Ligand-Induced Changes in Membrane-Bound Acetylcholine Receptor Observed by Ethidium Fluorescence. 1. Equilibrium Studies†

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ABSTRACT: The interactions between the fluorescent probe ethidium and acetylcholine receptor enriched membranes from *Torpedo californica* are described. One class of saturable ethidium sites was blocked by α-bungarotoxin and therefore reflects direct binding to the receptor ($K_d \sim 3$ μM; stoichiometry—one ethidium site per two α-bungarotoxin sites). The second class of sites was nonsaturable and unaffected by α-toxin and was therefore considered nonspecific in nature. The increase in fluorescence intensity observed upon addition of cholinergic agonists and antagonists accurately reflects the dissociation constant and stoichiometry of the high-affinity receptor sites for these ligands. The effects of local anaesthetics are complex in nature and depend on the structure of the ligand. For carbamylcholine, the increase in fluorescence intensity was due to an increase in the quantum yield of the dye bound to the membrane rather than a dye uptake. In general, ethidium appears not to strongly alter the properties of the membrane-bound acetylcholine receptor and can therefore be profitably used as a spectroscopic probe.

The development of techniques that permit preparation of membrane fragments highly enriched in AcChR[1] proteins from *Torpedo californica* (Duguid & Raftery, 1973) has made it possible to study the interactions of cholinergic ligands with the membrane-bound AcChR in vitro. The membrane-bound AcChR undergoes a conformational change upon ligand binding that is thought to resemble the in vivo phenomenon of desensitization. This has been shown with the technique of inhibition of the rate of radiolabeled toxin binding to the AcChR (Weber et al., 1975; Weiland et al., 1976, 1977; Lee et al., 1977; Quast et al., 1978a). An agonist-induced isomerization also attributed to desensitization has been detected by using intrinsic protein fluorescence (Bonner et al., 1976; Barrantes, 1976). Using the fluorescent local anaesthetic quinacrine in stopped-flow studies, Grünhagen & Changeux (1976) and Grünhagen et al. (1977) interpreted their kinetic observations as corresponding to the activation of the ion channel, followed by desensitization. In preliminary publications (Schimerlik & Raftery, 1976; Quast et al., 1978b), the detection of conformational changes in the membrane-bound AcChR using the extrinsic fluorescence probe Eth has been reported.

It is the purpose of this paper to describe the interaction of the fluorescent probe Eth with the membrane-bound AcChR. Synthesis of [^{3}H]Eth allowed us to examine the interaction of the dye with the receptor, and it was possible to estimate whether the observed increase in fluorescence intensity upon addition of Carb was due to a change in (1) the concentration of that fraction of Eth bound to the membrane fragments in a nonsaturable manner (nonspecifically bound Eth), (2) the fraction of Eth that is displaced by α-BuTx (specifically bound Eth), or (3) the quantum yield of the bound dye. A comparison of the apparent dissociation constants for cholinergic ligands, obtained by the concentration dependence of the fluorescence increase of the AcChR–Eth complex after addition of ligands, with those obtained from the inhibition of the rate of [^{125}I]-α-BuTx binding to the receptor showed that Eth did not radically change the ligand binding properties of the AcChR. It was possible to obtain the stoichiometry of high-affinity sites for Carb, *d*-Tc, and Deca as compared to α-BuTx sites directly by fluorescence titration. The following two papers (Quast et al. 1979; Schimerlik et al., 1979) describe kinetic studies of the interaction of the membrane-bound AcChR with cholinergic ligands and histrionicotoxin.

Experimental Section

Membrane fragments from *T. californica* enriched in AcChR were prepared as previously described (Duguid & Raftery, 1973; Reed et al., 1975). The concentration of α-BuTx sites was determined by the method of Schmidt & Raftery (1973). Labeled [^{125}I]-α-BuTx was prepared by a modification (Blanchard et al., 1979) of the method of Vogel et al. (1972) from α-BuTx purified from *Bungarus multicinctus* venom (Sigma Chemical Co.) by the method of Clark et al. (1972). Eth was purchased from Calbiochem; carbamylcholine chloride and *d*-tubocurarine chloride were from Sigma Chemical Co.; decamethonium bromide, gallamine triiodide, procaine, tetracaine, and dibucaine were from K & K Laboratories; hexamethonium chloride was from Schwarz/Mann; lidocaine was from Pfaltz and Bauer; and choline chloride was from Matheson Coleman and Bell. DAP was synthesized by a modification of the procedure of Mooser et al. (1972) and [^{3}H]carbamylcholine was a generous gift of Dr. Yuan Chao. All experiments were done in Hepes Ringer's buffer consisting of 20 mM Hepes, 250 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, and 4 mM $CaCl_2$, pH 7.4. [^{3}H]Eth containing the label in the protons of carbon-2 of the ethyl group on the quarternary nitrogen was prepared by adapting the procedure of Watkins (1952) to smaller quantities. Starting with 2,7-dinitro-9-phenylphenanthridine obtained from Boots

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[1] Abbreviations used: AcCh, acetylcholine; AcChR, acetylcholine receptor; α-BuTx, α-bungarotoxin; [^{125}I]-α-BuTx, the monoiodo ^{125}I derivative of α-bungarotoxin; Carb, carbamylcholine; *d*-Tc, *d*-tubocurarine; DAP, 1,10-bis(3-aminopyridinio)decane diiodide; Deca, decamethonium; Hexa, hexamethonium; Eth, ethidium; [^{3}H]Eth, [^{3}H]ethidium.

