Uses of Fluorescent Cholinergic Analogues to Study Binding Sites for Cholinergic Ligands in *Torpedo californica* Acetylcholine Receptor†

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ABSTRACT: A series of synthetic 1,*n*-bis(3-aminopyridinio)-alkane fluorescent probes have been used to determine the ligand binding properties of the acetylcholine receptor purified from *Torpedo californica* electroplax. At equilibrium, the probes bound to a single class of sites. The binding affinity of the fluorescent decamethonium analogues increased progressively as the number of methylene groups (*n*) increased from 4 to 12 and decreased in the range of 16–18 such groups. The receptor bound 1,12-bis(3-aminopyridinio)dodecane and 1,14-bis(3-aminopyridinio)tetradecane with the highest affinity while related monofunctional probes such as 1-(3-aminopyridinio)propane were bound with a substantially lower affinity. The data indicate that the receptor interacts strongly with both ends of a bifunctional probe such as 1,14-bis(3-aminopyridinio)tetradecane. Also, competition between bifunctional fluorescent probe binding and the binding of conventional cholinergic ligands was investigated and led to the conclusion that the probes, which are antagonists, form ternary complexes in the presence of acetylcholine.

The acetylcholine receptor protein purified from *Torpedo californica* (Schmidt & Raftery, 1973b) binds a variety of ligands with high affinity to half the number of α-BuTx[1] sites (Moody et al., 1973; Martinez-Carrion & Raftery, 1973). One such ligand is the fluorescent antagonist DAP. Recently, it has been shown (Witzemann & Raftery, 1977) that the likely location of the DAP binding site(s) is on one (M_r 40 000) of the four different polypeptides (M_r 40 000, 50 000, 60 000, and 65 000) which comprise purified *T. californica* AcChR (Raftery et al., 1974; Weill et al., 1974). A different approach (Karlin & Cowburn, 1973) with an affinity label for covalent attachment of the cholinergic analogue MBTA to DTT-treated AcChR has resulted in identification of the 40 000-dalton subunit as the site of attachment of this label, with a stoichiometry also corresponding to half the number of α-BuTx sites (Weill et al., 1974). The affinity labels used in these studies are antagonists. However, it has recently been demonstrated that the agonist bromoacetylcholine also can react covalently with the 40 000-dalton receptor subunit, following DTT reduction of the AcChR in both membrane-bound and solubilized, purified states (Moore & Raftery, 1979).

It is not known at the present time whether all agonists and antagonists bind to a single type of binding site and exert their differing physiological effects by means of conformational effects or lack thereof. The affinity labeling studies referred to above localize binding sites on the 40 000-dalton subunit. Furthermore, MBTA and BrAcCh labeling are likely to affect the same binding site since an additional requirement of a readily reducible disulfide in close proximity is required for covalent attachment.

We have studied the interactions of DAP and a homologous series of bis(onium) fluorescent compounds in an attempt to determine some basic features of the interactions of such antagonists with the AcChR. In addition, studies of the competition of more classical ligands such as AcCh, Carb, Deca, *d*-Tc, and α-BuTx with DAP and its analogues for AcChR binding sites have been conducted.

Experimental Section

Materials

3-Aminopyridine, 1,6-diiodohexane, 1,8-diiodooctane, 1,10-diiododecane, and 1,12-diiododododecane were obtained from K & K Laboratories. 1,14-Dibromotetradecane, 1,16-dibromohexadecane, 1,18-dibromoctadecane, and decamethonium dibromide were obtained from ICN Pharmaceuticals. 1,4-Diiodobutane, carbamylcholine chloride, *d*-tubocurarine, and acetylcholine chloride were obtained from Sigma Chemical Co. Decamethonium and hexamethonium were purchased from K & K Laboratories. Lyophilized *Bungarus multicinctus* venom was obtained from Sigma Chemical Co., and [^{125}I]-α-BuTx was prepared as described by Blanchard et al. (1979) from α-BuTx purified by the method of Clark et al. (1972). *Torpedo californica* was obtained locally, and the electric organs were excised and used directly or frozen at −90 °C.

