min did not change AcChR properties such as α-[^{125}I]Butx binding, ligand-induced inhibition of toxin binding, or subunit composition as determined by $NaDodSO_4$-gel electrophoresis (Witzemann and Raftery, 1977). $NaDodSO_4$-polyacrylamide gel electrophoresis was performed essentially according to Laemmli (1970), using 12.5% acrylamide separating and 3% acrylamide stacking gel. The gels were sliced with an SL 280 electric gel slicer (Hoefer Scientific Instruments) into 1-mm segments which were dissolved in 0.5 mL of H_2O_2 at 70 °C. Radioactivity was measured using Triton/toluene scintillation fluid in a Packard Scintillation counter optimized for ^{3}H.

Fluorescence measurements were performed on a Perkin-Elmer MPF-4 spectrofluorimeter, and absorption spectra were recorded on a Cary Model 118C spectrophotometer. All chemicals used were of the purest grade commercially available.

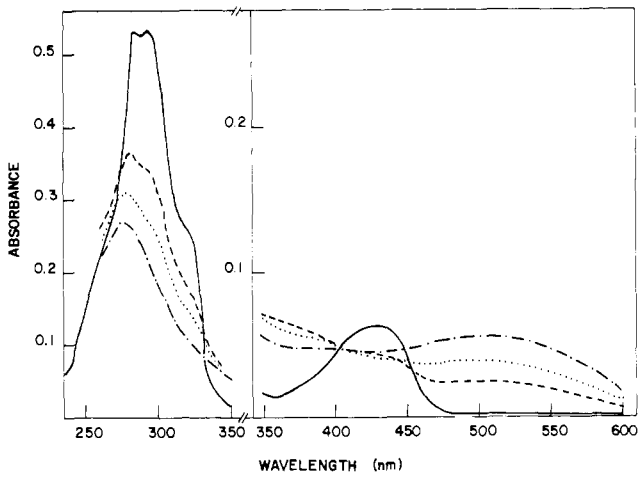

FIGURE 1: Absorption spectrum of ETA before and after photolysis. ETA (10^{-5} M) dissolved in 10% acetic acid (—) was irradiated at room temperature with the short-wavelength range of a UVSL-25 lamp for 30 s (- - -), 1 min (···), and 3 min (- • -).

Results

Binding of ETA to Purified AcChR in Solution. The extent of photolabeling of solubilized, purified AcChR as a function of increasing concentrations of [^{3}H]ETA is shown in Figure 2A. Incorporation of azide approached saturation at about 30 μM [^{3}H]ETA but a slight steady increase, attributed to nonspecific photolabeling, was observed by further raising the ligand concentration. When photolysis of AcChR and [^{3}H]ETA was performed in the presence of a large excess of ETBr, incorporation of radioactivity was prevented. In addition, the enhanced fluorescence of ETBr bound to AcChR (Schimerlik and Raftery, 1976) was decreased as a function of increasing ETA concentrations (Figure 2B) and, assuming identical affinities for the ETA and ETBr, a K_I of 5 μM for ETA was calculated.

Since the photolabeling showed saturation with increasing [^{3}H]ETA concentration, it seemed reasonable to determine whether photoinduced incorporation of [^{3}H]ETA resulted in labeling of a distinct polypeptide or if all four polypeptides of purified AcChR protein contained ETA (or ETBr) binding sites. At high [^{3}H]ETA concentrations, all four subunits showed the incorporation of [^{3}H]ETA (Figures 3A and 4D), as determined by $NaDodSO_4$-polyacrylamide gel electrophoresis. Labeling was prevented a large extent, but not completely, if ETBr (ninefold excess over [^{3}H]ETA) was present during the photolabeling process.

Photolysis carried out in the presence of α-Butx resulted in a decrease in radioactivity covalently bound to the 40 000-dalton subunit, whereas the other subunits were labeled as highly as in the control experiment (Figure 3A). It was also observed (Figure 3A) that not all of the [^{3}H]ETA sites appeared to overlap with toxin binding sites but that some toxin-independent [^{3}H]ETA sites were located on the 40 000-dalton subunit. The results shown in Figure 3B demonstrate that α-Butx clearly protected against photolabeling of the 40 000-dalton subunit.