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Pharmaceutical Co., the nitro groups were reduced and then blocked with 2 equiv of ethyl chloroformate as described in that reference. Tritiated ethyl tosylate was prepared from [1-^{3}H]ethanol (New England Nuclear; 100 mCi/mmol) and tosyl chloride as described by Fieser & Fieser (1967). In the quarternarization step, 0.43 mmol of the [^{3}H]ethyl tosylate plus 0.40 mmol of 2,7-bis(carbethoxyamino)-9-phenylphenanthridine was heated at 155–160 °C for 2.5 h in 2.5 mL of dry nitrobenzene. After the mixture was cooled to room temperature, it was divided into two 15-mL conical centrifuge tubes, and 9 mL of ice-cold ethyl ether was added to each tube, causing a precipitate to form. After the precipitate was washed with 5 mL of cold ethyl ether, the pellets were dissolved in 2–3 mL of H_2SO_4–H_2O (2:1) and heated for 2.5 h to remove the amino blocking groups. After the mixture was cooled to room temperature and neutralized with NH_4OH, solid NH_4Br was added to saturation. The mixture was filtered to remove excess NH_4Br, and the solution was flash evaporated to dryness. The residue was dissolved in acetone and applied to a preparative thin-layer silicic acid plate (Brinkmann, Sil Plate P20 F-22) and eluted with $CHCl_3$–MeOH–HAc–H_2O (40:10:1:1). Several (six) bands appeared, and the red band having an R_f of approximately 0.22 was scraped off and eluted with the MeOH–HCl–H_2O (48:1:1). The absorbance and fluorescence spectra of the purified [^{3}H]Eth were identical with those obtained for commerically available Eth, except that the extinction coefficient at 285 nm was about 5–10% too high, probably due to UV-absorbing impurities from the silicic acid. The concentrations of the [^{3}H]Eth determined by absorption at 480 nm (ϵ = 5300 M^{-1} cm^{-1}; Hudson & Jacobs, 1975) and by comparison of fluorescence at 610 nm (excitation at 483 nm) with standard nonradioactive solutions were in agreement. The labeled Eth cochromatographed with the nonradioactive compound, showing one spot in $CHCl_3$–MeOH–HAc–H_2O (40:10:1:1) (R_f = 0.41) on Merck 60 silicic acid thin-layer plates and on neutral aluminia plates, Type E (Merck, pore size 60 Å), in NH_4OH–ethyl acetate–MeOH (1:3:6) (R_f = 0.68) with over 95% of the radioactivity in the fluorescent spot. The specific activity of the [^{3}H]Eth was 100 mCi/mmol as expected, and the yield based on [^{3}H]ethyl tosylate was approximately 5%. Uncorrected fluorescence spectra were recorded on a Perkin-Elmer MPF-4 spectrofluorimeter at 26 °C. Fluorescence titrations to measure the dependence of the fluorescence increase on the concentration of cholinergic ligand added to the AcChR–Eth complex were done with an excitation wavelength of 483 nm, measuring the fluorescence emission at 610 nm. A 430-nm emission filter was used to lower the contribution from light scattering. Fluorescence intensity is expressed as arbitrary units of fluorescence and enhancements as $\Delta F/\mu M$.

Ligand binding studies with [^{3}H]Eth and [^{3}H]Carb were done with a Beckman airfuge. The membrane fragments were equilibrated with the radioactive ligand for 20 min in the airfuge tube, and duplicate 50-μL samples were withdrawn for liquid scintillation counting to give the total ligand concentration. The mixtures were then pelleted for 10 min at 105000g, and duplicate aliquots were withdrawn from the supernatant to give the concentration of free ligand. The concentration of bound ligand was calculated from total minus free concentrations. Samples were counted in 10 mL of Aquasol 2 in a Packard Tricarb scintillation counter (Model 3375) optimized for tritium.

The determination of the apparent dissociation constant for cholinergic ligands based on the inhibition of the initial rate of [^{125}I]-α-BuTx–AcChR complex formation has been described in earlier publications (Raftery et al., 1975; Quast et al., 1978a). The method for the determination of the rate of the transition of the AcChR from low to high affinity toward Carb under the conditions of α-BuTx sites in excess over [^{125}I]-α-BuTx has also been previously described (Lee et al., 1977; Quast et al., 1978a).

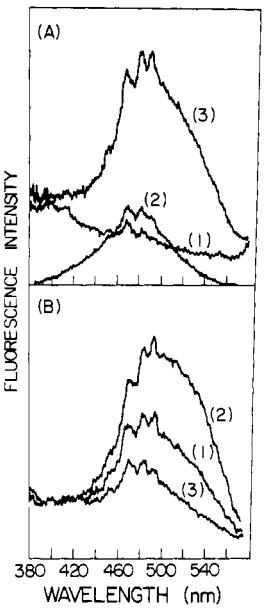

FIGURE 1: (A) Uncorrected fluorescence excitation spectra of membrane fragments, free ethidium, and ethidium plus membrane fragments. (1) Membrane fragments 1 μM in α-BuTx sites and 0.85 mg/mL of total protein. (2) Ethidium (1 μM) (spectrum 2) or membrane fragments 1 μM in α-BuTx sites plus 1 μM ethidium (spectrum 3). All solutions were in Hepes Ringer's buffer, pH 7.4. Emission was measured at 610 nm. Slit widths were 5 nm for excitation and 8 nm for emission. (B) Effects of Carb and α-BuTx on the excitation spectra of membrane fragments plus ethidium. Spectrum 1 was obtained with AcChR-enriched membrane fragments 1 μM in α-BuTx sites plus 1 μM Eth. Spectrum 2 was obtained after addition of 2 μM Carb to AcChR plus Eth [same concentrations as (1)]. From the law of mass action, with a dissociation constant of 0.05 μM for Carb (see text and Table I), this concentration corresponds to 96% of saturation of the high-affinity sites for Carb. Spectrum 3 has same concentration of α-BuTx sites and Eth as (1) plus 10 μM α-BuTx. Emission was measured at 610 nm (slit width 6.5 nm).