Methods

Fluorescent Probe Synthesis. Fluorescent probes were synthesized according to the method of Mooser et al. (1972). The bifunctional probes were synthesized by refluxing ap-

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[1] Abbreviations used: AcCh, acetylcholine; α-BuTx, α-bungarotoxin; BAP, 1,4-bis(3-aminopyridinio)butane; HAP, 1,6-bis(3-aminopyridinio)hexane; OAP, 1,8-bis(3-aminopyridinio)octane; DAP, 1,10-bis(3-aminopyridinio)decane; DoDAP, 1,12-bis(3-aminopyridinio)decane; TetraDAP, 1,14-bis(3-aminopyridinio)tetradecane; HecaDAP, 1,16-bis(3-aminopyridinio)hexadecane; OctaDAP, 1,18-bis(3-aminopyridinio)octadecane; NaDodSO$_4$, sodium dodecyl sulfate; AcChR, acetylcholine receptor; AcChE, acetylcholinesterase; Carb, carbamylcholine; Deca, decamethonium; BrAcCh, bromoacetylcholine; DTT, dithiothreitol; MBTA, 4-(*N*-maleimido)benzyltrimethylammonium iodide; Hexa, hexamethonium; *d*-Tc, *d*-tubocurarine.

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Table I: Analysis of the Synthetic 3-Aminopyridinium Salts

probe	% C found	% C theoretical	% N found	% N theoretical	% H found	% H theoretical	mp (°C)
3-amino-1-methylpyridinium iodide	30.69	30.52	11.80	11.87	3.87	3.84	119.5–121.5
3-amino-1-propylpyridinium iodide	36.42	36.38	10.57	10.61	4.97	4.96	119–120
BAP diiodide	33.80	33.76	11.27	11.25	4.21	4.05	231–233
HAP diiodide	36.63	36.52	10.58	10.65	4.53	4.60	253.5–255.5
OAP diiodide	39.12	39.01	9.97	10.11	5.14	5.09	181.5–183
DAP diiodide	41.40	41.25	9.46	9.62	5.41	5.54	178.5–181[a]
DoDAP diiodide	43.45	43.29	9.12	9.18	5.89	5.95	175–176.5
TetraDAP dibromide	51.09	52.95	9.89	10.29	7.46	7.41	161.5–163
HexaDAP dibromide	53.89	54.55	9.48	9.79	7.78	7.75	159–161.5
OctaDAP dibromide	55.64	56.00	9.17	9.33	8.04	8.06	163–164.5

[a] Mooser et al. (1972) observed that the melting point of DAP diiodide was 180–191.5 °C.

proximately 4 g of the dihaloalkane with 8 g of 3-aminopyridinium for 85 h in 60 mL of acetone. The monofunctional probes were synthesized by refluxing equimolar amounts of iodoalkane and 3-aminopyridine for 18 h in 60 mL of acetone. The acetone solutions were reduced to near dryness on a rotary evaporator. The dark-colored residues were dissolved in absolute ethanol and treated with decolorizing carbon. The products were recrystallized three to five times from ethanol. Off-white crystals were recovered in 20–50% yield. An elemental analysis and melting point determination were performed on each final product. Table I shows that the observed and theoretical elemental analyses were in good agreement. Thin-layer chromatographic analyses were performed with cellulose plates obtained from Kodak. The solvent contained butanol–ethanol–water–acetic acid (4:2:1:1 v/v), and each product ran as a single spot. Proton NMR yielded spectra which were consistent with those predicted for each product.

Purification of the AcChR. Receptor was purified from *T. californica* electroplax with affinity chromatography techniques as described by Schmidt & Raftery (1972). Proteolysis of subunits was minimized by taking the precautions outlined by Raftery et al. (1974) and Vandlen et al. (1976). The solubilized receptor solution was concentrated with an Amicon ultrafiltration cell with a PM-30 membrane. The concentrated protein solution was dialyzed at 4 °C against buffer containing 10 mM sodium phosphate and 0.03% Triton X-100 (pH 7.4) for use in binding studies under conditions of low ionic strength (buffer A) or 10 mM sodium phosphate, 0.03% Triton X-100, and 100 mM NaCl (pH 7.25) for use in binding studies under conditions of high ionic strength (buffer B). The solubilized receptor solution was then filtered through a 0.45-μm HAWP Millipore filter to remove particulate matter and stored at 4 °C until use.