The effects of propidium iodide, a compound related to ETBr, on the photolabeling process were tested at low [^{3}H]-ETA concentrations (10^{-5} M) to limit the amount of non-

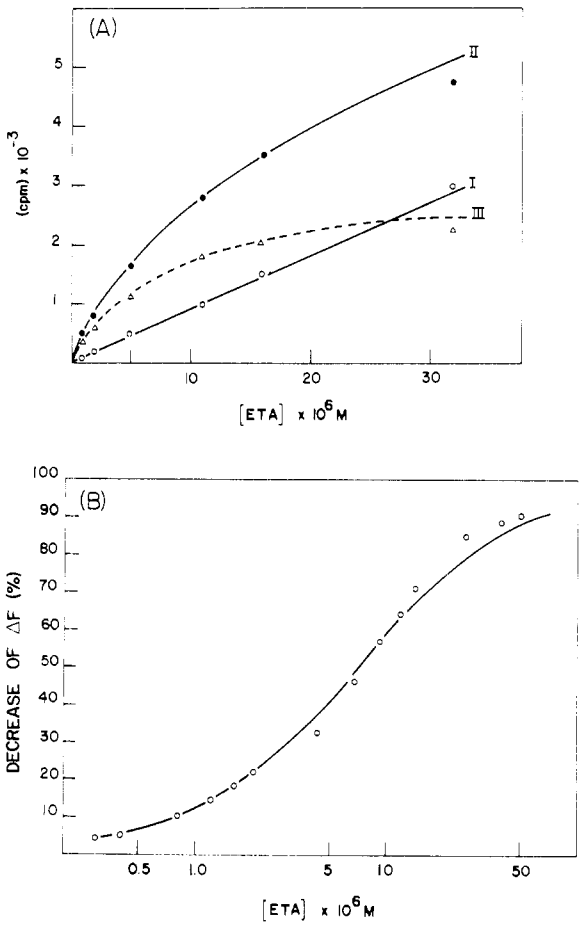

FIGURE 2: (A) Photolabeling of purified AcChR with [^{3}H]ETA. AcChR (6.4×10^{-7} M in α-Butx sites) in 10 mM sodium phosphate buffer, 100 mM NaCl, 0.1% Triton (pH 7.5) was irradiated in the presence of increasing [^{3}H]ETA concentrations with the short-wavelength range of UVSL-25 mineral-light lamp for 1 min at room temperature. One-hundred-microliter aliquots were taken before (curve I) and after photolysis (curve II), pipetted on DEAE filter disks and washed as described under Materials and Methods. The difference (curve II − curve I) was plotted in curve III and represents the amount of radioactively labeled AcChR (expressed in cpm). Curve I represents radioactivity nonspecifically bound to the disks. (B) Displacement of ETBr by ETA. Solutions used were AcChR (10^{-6} M in α-Butx sites) and ETBr (2×10^{-6} M) in 10 mM sodium phosphate buffer, 100 mM NaCl, 0.1% Triton (pH 7.5). The total volume was 2.5 mL. Excitation wavelength at 490 nm, emission 610 nm, using a Perkin-Elmer MPF-4 spectrofluorimeter to monitor enhanced ETBr fluorescence in the presence of AcChR (Schimerlik and Raftery, 1976). The solid line represents a theoretical titration curve and was fitted to the experimental data, assuming identical K_d values (5 μM) for ETA and ETBr.