Data Fitting. The initial rate of [^{125}I]-α-BuTx binding to the receptor in the presence of cholinergic ligands, k_{obsd}, was determined from the slope of a nonweighted least-squares fit to semilogarithmic plots of ln ($C_\infty - C_t$) vs. time. C_∞, counts per minute at equilibrium, corresponded to the concentration of [^{125}I]-α-BuTx–AcChR complex at equilibrium, and C_t corresponded to the concentration at time t. A constant additive background contained in each term thereby canceled. The data in Figure 3A and 3B were fitted by a weighted least-squares method using weighting factors in the single-reciprocal plot 3A recalculated according to Bevington (1969, Chapter 9.3). For the titration curves of observed fluorescence increase vs. total ligand concentration, the fits were drawn by calculating the percent saturation from the law of mass action. The K_d was determined from the ligand concentration at half-saturation, L_m, of the curve where the equation $L_m = K_d + R_0/2$ holds with R_0 equal to the concentration of ligand binding sites at saturation.

Results

Spectroscopic Observations. The excitation spectra in Figure 1A show that there was an increase in fluorescence intensity upon Eth binding to AcChR-enriched membrane fragments from *T. californica*. There were, however, no

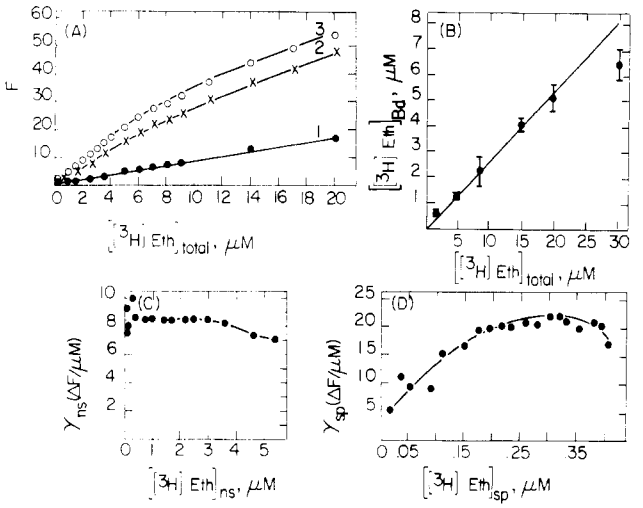

FIGURE 2: (A) Fluorescence titration with [^{3}H]Eth. The observed fluorescence enhancements were measured as a function of total [^{3}H]Eth concentration in (1) Hepes Ringer's buffer, (2) membrane fragments 1 μM in α-BuTx sites, plus 7.5 μM α-BuTx, and (3) membrane fragments alone. Excitation was at 483 ± 5 nm, and the emission was measured at 610 ± 10 nm. (B) Binding of [^{3}H]ethidium to membrane fragments containing AcChR–α-BuTx complex. A plot of [^{3}H]Eth (the concentration is shown) with membrane fragments (1 μM α-BuTx sites) incubated with 7.5 μM α-BuTx for 10 h at 4 °C prior to the experiment. Measurements of bound and free [^{3}H]Eth were done by equilibrium centrifugation (see Experimental Section). (C) Concentration dependence of the fluorescence enhancement γ_{NS} of nonspecifically bound [^{3}H]Eth. γ_{NS} was calculated as described in the text (eq 1) from the data in Figure 2A, curves 1 and 2, and Figure 2B. (D) Concentration dependence of the fluorescence enhancement γ_{SP} of specifically bound [^{3}H]Eth. γ_{SP} was calculated from eq 2 (see text). We determined the concentration of specifically bound dye from the law of mass action by using a dissociation constant of 3.2 μM (Table I) and assuming that the number of specific Eth sites is half the number of toxin sites (see text). The concentration of nonspecifically bound dye was determined from Figure 2B, and the fluorescence contribution of specific, nonspecific and free dye was evaluated from Figure 2A.

detectable shifts in the excitation (Figure 1A) or emission (broad peak, maximum at 610 nm) maxima. The strong absorbance of the dye at 285 nm (Hudson & Jacobs, 1975) with emission also at the 610-nm band prevented use of the energy transfer technique where excitation of the tryptophan band of the protein would result in a specific excitation of the dye bound to the protein. Therefore, excitation at 483 or 493 nm, and emission at 610 nm, was routinely used. In Figure 1B, the effects of the addition of 2 μM Carb to the AcChR–ethidium complex (96% of saturation of the AcChR; see caption) or a 10-fold molar excess of α-BuTx over AcChR are shown. The addition of 2 μM Carb resulted in a 45–53% increase in fluorescence emission at 610 nm as seen from the increase in the excitation maxima at 483 and 493 nm, respectively (spectra 1 and 2, Figure 1B), while saturation of the AcChR with α-BuTx resulted in a 32% decrease in fluorescence intensity (spectra 1 and 3, Figure 1B).

Interaction between Ethidium and AcChR-Enriched Membrane Fragments. In order to characterize the interaction between the dye and the AcChR-enriched membrane fragments, we undertook an experiment correlating [^{3}H]Eth fluorescence in the membranes with the amount of radioactive dye bound to the membrane fragments (by equilibrium centrifugation; see Experimental Section). Figure 2A shows a plot of fluorescence intensity vs. total [^{3}H]Eth concentration (a) in buffer alone [curve 1 (●)], (b) in a solution of membrane fragments incubated with an excess of α-BuTx [curve 2 (×)], and (c) in a solution of membrane fragments alone [curve 3 (○)]. At all [^{3}H]Eth concentrations measured, the fluorescence intensity of the dye was greater in the presence of membrane fragments without added toxin. Since the fluorescence of [^{3}H]Eth in buffer alone (curve 1, Figure 2A) was linear to a concentration of 20 μM, a fluorescence intensity of 0.95 ΔF (arbitrary units) per μM concentration of the free dye was calculated.

