A FLUORESCENCE PROBE OF ACETYLCHOLINE RECEPTOR CONFORMATION AND LOCAL ANESTHETIC BINDING*

M. Schimerlik and M. A. Raftery**

Church Laboratory of Chemical Biology
Division of Chemistry and Chemical Engineering**
California Institute of Technology
Pasadena, California 91125

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SUMMARY. The fluorescent dye ethidium bromide binds to the acetylcholine receptor with an apparent K_d of 3 μM and a stoichiometry of 1 molecule of ethidium per α-bungarotoxin site. Time dependent fluorescent increases were observed upon addition of carbamylcholine, the amplitude and half-time of which were dependent on the Carb[1] concentration. It appeared that these fluorescence increases resulted from a lowering of the K_d for ethidium as the AcChR-Carb complex underwent an isomerization from low to high affinity form(s) for carb, and more ethidium was bound. Titration with the local anesthetic procaine led to ethidium fluorescence increases at low procaine concentrations, followed by a fluorescence decrease at higher procaine concentrations to that level induced by saturating α-bungarotoxin. Thus it appeared that the ethidium binding site either interacted with or was identical with local anesthetic binding site(s).

The acetylcholine receptor protein has been purified from *Torpedo californica* (1-3) and the binding of cholinergic agonists and antagonists studied by means of their inhibition of the rate of ^{125}I-α-Bgt-AcChR complex formation (4, 5) or using equilibrium techniques (6). In addition, the technique of ligand inhibition of the time course of receptor-α-Bgt complex formation has been used to show the isomerization of the AcChR-ligand complex from low affinity to high affinity form(s) in the presence of agonists (7) or in the presence of either agonists or antagonists (8) corresponding to an *in vitro* model of the *in vivo* phenomenon of desensitization (9-11). The use of fluorescence probes such as DAP [bis(3-amino-pyridium)1,10-decane diiodide] (12,13), Dns-Chol (1-(5-dimethyl-aminonaphylene-1-sulfonamide)propane-3-trimethyl-ammonium iodide) (14) and quinacrine (15,16) has contributed a great deal of information concerning the topography of AcChR ligand binding sites and possible states of the AcChR-ligand

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[1]Abbreviations: AcCh, acetylcholine; AcChR, acetylcholine receptor; Carb, carbamylcholine; α-Bgt, α-bungarotoxin; ^{125}I-α-Bgt, ^{125}I-α-bungarotoxin; ANS, 1-anilino-8-naphthalene sulfonate.

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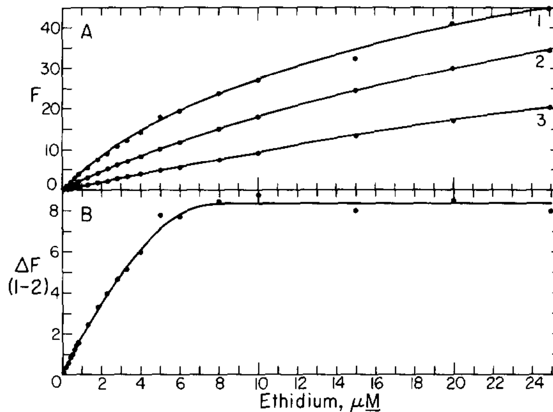

Figure 1A. Curve 1, membrane fragments 2.3 μM in α-Bgt binding sites; curve 2, membrane fragments preincubated with 10 μM α-Bgt; curve 3, Ringers buffer 2.5 ml total volume. Excitation 483 nm, emission 610 nm,

Figure 1B. Fluorescence difference, curve 1 minus curve 2, sensitivity 1.

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complexes. In this communication we present data concerning the use of ethidium as a fluorescent probe of the AcChR in its membrane environment.

MATERIALS AND METHODS

Torpedo californica was obtained live locally and maintained in a tank at 16°C or the electric organs were removed and stored at -90°C. Occasionally, the animal was killed after lowering the tank temperature to 2°C over 48 hrs. AcChR enriched membrane fragments were prepared by homogenization of electric organs in Ringers solution (5 mM Tris, 250 mM NaCl, 2 mM $MgCl_2 \cdot 6H_2O$, 4 mM $CaCl_2 \cdot 2H_2O$, 5 mM KCl, 0.02% w/v NaN_3, pH 7.4) and fractionated using previously developed procedures (17,18) except that the zonal sucrose gradient was 25 - 50% w/v sucrose in Ringers.

The concentration of α-Bgt binding sites was determined by a DEAE-cellulose filter-disc assay procedure (2) using ^{125}I-α-Bgt prepared from α-Bgt purified from Bungarus multicinctus venom (Sigma Chemical Co.) by the procedure of Clark *et al.* (19). Membrane fragments were routinely assayed to determine whether they were of high or low affinity form for Carb and capable of undergoing the transition from low to high affinity form(s) upon preincubation with that ligand (7,8).