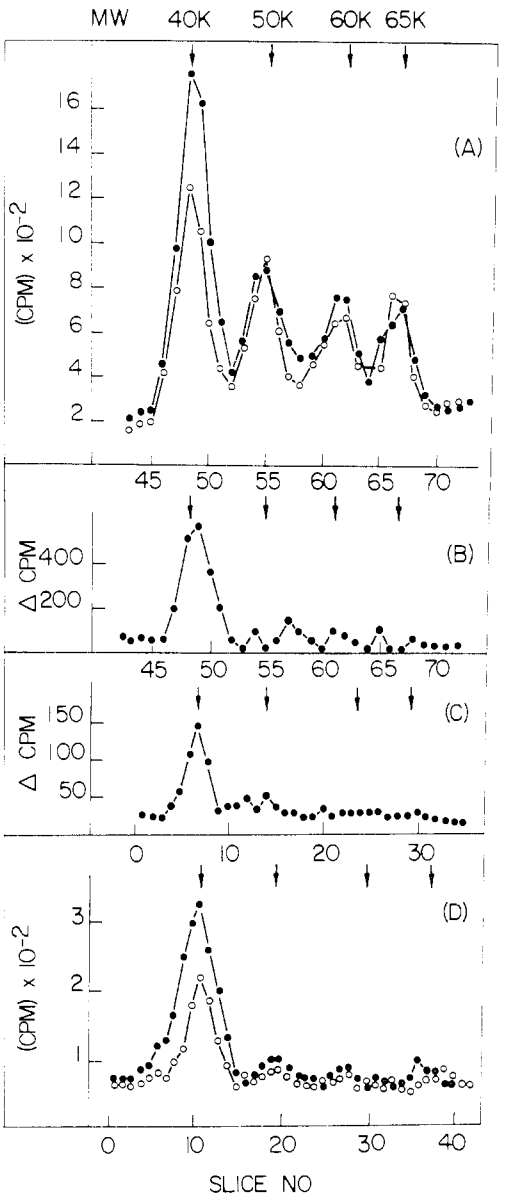

FIGURE 3: (A) NaDodSO$_4$-polyacrylamide gel electrophoresis of [^{3}H]ETA-labeled purified AcChR. AcChR (4×10^{-6} M in α-Butx sites) in 10 mM sodium phosphate buffer, 100 mM NaCl, 0.1% Triton (pH 7.5) was irradiated in the presence of 6×10^{-5} M [^{3}H]ETA (●-●) or AcChR was incubated with α-Butx (10^{-4} M) (○-○) for 1 h prior to irradiation. (B) Inhibition of [^{3}H]ETA labeling of purified AcChR by α-Butx. The difference in the distribution of radioactivity (as determined in A) obtained after labeling of purified AcChR in the absence or presence of α-Butx. (C) Inhibition of [^{3}H]ETA labeling of purified AcChR by propidium iodide. Purified AcChR (2.9×10^{-6} M in α-Butx sites) and [^{3}H]ETA (10^{-5} M) were irradiated in the absence or presence of propidium iodide (9×10^{-5} M). The difference in the distribution of radioactivity determined after NaDodSO$_4$-gel electrophoresis is shown. (D) NaDodSO$_4$-polyacrylamide gel electrophoresis of [^{3}H]ETA labeled membrane-bound AcChR. AcChR-enriched membrane fragments (5×10^{-7} M in α-Butx sites) were irradiated in the presence of [^{3}H]ETA (2×10^{-6} M) (●-●); membrane fragments were incubated with α-Butx (4×10^{-6} M) for 30 min prior to photolabeling with [^{3}H]ETA (○-○). The arrows indicate the positions of the polypeptides with apparent molecular weights of 40 000, 50 000, 60 000, and 65 000.

specific labeling. At 9×10^{-5} M propidium iodide, most of the 40 000-dalton subunit labeling was prevented, indicating that this ligand interfered with ETA binding sites (Figure 3C). It should be noted that the concentration of propidium iodide used here was considerably higher than the K_d value for propidium binding (1.8×10^{-6} M) to specific sites on the purified AcChR (Sator et al., 1977).

Photolabeling of AcChR-Enriched Membrane Fragments. When membrane fragments enriched in AcChR were irradiated in the presence of 90 μM [^{3}H]ETA (Figure 4C), extensive labeling of the 40 000-dalton polypeptide resulted. Because of the relatively high background radioactivity (ca. 200 cpm) in these experiments, it was not possible to decide whether the 50 000- and 60 000-dalton species had reacted to a small extent with [^{3}H]ETA, but it was clear that of the subunits other than the 40 000-dalton polypeptide only the 65 000-dalton subunit was labeled significantly above the background level. Protection by α-Butx under these conditions amounted to only about a 15-20% decrease in labeling of the 40 000-dalton subunit, indicating labeling of sites other than those occupied by the toxin. No difference in labeling of the 65 000-dalton subunit in the presence or absence of α-Butx was detected (not shown) in agreement with the results obtained with solubilized, puri-