When α-BuTx was added to a preparation of membrane fragments plus [^{3}H]Eth, there was an increase in the concentration of free [^{3}H]Eth as measured by centrifugation assay (see Experimental Section). This indicated that a fraction of the dye bound to the membranes was displaceable by α-BuTx. This fraction of dye will be referred to as specifically bound Eth. The nonspecific binding of the dye was investigated by ultracentrifugation with the same membrane preparation and an identical concentration of AcChR–α-BuTx complex as in the spectroscopic experiments (curve 2, Figure 2A). These data are shown in Figure 2B. The binding was linear up to a total concentration of 20 μM [^{3}H]Eth, showing that the receptor–toxin complex had no saturable class of binding sites in that concentration range. At a receptor–toxin complex concentration of 1.0 μM, the fraction of the dye nonspecifically bound corresponded to 27% of the total dye.

The fluorescence enhancement of the nonspecifically bound dye can be estimated for two limiting cases. In the first case, it was assumed that there is no loss of light reaching the photomultiplier tube due to light scattering by the membrane fragments. Thus, the observed fluorescence intensity per micromolar of free ethidium, γ_F, was the same in buffer alone as in the presence of membrane fragments. Then the total fluorescence observed in Figure 2A, curve 2 (after subtraction of the small scattering contribution of the membranes), could be written

$$F_{total} = \gamma_F[Eth]_F + \gamma_{NS}[Eth]_{NS} \quad (1)$$

where the subscripts F and NS refer to the free and the nonspecifically bound dye. Assuming $\gamma_F = 0.95\ \Delta F/\mu M$ (see above) and using the result $[Eth]_{NS} = 0.27[Eth]_{total}$, we could calculate γ_{NS} as a function of the nonspecifically bound Eth concentration. The data shown in Figure 2C indicated that the fluorescence of the nonspecifically bound [^{3}H]Eth deviated from linearity at $[[^3H]Eth]_{NS}$ greater than 3 μM ($[[^3H]Eth]_{total} \approx 12\ \mu M$). From the linear region of this plot, the γ_{NS} was calculated to equal 8.5 $\Delta F/\mu$M (see Figure 2C).

Assuming that the concentration of free plus specifically bound [^{3}H]Eth equaled $0.73[[^3H]Eth]_{total}$ (this approximation neglects the perturbation in the concentration of $[[^3H]Eth]_{NS}$ by the presence of a small fraction of specific binding sites), we calculated the concentration of specifically bound [^{3}H]Eth at any concentration of $[[^3H]Eth]_{total}$ from the law of mass action by using the dissociation constant for the specifically bound Eth (see Table I). When the fluorescence contribution from $[[^3H]Eth]_{NS}$ was subtracted (Figure 2C), the remaining fluorescence $\Delta F'_{total}$ equaled

$$\Delta F'_{total} = \gamma_F[[^3H]Eth]_F + \gamma_{SP}[[^3H]Eth]_{SP} \quad (2)$$

where the subscript SP refers to the specifically bound dye.

Figure 2D shows that γ_{SP}, calculated from eq 2, also appears to be concentration dependent, reaching a maximum (21–22 $\Delta F/\mu$M) at about 40–70% of saturation ($[[^3H]Eth]_{total}$ = 4–14 μM) and decreasing at higher concentrations. The maximum enhancement of the specifically bound dye was approximately 2.5 times greater than that of the nonspecifically bound dye and 23 times that of the free dye.

The second limiting case was that light scattering in the presence of membrane fragments significantly diminishes the fluorescence enhancements. In this case we assume that F_{total}

Table I: Equilibrium Constants for Ligands Interacting with AcChR

ligand	K_d[a] (μM)	K_i[b] (μM)
ethidium, 4 mM Ca^{2+}	3.2 ± 0.1[c]	2.5 ± 0.1
ethidium, no Ca^{2+}		4.9 ± 0.3
Carb	0.05	0.12 ± 0.02
Deca	0.14	0.18 ± 0.01
nicotine	0.40	0.93 ± 0.11
choline	27.5	51 ± 0.4
d-Tc	0.035	0.2 ± 0.04
gallamine	1.3	11.1 ± 1.0
Hexa	23.0	121.0 ± 14

[a] Determined from the dependence of the ethidium fluorescence increase on ligand concentration. [b] Determined from the inhibition of the rate of toxin binding. [c] Scatchard plot of [^{3}H]Eth binding. Mean value of five experiments.

in eq 1 comes only from the nonspecifically bound Eth (i.e., γ_F has become very small compared to that observed in the absence of light scattering). Under this assumption, one calculates γ_{NS} = 11 $\Delta F/\mu$M over the linear range of the fluorescence increase as compared to γ_{NS} = 8.6 in the other limiting case. γ_{SP} follows the same concentration dependence as shown in the previous case (see Figure 2D) with a maximum enhancement of 21 $\Delta F/\mu$M as compared to 22 $\Delta F/\mu$M found with the other limiting assumptions. Thus, even if the contribution of the free dye is neglected because of light scattering from the membrane suspension, γ_{SP}(max) was still about twice that of γ_{NS}(max).