Characterization of the Purified Acetylcholine Receptor. DEAE-cellulose disc assays were performed as described by Schmidt & Raftery (1973a) to determine the total concentration of [^{125}I]-α-BuTx binding sites at equilibrium. Protein concentration was determined by the method of Lowry et al. (1951) with BSA as standard. Routinely, the purified receptor bound 9–10 nmol of α-BuTx/mg of protein. The integrity of the purified receptor was assessed by $NaDodSO_4$–polyacrylamide gel electrophoresis in the presence of 1% mercaptoethanol by using the procedure of Laemmli (1970). Routinely, Coomassie blue stained bands of apparent molecular weights 40 000, 50 000, 60 000 and 65 000 were observed, indicating that little or no proteolysis had occurred (Raftery et al., 1974; Weill et al., 1974; Vandlen et al., 1976).

Fluorescent measurements were made on Perkin-Elmer spectrofluorimeter Models MPF-2A, MPF-3, or MPF-4 or on a Schoffel spectrofluorimeter. Fluorescence emission was monitored at 403 nm with an excitation wavelength of 330 nm. In all cases, buffers were 5 mM Tris, pH 7.4, with less than 0.1% Triton X-100 (from dilution) present in purified AcChR preparations to facilitate detection of excitation and emission maxima. Four 10-mm cuvettes were used in thermostated cell compartments at 25 °C. The dissociation constant for DAP binding to purified AcChR was determined from a Scatchard plot of the fluorescence data obtained by titrating samples of AcChR (2.4×10^{-7} M) in α-BuTx sites with 5-μL aliquots of a 10^{-5} M DAP stock solution (Martinez-Carrion & Raftery, 1973). A similar method was used for AcChR-enriched membrane preparations (Duguid & Raftery, 1973). Two-milliliter samples were used throughout. Details of the concentrations of AcChR and DAP used in the competition experiments with other cholinergic ligands are given in the legends to the figures.

Centrifugation of solutions of AcChR complexed with DAP, to which AcCh or Deca was added to displace DAP from its binding sites, was carried out at 100000*g* for 5 h in a Beckman 41 rotor, a condition sufficient to pellet the AcChR. Samples (2 mL) of the supernatants from such centrifugations were monitored for free DAP by fluorescence under the conditions outlined above, and these samples were compared with noncentrifuged samples of the same volume with the same conditions (see Figure 7). AcChR was assayed in all cases by the method of Schmidt & Raftery (1973a).

Results

Direct Binding Studies. In Figure 1A the relative fluorescence intensity of DoDAP vs. total probe concentration is shown (a) in the presence of receptor, (b) with receptor pretreated with a twofold excess of α-BuTx, and (c) in the absence of receptor. The fluorescence intensity vs. total probe concentration in the receptor-free and the α-BuTx-treated receptor solutions was linear, but a measurable quantity of fluorescent probe was quenched in the latter. This quenching may result from probe binding with low affinity to sites not blocked by α-BuTx. The fluorescence intensity vs. total probe concentration in the receptor solution increased slowly with a hyperbolic lag and then increased linearly with the same apparent slope as solution b. This indicates that, at low probe concentrations, the bulk of the total probe added was quenched due to AcChR interaction, and the Figure 1A insert shows that a replot of the amount of bound DoDAP vs. free DoDAP was hyperbolic. At high probe concentrations no further saturable components were detected. Figure 1B is a Scatchard replot of a similar experiment which was conducted with OAP. The data were fit by a straight line, indicating that OAP bound