Fluorescence measurements were made in a Perkin-Elmer MPF-4 spectrofluorimeter thermostated at 25° ± 1°C using an excitation wavelength of 483 nm and monitoring emission at 610 nm. Fluorescence due to ethidium binding to α-Bgt sites was obtained by subtracting the fluorescence increase from membrane fragments reacted with an excess of α-Bgt from that obtained from membrane fragments in the absence of α-Bgt.

Carbamylcholine and procaine HCl were purchased from Sigma Chemical Co. and ethidium bromide from Cal. Biochem. All experiments were done in Ringers buffer at 25°C.

RESULTS

A typical ethidium bromide fluorescence titration is shown in Fig. 1A; the top curve represents the fluorescence of membrane fragments (2.3 μM in α-Bgt

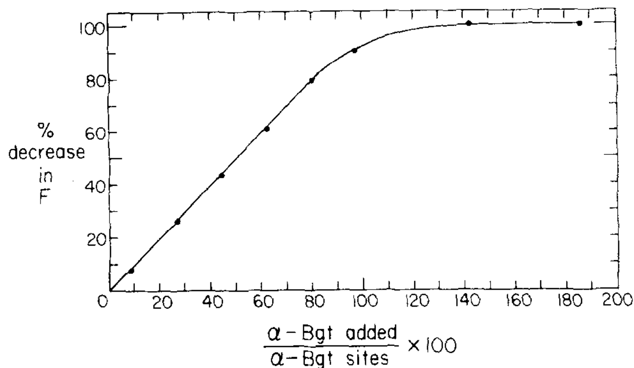

Figure 2. Displacement of ethidium by α-Bgt. Initially 2.3 μM in α-Bgt sites, 25 μM ethidium in Ringers buffer.

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sites) corrected for light scattering, while the second curve is that of membrane fragments pretreated with a ten-fold excess of α-Bgt. Curve 3 is the fluorescence of ethidium in buffer alone. The difference in fluorescence between curves 1 and 2, attributed to ethidium binding to α-Bgt sites, plotted *versus* total ethidium concentration in Fig. 1B, was hyperbolic and showed that this binding was a saturable function of the ethidium concentration. The midpoint of the curve yielded an apparent K_d (uncorrected for non-specific binding) of 2.5 μM for ethidium bound at α-Bgt sites.

In order to determine the stoichiometry of ethidium sites to α-Bgt sites a cuvette containing AcChR (2.3 μM of α-Bgt sites) and 25 μM ethidium was titrated with α-Bgt and the percent fluorescence decrease *versus* percent α-Bgt sites occupied was plotted (Fig. 2). This data clearly showed a linear displacement of ethidium by α-Bgt until nearly 90% of the toxin sites were occupied. Thus, it appeared that one molecule of ethidium bound per α-Bgt site.

When 1 μM Carb was added to a mixture of 0.5 μM ethidium plus membrane fragments (0.5 μM α-Bgt sites) in the low affinity form (not desensitized) a time dependent fluorescence increase was observed (Fig. 3). The half-time for the process, shown in Fig. 4, was approximately 1.2 min, becoming shorter at higher Carb concentrations. In addition, the amplitude of this fluorescence increase was titratable by Carb with the response at saturation (usually 2 - 3-fold molar excess of Carb over toxin sites) equal to the sum of the individual increases. There was no time-dependent increase in maximum fluorescence upon saturation with ethidium in the presence of a hundred-fold molar excess of Carb (data not shown) nor were there shifts in the emission or absorption spectra of the bound dye. Since both Carb and ethidium binding were rapid on

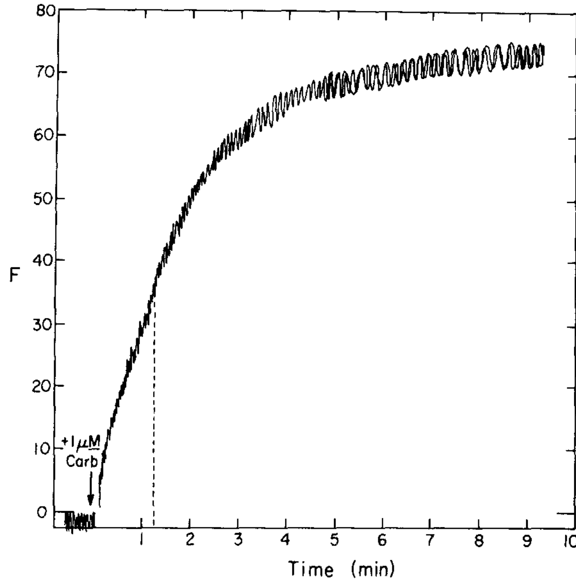

Figure 3. Time dependence of ethidium fluorescence change upon addition of Carb. Membrane fragments, 0.5 M in α-Bgt sites, plus 0.5 μM ethidium. 1 μM Carb added at time indicated above. Sensitivity 30.