Interactions of Ethidium with the Membrane-Bound AcChR. From the data presented above, it was apparent that there were two classes of Eth bound to the membrane fragments: Eth that was displaceable by α-BuTx (specifically bound to the AcChR) and a nonsaturable component not displaceable by α-BuTx (nonspecifically bound). Interactions between the specifically bound Eth and the AcChR were examined by using two approaches: (1) the inhibition of the kinetics of [^{125}I]-α-BuTx–AcChR complex formation and (2) construction of a Scatchard plot (Scatchard, 1949) from the difference of [^{3}H]Eth binding to membrane-bound AcChR and AcChR–α-BuTx complex.

Figure 3A shows the inhibition of the rate of toxin binding by Eth. The data were fitted to eq 3 since α-BuTx was found

$$k_{obsd} = \frac{d}{dt}[RT]|_{t=0} = kT_0R_0\left[1 + \frac{E_0}{K_i}\right]^{-1} \quad (3)$$

to directly displace Eth (see above). k_{obsd} is the initial slope of a semilogarithmic plot of ln $(C_\infty - C_t)$ vs. time (see Experimental Section), and k is the second-order rate constant for combination of [^{125}I]-α-BuTx with the AcChR. R_0, T_0, and E_0 are the initial concentrations of receptor, toxin, and Eth, and K_i is the apparent equilibrium dissociation constant of the AcChR–Eth complex (the correction for bound dye is negligible at the low concentration of membrane fragments used in these studies). The abscissa intercepts of the fit to the single-reciprocal plot of the data according to eq 3 gave values of 2.5 ± 0.1 μM and 4.9 ± 0.3 μM for the apparent dissociation constant of Eth in the presence and absence of 4 mM Ca^{2+}, respectively (see Figure 3A).

The specific binding of [^{3}H]Eth to the AcChR in the presence of 4 mM Ca^{2+}, measured by ultracentrifugation, is shown in Figure 3B. These data presented in the form of a Scatchard plot (Scatchard, 1949) indicate a single homogeneous class of sites with a dissociation constant equal to 4.4 ± 0.1 μM. The weighted mean of five experiments gave a slightly lower value of 3.2 ± 0.1 μM (Table I) for the dissociation constant of the specifically bound Eth. The abscissa intercept in Figure 3A indicated a stoichiometry of 0.53 ± 0.01 mol of Eth sites/mol of α-BuTx sites.

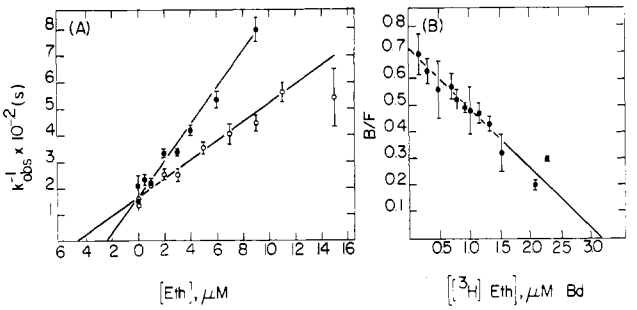

FIGURE 3: (A) Inhibition of the rate of [^{125}I]-α-BuTx binding at various concentrations of ethidium in the presence and absence of Ca^{2+}. Membrane fragments, 0.04 μM in α-BuTx sites, were equilibrated with Eth for 20 min, and 0.4 μM [^{125}I]-α-BuTx was added at time zero. The observed rate constant of toxin binding was determined from normalized semilogarithmic plots (see Experimental Section). The data in the presence of 4 mM Ca^{2+} (●) or Hepes Ringer's buffer minus Ca^{2+} (○) were fitted to the reciprocal form of eq 3 as described under Data Fitting. The values for K_i determined from the abscissa intercept were 2.5 ± 0.1 μM at 4 mM Ca^{2+} and 4.9 ± 0.3 μM in the absence of Ca^{2+}. (B) Scatchard plot of specific binding of [^{3}H]Eth to AcChR-enriched membrane fragments. The concentrations of bound and free [^{3}H]Eth were determined by equilibrium centrifugation (see Experimental Section). The concentration of specifically bound [^{3}H]Eth was determined by subtracting the concentration of nonspecifically bound dye (determined as in Figure 2B) from the total dye bound to the membrane fragments in the absence of α-BuTx. The membranes were 6.0 μM in α-BuTx sites, and the nonspecifically bound dye was determined after incubating the AcChR with a threefold molar excess of α-BuTx for 24 h at 4 °C. A linear least-squares fit to the data gave a slope, equal to the reciprocal of the dissociation constant, of 4.4 ± 0.1 μM and an abscissa intercept, equal to the total [^{3}H]Eth sites, of 3.2 ± 0.1 μM, indicating a stoichiometry of 0.53 mol of [^{3}H]Eth sites/mol of α-BuTx sites.

Ethidium as an Indicator of Cholinergic Ligand Binding. To evaluate the effect of cholinergic ligands on the fluorescence of the AcChR–Eth complex, a determination of the number of high-affinity sites, e.g., for Carb, was done under the condition where the concentration of Carb sites was in large excess over the dissociation constant for Carb. The number of high-affinity Carb sites was first determined by measuring [^{3}H]Carb binding to the AcChR in an equilibrium centrifugation experiment (Figure 4A) and was found to equal half of the total α-BuTx sites, in agreement with data previously reported (Raftery et al., 1975; Quast et al., 1978a,b). In Figure 4B nonradioactive Carb was used to titrate the observed fluorescence increase of Eth bound to the membrane fragments at high AcChR concentration. A break point equal to half of the total α-BuTx sites was again observed. Similar results were found with *d*-Tc (Figure 4C) and Deca (Figure 4D) in agreement with earlier equilibrium dialysis studies on the solubilized purified AcChR (Moody et al., 1973). The concentration dependence of the observed fluorescence increase of Eth for several cholinergic ligands (where the $[AcChR]_0$ was approximately equal to the K_d of the ligand) is shown in Figure 5A,B. The apparent dissociation constants obtained from these curves are compared with the inhibition constant K_i, obtained by the inhibition of the rate of toxin binding by these ligands fitted to eq 3 (see Table I).