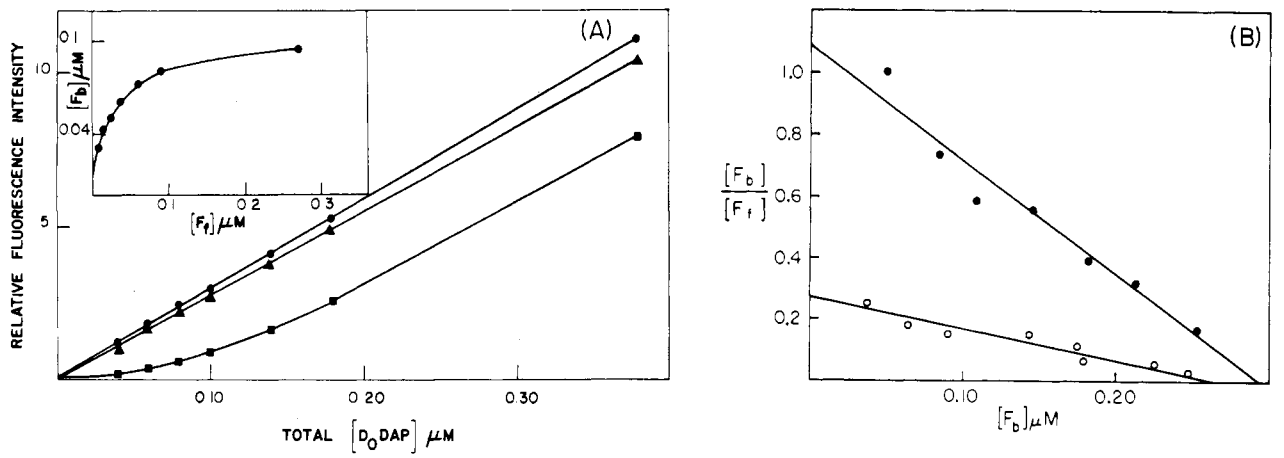

FIGURE 1: (A) Plot of the relative fluorescent intensity of DoDAP (corrected for background) as a function of the total amount of fluorescent probe added. The experiment was performed as described under Methods with buffer A. The receptor concentration was 0.2 μM in α-BuTx binding sites for the bottom curve and for the middle curve which was also preincubated with 0.4 μM α-BuTx. The top curve represents buffer alone. (B) Scatchard plot of OAP binding when the receptor concentration was 0.6 μM in α-BuTx binding sites. The lines drawn result from a linear least-squares regression analysis of the data obtained in buffer A (●) and in buffer B (○).

Table II: Ligand Binding Parameters

probe	K_d (μM)[b] buffer A	buffer B
OctaDAP	0.175 ± 0.075 (4)	0.67 (2)
HexaDAP	0.047 ± 0.012 (5)	0.15 (1)
TetraDAP	0.032 ± 0.011 (5)	0.11 (1)
DoDAP	0.034 ± 0.010 (7)	0.19 (2)
DAP	0.061 ± 0.011 (7)	0.42 ± 0.09 (4)
OAP	0.295 ± 0.045 (5)	0.95 (2)
HAP	1.03 ± 0.29 (5)	3.7 (3)
BAP	3.35 ± 1.0 (5)	>10
3-amino-1-methylpyridinium	>10	nd[a]
3-amino-1-propylpyridinium	>10	nd

[a] nd represents not determined. [b] The experiments were performed as described under Methods with the fluorimetric assay. The K_d values expressed represent a mean value based upon the number of experiments indicated in parentheses. If four or more experiments were performed a standard deviation was determined. In some experiments fluorescence quenching was observed, but the resolution of the data was not sufficient to accurately determine a K_d value (K_d > 10 μM).

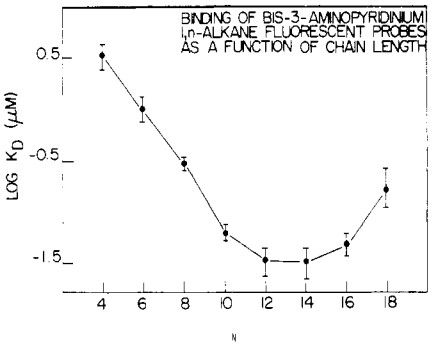

FIGURE 2: Plot of log K_d for the synthetic bis(3-aminopyridinium) probes vs. the number of methylene groups (n) between the two 3-aminopyridinium rings when buffer A was used.