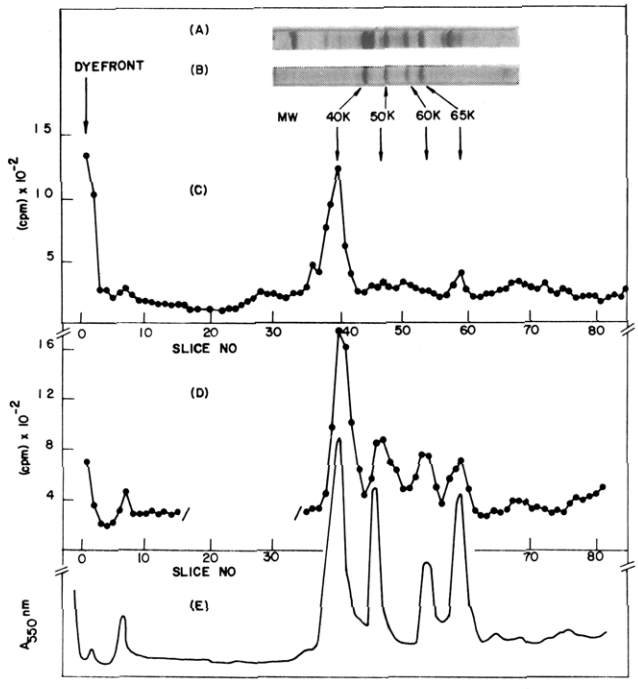

FIGURE 4: $NaDodSO_4$–polyacrylamide gel electrophoresis of AcChR. Typical Coomassie blue stained gels after electrophoresis of membrane-bound (A) and solubilized purified (B) AcChR. (C) distribution of ^3H after $NaDodSO_4$–polyacrylamide gel electrophoresis of membrane-bound AcChR (4×10^{-6} M in α-Butx binding sites) irradiated in the presence of 9×10^{-5} M [^{3}H]ETA or (D) of solubilized AcChR (4×10^{-6} M in α-Butx binding sites) irradiated in the presence of 6×10^{-5}M [^{3}H]ETA. (D) Scan at 550 nm of a Coomassie blue stained gel of solubilized purified AcChR after electrophoresis.

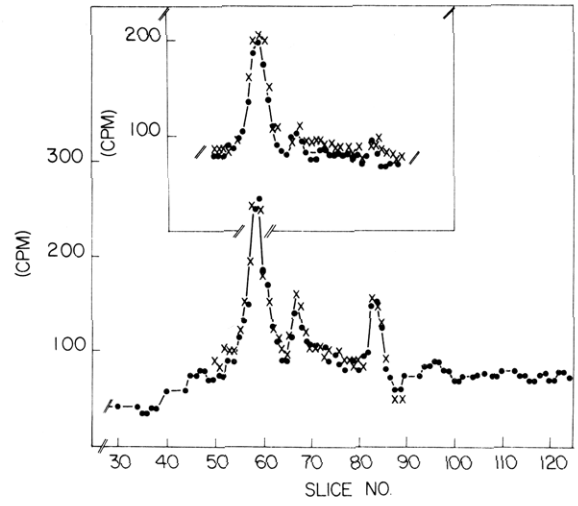

FIGURE 5: Distribution of radioactivity after $NaDodSO_4$–polyacrylamide gel electrophoresis of [^{3}H]ETA-labeled membrane-bound AcChR. Membrane-bound AcChR (5×10^{-7} M in α-Butx binding sites) was photolabeled in the presence of [^{3}H]ETA (2×10^{-6} M) upper, or membrane-bound AcChR was incubated with 5×10^{-6} M Carb for 10 min to convert the AcChR to the high ligand affinity form prior to irradiation, lower curve. (Note the increased labeling on the 40 000-, 50 000-, and 65 000-dalton polypeptides.)

fied AcChR. Lowering the azide concentration to 6–10 μM resulted in lower background counts and revealed that minor labeling occurred on the 50 000- and 65 000-dalton subunits but not on the 60 000-dalton polypeptide. No protein components of the membrane fragment preparations other than the AcChR showed significant incorporation of radioactivity, even when 90 μM [^{3}H]ETA was used in the labeling reaction.