In order to determine whether the fluorescence increase observed upon addition of Carb was due to an increase in the concentration of dye bound to the membrane fragments or to a change in the quantum yield of the dye already bound, we examined the effects of the addition of Carb on the concentration of membrane-bound [^{3}H]Eth (Figure 6). After the nonspecifically bound Eth (1.4 μM) was corrected for, the

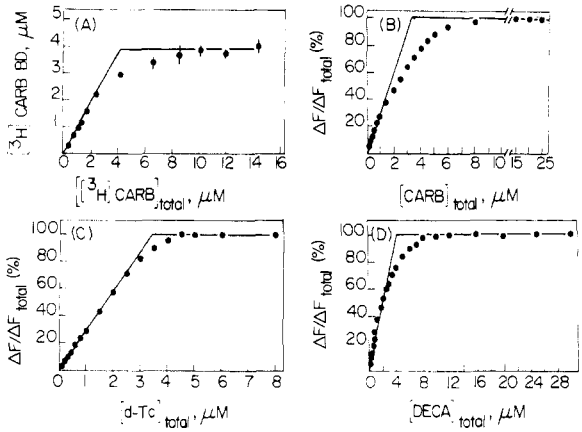

FIGURE 4: (A) Binding of [^{3}H]Carb to AcChR-enriched membrane fragments. The binding of [^{3}H]Carb to membrane fragments (7.3 μM in α-BuTx sites) was determined by the equilibrium centrifugation assay (see Experimental Section). At saturation, 3.85 μM [^{3}H]Carb was bound to the membrane fragments corresponding to 0.53 mol of high-affinity [^{3}H]Carb sites/mol of α-BuTx sites. The abscissa value of the break point, 4.15 μM [^{3}H]Carb sites (0.57 mol of [^{3}H]Carb sites/mol of α-BuTx sites), was somewhat higher, probably reflecting a slight discrepancy in the determination of the specific activities of the labeled Carb and toxin. (B–D) Titration of the increase in ethidium fluorescence caused by Carb (B), *d*-Tc (C), and Deca (D). The normalized fluorescence increase is plotted vs. total ligand concentration, showing a sharp break point when the high-affinity sites of the receptor for the corresponding ligand were saturated. (B) Carb: $[AcChR]_0$ = 5.96 μM in α-BuTx sites. The break point at 3.2 μM Carb indicates 0.54 mol of high-affinity Carb sites/mol of toxin sites. (C) *d*-Tc: $[AcChR]_0$ = 6.6 μM, break point at 3.35 μM *d*-TC (51% of toxin sites). (D) Deca: $[AcChR]_0$ = 8 μM, break point at 3.8 μM Deca (48% of toxin sites). The concentration of Eth was 1 μM in all cases.

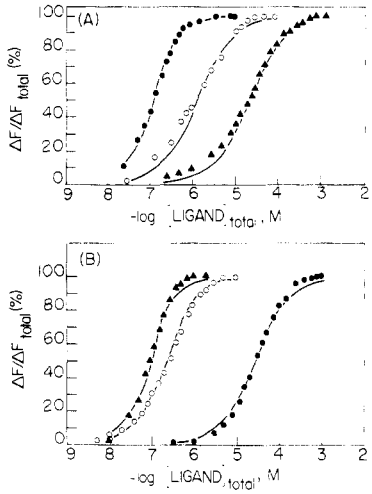

FIGURE 5: Titration curves of normalized ethidium fluorescence increase by cholinergic ligands. (A) Experimental results for Carb (●), Hexa (▲), and gallamine (○). The concentration of Eth was 1 μM and of membrane fragments was 0.3 μM in α-BuTx sites. The theoretical curves were calculated (see Data Fitting) from the law of mass action and dissociation constants of 0.05, 23, and 1.3 μM for Carb, Hexa, and gallamine, respectively. The number of high-affinity sites for these ligands was assumed to equal half of the total number of α-BuTx sites. Changing this number by a factor of 2 would affect only the titration curve for Carb. (B) Experimental results for choline (●), *d*-Tc (▲), and Deca (○). The concentration of α-BuTx sites was 0.3 μM for choline and Deca and 0.22 μM for *d*-Tc while $[Eth]_{total}$ = 1 μM in all cases. The theoretical curves were calculated from dissociation constants of 27.5, 0.035, and 0.14 μM for choline, *d*-Tc, and Deca, respectively. The number of ligand sites was again assumed to equal half the number of α-BuTx sites.

concentration of free plus specifically bound dye equaled 3.6 μM, and the number of specific Eth sites equaled 1.7 μM. Under these conditions, 0.8 μM [^{3}H]Eth sites were occupied

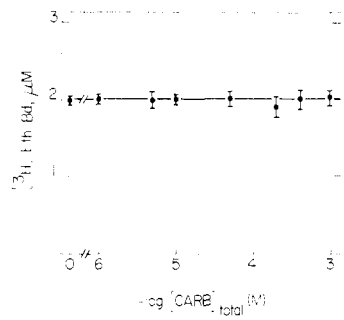

FIGURE 6: Effect of Carb on the concentration of [^{3}H]Eth bound to AcChR-enriched membrane fragments. Membrane fragments were 3.4 μM in α-BuTx sites (1.7 μM in Eth sites), and the total [^{3}H]Eth concentration equaled 5 μM. In an experiment similar to that in Figure 2B, the concentration of nonspecifically bound dye was found to equal 1.4 μM. Thus, the concentration of free plus specifically bound dye was 3.6 μM. From the law of mass action, with a dissociation constant of 3.2 μM (Table I), the specifically bound [^{3}H]Eth was calculated to equal 0.8 μM. Thus, about 50% of the total Eth sites were occupied. Carb was varied from 0 to 1 mM, and the concentration of bound and free [^{3}H]Eth was determined by equilibrium ultracentrifugation (see Experimental Section).