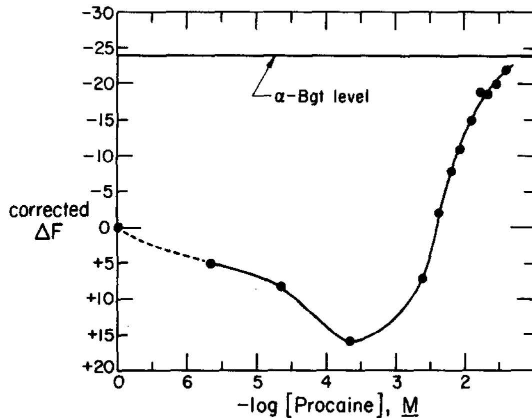

Figure 4. Effect of procaine on ethidium fluorescence. Membrane fragments 0.42 μM in α-Bgt sites plus 1.0 μM ethidium. Fluorescence corrected for non-specific effect.

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this time scale, it seemed likely that there was a slow isomerization of the receptor-carb complex taking place to a form which had an increased affinity for ethidium and the increase in fluorescence was due to an increase in total dye bound. In the presence of an excess of α-Bgt no fluorescence increase was observed, indicating that this phenomenon was specific for α-Bgt binding sites.

The local anesthetic procaine had a dual effect on ethidium fluorescence. This is shown in Fig. 4 where the fluorescence change is plotted *versus* the concentration of procaine added, corrected for nonspecific effects. At low concentrations of procaine (2×10^{-6} - 2×10^{-4} M) there was a fluorescence increase while at higher procaine concentrations (2×10^{-4} - 3.6×10^{-2} M) a fluorescence decrease was observed that titrated up to the amount of ethidium displaced by saturating α-Bgt. In membrane fragments pretreated with α-Bgt no fluorescence increase was observed; however, there was a smaller fluorescence decrease observed at higher procaine concentrations. Thus, it appeared that at low concentrations procaine either caused an effect similar to Carb, or an increase in quantum yield of specifically bound ethidium, while at higher concentrations it displaced (or quenched) both the ethidium bound at the α-Bgt binding sites and nonspecifically bound ethidium.

DISCUSSION

The data presented in Figs. 1 and 2 indicate that one molecule of ethidium binds per α-Bgt site with an apparent dissociation constant of about 3 μM. Since ethidium was displaced linearly by α-Bgt it seems unlikely that this dye exhibits the half-site phenomena shown by DAP(Bis-(3-aminopyridinium)1,-10-decane diiodide) (13) or by the close analog propidium (20).

Upon addition of the agonist carbamylcholine to membrane fragments in the low affinity form towards this ligand, a slow fluorescence increase was observed (Fig. 3) and both the amplitude and the half-time of this process were dependent on the concentration of added ligand. The fact that there was no change in the fluorescence enhancement of bound ethidium in the presence of a 100-fold excess of Carb and that membrane fragments pretreated with α-Bgt did not exhibit this phenomenon imply that this fluorescence change was specific for α-Bgt binding sites and was due to an increase in the amount of specifically bound dye. When membrane fragments were irreversibly changed to the higher affinity form for Carb upon standing (7) as measured by inhibition of ^{125}I-α-Bgt binding (8) only small fluorescence increases were observed when this ligand was added. This may be due to a small population of membrane bound AcChR still in the low affinity state. Thus, it seems likely that the time dependent fluorescent increase described here reflects the isomerization of the AcChR from the low affinity (nondesensitized) to the high affinity (desensitized) from which then exhibits a lower K_d for ethidium.

The effects of procaine on bound ethidium appear to be rather complex (Fig. 4). At low concentrations (2×10^{-6} - 2×10^{-4} M) a net fluorescence increase was observed only in membrane fragments not treated with α-Bgt. At this time, it is not possible to differentiate between the possibility of an

effect similar to that of Carb or a change in quantum yield of specifically bound ethidium either by procaine binding to a nearby site or to a distant AcChR site causing a change in the ethidium environment. Along these lines, Levy and Cheng (21) have observed fluorescence increases in ANS bound to hepatocyte plasma membranes without an increase in total ANS sites when procaine was added, and they also observed a decrease in the dissociation constant for this dye. At higher concentrations (2×10^{-4} - 3.6×10^{-2} M) of procaine the fluorescence decreased (midpoint of 10^{-2} M) to the level attained when saturating amounts of α-Bgt were added.

The results presented here indicate that ethidium bromide is an extremely sensitive probe of the AcChR in its membrane environment. Its ability to reflect changes in the state of the AcChR after ligand binding and the decrease in fluorescence observed upon addition of local anesthetics indicate that ethidium may be useful in exploring ligand induced isomerizations of the AcChR as well as the topography of local anesthetic binding sites. Such studies, as well as experiments to determine whether local anesthetics quench ethidium fluorescence or displace the dye from the AcChR, are currently in progress.

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