Ligand Induced Subunit Interactions. Since AcChR-enriched membrane fragments can undergo a change from low ligand affinity to high affinity form(s) (Weber et al., 1975; Weiland et al., 1976: Lee et al., 1977), presumably involving conformational changes (Raftery et al., 1975; Changeux et al., 1975; Bonner et al., 1976; Quast et al., 1978), the question arises as to whether these different receptor forms also react with ETA in a distinctive manner, since ETBr fluorescence is affected by the presence of Carb (Schimerlik and Raftery, 1976) but is not displaced (Schimerlik et al., in preparation). The affinity for Carb of membrane-bound AcChR was measured by comparing the initial rates of α-[^{125}I]Butx complex formation in the presence of 1 μM Carb, under conditions where α-Butx was in excess of AcChR binding sites, with and without preincubation with 1 μM Carb (Lee et al., 1977). Membrane fragments were considered completely in the low-affinity form when the initial rate of toxin binding was the same in the absence or presence of Carb without preincubation of receptor and ligand. Preparations where the initial rate of AcChR–α-[^{125}I]Butx complex formation in the presence of Carb approached that found after preincubation with Carb (receptor converted to high affinity form) were discarded.

Modification experiments were carried out in the presence of 2 μM [^{3}H]ETA which was slightly below the estimated K_d of the azide, to minimize nonspecific labeling. Under these conditions, the 40 000-dalton subunit was preferentially labeled, and incorporation of radioactivity could be prevented to an extent of about 70% by incubation of the membrane fragments with excess α-Butx for 30 min prior to photolabeling (Figure 3D). Very little label was detectable on the 50 000- and 65 000-dalton subunits, and no significant difference was observed for these subunits in the absence or presence of toxin. The same preparation was incubated with 5 μM Carb for 10 min to convert the receptor to a high-affinity state(s) and then it was labeled under identical conditions. Upon $NaDodSO_4$–gel electrophoresis a striking difference was observed between the two samples when comparing the amount of radioactivity bound to the individual receptor subunits: an increased incorporation of [^{3}H]ETA of 1.4-, 2.4-, and 5-fold was recorded for the 40 000-, 50 000-, and 65 000-dalton subunits, respectively (Figure 5). Qualitatively similar effects on the labeling pattern were observed with another agonist, nicotine, and even antagonists such as *d*-tubocurarine or DAP (not shown) when the membrane fragments were incubated in the presence of these ligands prior to photolabeling.

Effects of Histrionicotoxin. H_{12}-HTX, a neurotoxin supposedly specific for blocking the cholinergic ionophore (Albuquerque et al., 1973) which binds (K_D ~0.4 μM) to AcChR-rich membrane fragments (Eldefrawi et al., 1977; Elliott and Raftery, 1977), was tested for its ability to interfere with [^{3}H]ETA labeling, since it has been shown to affect AcChR-bound ETBr fluorescence (Elliott and Raftery, 1978, in preparation). Membrane fragments in the low ligand affinity form (ca. 0.6 μM in toxin binding sites) and in the presence of 2 μM [^{3}H]ETA showed no difference in the amount and distribution of [^{3}H]ETA incorporated upon irradiation in the presence or absence of 1 μM H_{12}-HTX (Figure 6). However, if 1 μM H_{12}-HTX was present in addition to 5 μM Carb, the labeling pattern was changed significantly. Radioactivity bound to the 40 000-dalton subunit was decreased to about 30–40%, and almost no labeling occurred on the 65 000-dalton subunit, whereas only minor changes were found on the 50 000-dalton subunit.

Discussion

The nicotinic acetylcholine receptor in solubilized, purified form is a macromolecular assembly of four different types of