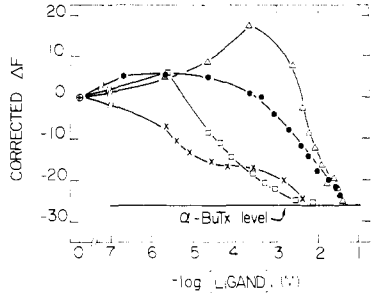

FIGURE 7: Effect of local anaesthetics on ethidium fluorescence in membrane fragments. Conditions used were 1 μM Eth, 0.42 μM α-BuTx sites. Corrected ΔF equals ΔF for membrane fragments alone minus that for membrane fragments incubated with a 10-fold molar excess of α-BuTx. α-BuTx level is that level of fluorescence decrease caused by the addition of a 10-fold molar excess of α-BuTx. The local anaesthetics were lidocaine (●), procaine (△), tetracaine (×), and dibucaine (□); (+) is the fluorescence level in the absence of ligands. The curves drawn through the data points have no theoretical significance.

and 0.9 μM were empty; thus, the concentration of bound dye should be sensitive to any change caused by Carb. The data in Figure 6 indicated that there was no change in the concentration of [^{3}H]Eth bound to the membrane fragments (to within 5%) as the Carb concentration was increased from zero to 1 mM. Thus, the fluorescence increase was largely, if not totally, due to a change in quantum yield of the bound dye. At higher concentrations of Carb (>1 mM), *d*-Tc (>10 μM), and DAP (>10 μM), [^{3}H]Eth was displaced from the membrane fragments, and a concomitant decrease in fluorescence intensity was observed [see Quast et al. (1979) (following paper) and Figure 7]. The ligand HTX did not displace [^{3}H]Eth at concentrations up to 30 μM, in agreement with the observation of Elliott & Raftery (1978) who found that Eth did not displace [^{3}H]HTX.

Effects of Local Anaesthetics. Figure 7 shows the effect of four local anaesthetics on the fluorescence properties of membrane-bound Eth. The fluorescence intensity has been corrected for nonspecific effects that were observed when the anaesthetics were added to the receptor–toxin complex. The nonspecific fluorescence decrease occurred for procaine and tetracaine at concentrations greater than 1 mM and was negligible for dibucaine and lidocaine. The concentration dependence of the nonspecific fluorescence decrease agreed with displacement of [^{3}H]Eth, determined by equilibrium

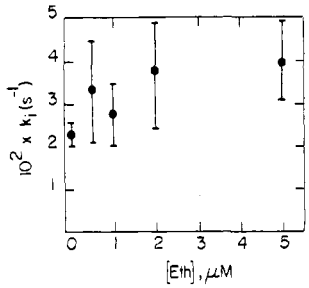

FIGURE 8: Ethidium concentration dependence of the rate of transition of AcChR from low- to high-affinity form. The receptor (0.88 μM in toxin sites) was incubated with the concentration of Eth indicated for 20 min. Then [^{125}I]-α-BuTx and Carb were simultaneously added ($[toxin]_0$ = 94 nM, $[Carb]_0$ = 5 μM), and the kinetics of toxin binding were followed by the DEAE filter disc assay (Schmidt & Raftery, 1973). At the chosen concentration, the kinetics were strongly biphasic with the fast phase, reflecting the Carb-induced isomerization of the receptor from low- to high-affinity form, and the slow phase, showing toxin binding in the presence of Carb to high-affinity receptor (Quast et al., 1978a). After we subtracted out the slow phase in a semilog plot, a replot of the difference in semilog form yielded a straight line. The rate of transition, k_i, was determined by an unweighted linear least-squares fit of these data. The error bars indicate the statistical error in k_i.

centrifugation, induced by local anaesthetics at concentrations greater than 500 μM. The curves shown in Figure 7 are complex in nature and appear to be a superposition of several different effects varying with concentration. Procaine, lidocaine, and dibucaine showed an initial increase in fluorescence intensity, followed by a quenching of Eth fluorescence approaching the level found when Eth is displaced by α-BuTx. Tetracaine appeared to only quench Eth fluorescence in a noncompetitive manner. Since the effects of tetracaine, dibucaine, and lidocaine occurred at much lower concentrations than the displacement of the ^{3}H-labeled dye from the membrane fragments, it seems likely that these local anaesthetics cause a conformational change in the membrane-bound receptor, resulting in a decrease of the quantum yield of the AcChR–Eth complex. The fluorescent increase, most pronounced in the case of procaine, appears to indicate that the conformation of the AcChR–Eth complex undergoes several transitions, depending on the concentration and structure of the local anaesthetic binding to the receptor (or to the membrane in the vicinity of the protein).

Since Eth fluorescence was affected by local anaesthetics (Figure 7), it was important to determine whether Eth itself acted strongly as a local anaesthetic. Since some such compounds are known to dramatically affect the rate of transition of the AcChR from the low- to high-affinity state (Weiland et al., 1977; Briley & Changeux, 1978), the rate constant of this process, k_i, was determined as a function of Eth concentration (Figure 8). The value of k_i showed only a slight variation from $(2.3 \pm 0.3) \times 10^{-2}$ s^{-1} to $(4 \pm 1) \times 10^{-2}$ s^{-1} at Eth concentrations from 0 to 5 μM. The value at 2 μM [the concentration used in the stopped-flow studies (see papers two and three of this series)] showed an increase in k_i of (1.65 ± 0.64)-fold. Since the limiting value of k_i is approximately 0.14 s^{-1} (Quast et al., 1978a,b, 1979), these changes appear to be rather small in nature and suggest that Eth does not strongly affect the observed rate of receptor transition from low to high affinity. In addition, it was found that Eth at concentrations up to 10 μM did not affect the neurally evoked postsynaptic potential in *Electrophorus* electroplax (H. L. Lester, personal communication).

Discussion

This communication describes the interactions of the fluorescent probe Eth with AcChR-enriched membrane fragments from *T. californica*. There appear to be two populations of dye bound to the membrane: (1) nonspecifically bound Eth which binds in a nonsaturable manner up to 20 μM total dye and is not affected by α-BuTx and (2) Eth that shows saturable binding and is displaced by α-BuTx (i.e., dye bound to the AcChR). The fluorescence enhancement of the specifically bound dye was estimated to be approximately two to three times that of the nonspecifically bound dye under the two limiting approximations of neglecting (1) the light scattering from the membranes and (2) a large inner filter effect due to light scattering.

The specifically bound dye was characterized by kinetic studies of the inhibition of the rate of toxin binding to the receptor (Figure 3A). The results were consistent with Eth acting as a competitive inhibitor that bound about twofold more tightly in the presence of Ca^{2+} (K_i = 2.5 μM) than in its absence (K_i = 4.9 μM). The value obtained in the presence of calcium agrees well with that determined by equilibrium centrifugation experiments with the labeled dye (K_d = 3.2 μM). The stoichiometry obtained from the abscissa intercept of the Scatchard plot in Figure 3B was 0.5 mol of [^{3}H]Eth/mol of α-BuTx sites. The phenomenon of a ligand appearing to linearly inhibit the rate of toxin association while binding with high affinity to half of the total α-BuTx sites has also been observed for carbamylcholine (Quast et al., 1978a) and remains mechanistically unexplained.

The data in Figure 4 indicated that the stoichiometries of high-affinity sites for Deca, Carb, and *d*-Tc are accurately reflected in the titration of Eth fluorescence by these ligands. Determination of the apparent dissociation constant by this method (Figure 5 and Table I) yields results that are within a factor of 2 for agonists and 5–10 for antagonists of those determined from the inhibition of the rate of toxin binding to the AcChR. Since the fluorescence experiments were done with different preparations of membrane fragments from the kinetic experiments, we feel that the agreement between the results from such different methods is, in general, quite good. Thus, the determination of apparent K_d for cholinergic ligands by titration of the fluorescence increase of Eth is very convenient when compared to methods such as equilibrium centrifugation with radioactive ligand and the inhibition of the rate of toxin binding.

The data in Figure 6 indicate very strongly that the fluorescence increase observed upon addition of Carb to a preparation of membrane fragments plus Eth does not come from an increase in the concentration of either the specifically or nonspecifically bound Eth. If the total fluorescence increase is attributed to a 5% uptake of the dye, which cannot be ruled out, the enhancement of this fraction must be about 20 times higher than that of the Eth bound before the addition of ligand and at least 150 times that of the free dye. Since this seems unlikely, we attribute the fluorescence increase to an increase in the enhancement of dye already bound to the membranes. Although it would be most appealing to attribute the change in quantum yield only to the class of specifically bound dye, the evidence presented above does not permit that distinction to be made since α-BuTx, which displaces the specifically bound Eth, also prevents cholinergic ligands from binding. Only in the case of HTX, where a ternary complex (HTX–α-BuTx–AcChR) can be formed (Elliott & Raftery, 1977), can an unambiguous assignment be made since over 90% of the amplitude of the fluorescence change caused by HTX was lost (Schimerlik et al., 1979) in the presence of α-BuTx, and HTX did not displace [^{3}H]Eth. The simplest explanation of

these results was that the fraction of Eth that was displaced by α-BuTx was responsible for over 90% of the amplitude of the fluorescence change. Thus, in the case of HTX, we could directly demonstrate that the fluorescence signal did indeed arise from the Eth specifically bound to the AcChR.

The effects of local anaesthetics on the AcChR–Eth complex indicate that these compounds interact with the membrane-bound receptor in a very complicated manner. At low concentrations, all except tetracaine appeared to cause a conformational change that had the same fluorescence sign as that caused by Carb, while at higher concentrations a decrease in Eth fluorescence occurred. This decrease could be assigned largely to a decrease in quantum yield for all the local anaesthetics except procaine since it occurred in a concentration range where little if any [^{3}H]Eth was displaced. It is not possible from our data to determine if these diverse effects are caused by binding of several molecules of local anaesthetic to different classes of discrete receptor sites or are the result of changes in the membrane environment of the protein. These data do, however, indicate that the conformation of the AcChR–Eth complex depends on the nature as well as the concentration of local anaesthetic present.

The interactions of Eth with *T. californica* membrane fragments enriched in AcChR appear to be rather complex. However, the titration of the observed fluorescence increases at constant low Eth (i.e., 1–2 μM) concentrations does give results compatible with those found with other methods (Figure 5), indicating that this dye accurately reflects the interactions of cholinergic ligands with the AcChR. The following two communications (Quast et al., 1979; Schimerlik et al., 1979) present kinetic analyses of these interactions as monitored by stopped-flow techniques.

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