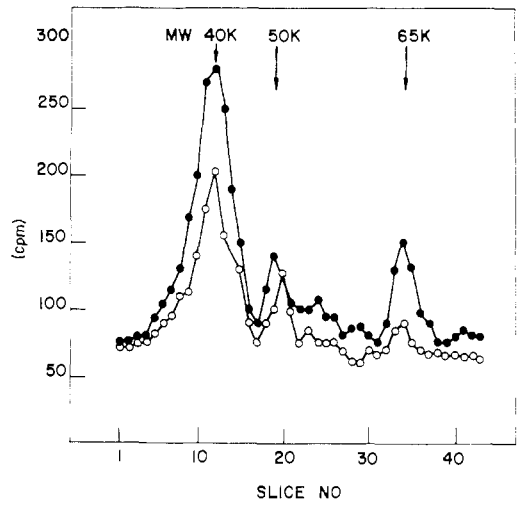

FIGURE 6: Effect of H_{12}-HTX on photolabeling of membrane-bound AcChR. Membrane-bound AcChR (5×10^{-7} M in α-Butx binding sites) was incubated for 10 min with Carb (5×10^{-6} M) to convert AcChR to its high ligand affinity form(s). H_{12}-HTX was added to a final concentration of 10^{-6} M. After another 10-min incubation at room temperature, photolabeling was carried out in the presence of 2×10^{-6} M [^{3}H]ETA (O-O). The control was performed under identical conditions but without H_{12}-HTX (●-●).

polypeptide chains, the individual function(s) of which we as yet know little about. All of the polypeptide types, in the same apparent stoichiometry (Duguid and Raftery, 1973; Raftery et al., 1974, 1975; Reed et al., 1975), appear in highly enriched membrane preparations from *Torpedo californica electroplax*. A full understanding of the molecular mechanisms by which the AcChR molecule accomplishes synaptic transmission will depend on the assignment of structural and/or functional roles to all of these polypeptide types. To date a specific role has been assigned only to the polypeptide component of 40 000 molecular weight in that it appears to bind α-neurotoxins (Hucho et al., 1976) and three types of cholinergic affinity labels. The first of these is MBTA (Karlin and Cowburn, 1973; Karlin et al., 1975), a cholinergic antagonist which reacts covalently with AcChR treated with reducing agents. This reagent reacts with half of the number of sites occupied by α-[^{125}I]Butx (Weill et al., 1975). Witzemann and Raftery (1977) have described a photoaffinity label for the nicotinic AcChR, bis(3-azidopyridinium) **1**, 10-decane perchlorate, that also reacts covalently with half of the total number of α-[^{125}I]Butx binding sites. These authors suggested that the specific binding site for the photoaffinity reagent resides on the 40 000-dalton AcChR subunit and that a polypeptide of 50 000 daltons, which was also labeled by the reagent, is in close proximity to the binding site. The two affinity labels associate with half of the total number of AcChR sites as does α-Butx. This behavior is reminiscent of AcCH (Moody et al., 1973) or Carb (Raftery et al., 1974) binding to half of the total number of sites which can be determined for α-Butx. The third type of ligand which has been shown to interact with the 40 000-dalton subunit (Hsu and Raftery, 1977, unpublished) is BrAcCh, a cholinergic agonist (Silman and Karlin, 1969).

In the work described here, we have used a photolabel, [^{3}H]ETA, which was expected to have a broad range of reactivity such that several features of AcChR ligand binding sites and consequent conformational changes could be studied. In the case of the solubilized, purified AcChR it was found that all four subunit types were labeled with [^{3}H]ETA with the subunit of 40 000 molecular weight being labeled most extensively. These results showed that indeed the reagent had a generally wide range of reactivity. Despite this it was useful, since inhibition of the incorporation of label by α-Butx strongly implicates toxin binding to the 40 000 molecular weight subunit; however, the results do not preclude additional contacts between the neurotoxin and the other subunit types.

In contrast to the labeling of all four receptor subunit types of the purified AcChR, the labeling of receptor-enriched membrane fragments was more selective (Figure 5C,D). [^{3}H]ETA reacted preferentially with the 40 000-dalton subunit and only to a minor extent with the other subunits or with other proteins present in the membrane fragments. The purity of our best membrane preparations was such that 40–50% of the total protein was AcChR protein (Reed et al., 1975). Similar data were obtained using preparations that contained only 10% AcChR on a protein basis, again demonstrating the preferential labeling of the AcChR. The results clearly demonstrate differences in the topography of receptor subunits depending on whether the molecule is in detergent solution or in a membrane-bound state, and these findings agree well with those described by Witzemann and Raftery (1977) using a photolabel derived from the cholinergic antagonist DAP as well as with recent results of Hartig and Raftery (1977) using lactoperoxidase-catalyzed iodination of purified and membrane-bound AcChR.

Because of the preferential incorporation of photolabel into the 40 000-dalton subunit of the membrane-bound receptor, it was possible to conduct experiments designed to determine whether a variety of compounds such as α-Butx and cholinergic agonists or antagonists protected against incorporation of photolabel into this particular subunit. As shown in Figure 3D, it was clear that α-Butx could extensively protect against incorporation of photolabel; however, not all ethidium binding sites on the 40 000-dalton subunit were protected by inclusion of the toxin. Since α-Butx interacts irreversibly with the AcChR, it is reasonable to conclude that at the concentration of label used (2 μM) [^{3}H]ETA sites exist on 40 000 molecular weight subunit which are topographically distinct from the neurotoxin binding region.

In contrast to the results obtained with α-Butx, attempted protection by cholinergic ligands resulted in *increased incorporation* of label, and additionally the preferential modification of the 40 000 molecular weight subunit was no longer the case. The most dramatic effect was obtained with the cholinergic agonist Carb, as shown in Figure 6. The presence of 5×10^{-6} M Carb increased the incorporation of [^{3}H]ETA not only into the 40 000 molecular weight subunit but also caused significant labeling of the 50 000- and 65 000-dalton subunits (Figure 6), considerably above the control (Figure 6) and to an extent greater than when a tenfold excess of photolabel was used in the absence of Carb. This result is interpreted as representative of cholinergic ligand-induced conformational changes in the 40 000 molecular weight subunit, to which the ligand binds, and in addition as a demonstration of intersubunit conformational effects involved in structural changes generated at the 40 000 molecular weight subunit and communicated to the subunits of molecular weight 50 000 and 65 000. The observed increase in bound radioactivity could be explained by an increase in [^{3}H]ETA binding sites upon incubation with cholinergic ligands or by assuming a constant number of binding sites for the azide; however, due to conformational changes induced by Carb the enivronment of this binding site is now changed in such a way that the reactivity of the protein with the photoactivated nitrene is increased. (Such an increase in reactivity could be achieved by exclusion of H_2O molecules from the binding area.) Binding studies with [^{3}H]ETBr favor the second explanation, since it was shown that the number of

binding sites for ETBr does not change, whether the membrane fragments are in the low- or the high-affinity form for Carb (Schimerlik and Raftery, in preparation). Thus, the experiment supports the notion that extensive conformational changes occur in the AcChR macromolecular assembly. It further implicates three of the four types of receptor subunits as being responsive to the interaction of a small cholinergic ligand with a limited number of binding sites on a specific subunit.

The conformational changes which have been detected by our labeling procedure are most likely the result of structural changes that accompany conversion of the AcChR from its normal low-ligand affinity state to one of high-ligand affinity, as previously described by Katz and Thesleff (1957) and Rang and Ritter (1971) for in vivo studies and by Weber et al. (1975), Weiland et al. (1976), Lee et al. (1977), and Quast et al. (1978), for in vitro preparations. As described by Lee et al. (1977) and Quast et al. (1978), the conversion of AcChR from a state of low ligand affinity to one (or more) of high ligand affinity is not restricted to the action of cholinergic agonists, but antagonists such as *d*-tubocurarine or DAP can also effect this conversion, and we have observed effects of these ligands on the labeling process described here which lend further support to this line of reasoning.

One further type of ligand interaction with the AcChR was investigated by means of protection against photoinduced incorporation of [^{3}H]ETA. This involved the use of the ligand H_{12}-HTX, which is thought to interact directly with or to modulate the properties of the ion channel associated with the nicotinic AcChR (Albuquerque et al., 1973). This neurotoxin has been shown not to interact with the acetylcholine (Kato and Changeux, 1976) or carbamoylcholine binding sites nor to affect the rate of conversion from low- to high-affinity ligand forms (Elliott and Raftery, 1977). Furthermore [^{3}H]-H_{12}-HTX binds to one-fourth the number of binding sites occupied by α-[^{125}]Butx; i.e., it appears to bind one to one with the membrane-bound AcChR. It may therefore be a potentially useful ligand for investigation of the properties of the molecular apparatus involved in cation flux associated with the AcChR. In the presence of H_{12}-HTX, no protection or enhancement of photoinduced incorporation of [^{3}H]ETA was obtained. However, H_{12}-HTX in the presence of Carb afforded significant protection against the incorporation of photolabel into the 40 000-dalton subunit and in addition almost eliminated incorporation induced (by Carb) into the 65 000-dalton subunit. It cannot be concluded from these results whether H_{12}-HTX interfered directly with [^{3}H]ETA-binding sites or, indirectly, by means of additional conformational changes.

With regard to distinguishing between the possibilities, recent results (Schimerlik and Raftery, in preparation) have shown that HTX does not induce either an increase or a decrease of AcChR-bound [^{3}H]ETBr binding but does cause a large change in the quantum yield of the bound ethidium (Schimerlik, Elliott, and Raftery, 1978, to be published). These effects taken together with the labeling results presented here suggest that binding of HTX can cause conformation changes in membranes enriched in the AcChR but that these structural transitions are not identical with those induced by Carb, *d*-tubocurarine, or DAP.

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Influence of Membrane Lipids on Acetylcholine Receptor and Lipid Probe Diffusion in Cultured Myotube Membrane†

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ABSTRACT: We measured how alterations of the fatty acyl chains in the plasma membranes of cultured chick embryo myotubes affect the lateral mobility of the fluorescent membrane lipid probe dioctadecylindocarbocyanine (diI) and of acetylcholine receptors (AChR) labeled with fluorescent tetramethylrhodamine-α-bungarotoxin (TMR-αBgt). Membrane phospholipid fatty acyl chains were altered by manipulating the fatty acid composition of the growth medium; lateral mobility was measured by the fluorescence photobleaching recovery (FPR) technique. In general, our results demonstrate that substantial membrane lipid acyl changes need not substantially affect the lateral mobility of cell-membrane components. We found that membrane fatty acyl changes affected lateral motion by less than a factor of 1.5 to 2 for diI and less than a factor of about 3 to 4 for AChR, at both low (12 °C) and high (31 °C) temperatures. For all the altered cell types tested, an increase of temperature from 12 to 31 °C resulted in (a) a threefold increase in the diI diffusion constant with almost all the diI mobile throughout the temperature range, and (b) an increase in the fraction of AChR which is mobile from about 20 to about 60%. At 31 °C, the diI diffusion constant is almost two orders of magnitude larger than the mobile AChR diffusion constant. The fatty acyl changes do not affect the optically observed distribution of either diI or AChR in the membrane.

Animal cell membranes possess a complex lipid composition consisting primarily of several different phospholipids and fatty acyl groups, cholesterol, and sphingolipids. The functional role of the diverse lipid composition remains, in large part, a mystery. One speculation is that it modulates the mobility and distribution of membrane components. The combination of two recent developments makes feasible the investigation of this possibility. First, techniques are now available for manipulating the lipid composition of cells grown in culture (Horwitz, 1977). Second, a method now exists for measuring the lateral mobility and distribution of fluorescence-labeled membrane components (Axelrod et al., 1976a; Koppel et al., 1976).

With these experiments, we observed that substantial membrane lipid fatty acyl changes in primary cultures of myotubes from chick did not substantially affect the lateral mobility of either a membrane lipid probe or the acetylcholine receptor (AChR), an integral membrane protein. We report the lateral mobilities of the lipid probe and AChR as a function of temperature.

We have altered the lipid composition of cultured cells by growing them in a medium depleted of lipid and biotin and supplemented with a selected fatty acid. The incorporation of those fatty acids that are not readily metabolized results in an appreciable alteration in the fatty acyl composition of the membrane phospholipids (Horwitz, 1977; Horwitz et al., 1978).

The exogenous phospholipid-like membrane fluorescent lipid probe employed was dioctadecylindocarbocyanine [diI-C_{18}-(3), or diI] (Sims et al., 1974; Schlessinger et al., 1977; Badley et al., 1973). AChR on the myotube surface was visualized by specific binding with fluorescent-tagged α-bungarotoxin (TMR-Bgt) (Ravdin and Axelrod, 1977; Axelrod et al., 1976b; Anderson and Cohen, 1974). We have measured lateral motion of fluorescent molecules on a cell surface by a technique called fluorescence photobleaching recovery (FPR) (Axelrod et al., 1976a, Koppel et al., 1976). In this technique, the fluorescence of a small region on the cell surface is bleached by a bright flash

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