to a single class of noninteracting sites. The Scatchard plots obtained with the other bifunctional probes were similar in form, and the binding data obtained with several bifunctional fluorescent probes are summarized in Table II. The bifunctional probes bound with increasing affinity to the AcChR as the length of the methylene bridge (n) between the two 3-aminopyridinium rings was increased from 4 to 12 and with decreasing affinity as n increased from 16 to 18, and the purified receptor bound DoDAP (n = 12) and TetraDAP (n = 14) with the highest affinity (Figure 2). The data in Table II show that the receptor did not bind the synthetic monoquaternized probes with appreciable affinity. The simplest interpretation is that the receptor interacts strongly with the bifunctional probes at two anionic binding subsites and that bifunctional probes in which n < 8–10 or monofunctional probes interact with only one of the receptor subsites and thus bind with lower affinity.

Previously a great body of work has been conducted on electrophysiological responses to polymethylenebis(trialkylammonium) compounds [for a recent review see Michaelson (1973)]. In the trimethylammonium series, two maxima in blocking activity were observed, corresponding to the C_{10} and C_{16-18} compounds (Patton & Zainis 1949; Barlow & Zoller, 1964) while in all preparations studied, using the corresponding triethylammonium series, only the second maximum (C_{16-18}) appeared. Since our series of fluorescent probes have bulky groups associated with the quaternary nitrogens, it is possible that the binding studies reported here reflect some of the same phenomena observed in the earlier physiological studies.

Figure 1B illustrates that the affinity of the receptor for OAP decreased dramatically as the ionic strength of the buffer increased (in buffer A, K_d = 0.26 μM, and in buffer B, K_d = 0.95 μM). A summary of the direct binding data of the bifunctional probes obtained at equilibrium with buffers A and B is presented in Table II. These data indicate that all bifunctional probes tested appeared to bind with four- to fivefold lower affinity in the buffer containing high concentrations of NaCl (buffer B).

Cholinergic Ligand Binding to Half of the Bungarotoxin Binding Sites. It was evident from Figure 1B that OAP bound to only half of the total α-BuTx binding sites. This result was identical with our earlier findings with DAP binding to *T. californica* purified AcChR (Martinez-Carrion & Raftery, 1973). We have also shown that AcCh, *d*-Tc, and Deca bound to half of the available α-BuTx sites (Moody et al., 1973). We therefore used other methods to substantiate this half of the sites binding by the bifunctional fluorescent probes.

A clear demonstration of DAP binding to half of the α-BuTx binding sites on purified *Torpedo* AcChR under equilibrium conditions is presented in Figure 3. A solution of AcChR (10^{-6} M in α-BuTx binding sites) was saturated with DAP and monitored by the increase in fluorescence at 403 nm [DAP fluorescence appears to be completely quenched upon association with the AcChR—see Martinez-Carrion & Raftery (1973)]. Minimal release of DAP was observed until

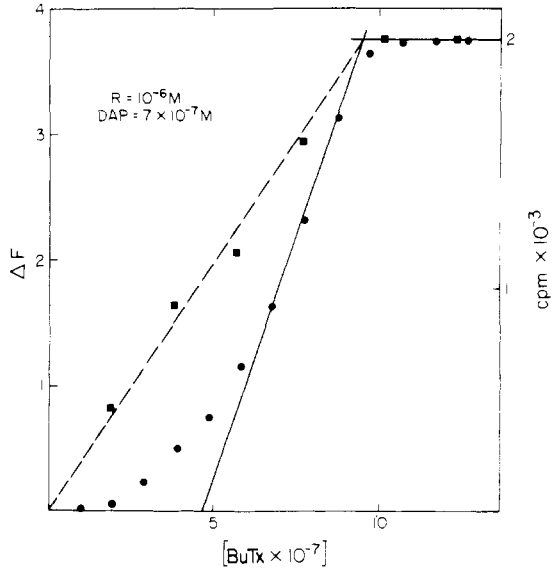

FIGURE 3: Release of DAP from its complex with AcChR as a function of added α-BuTx. [AcChR] = 10^{-6} M in α-BuTx binding sites; [DAP] = 7×10^{-7} M; K_d of DAP = 3×10^{-7} M. The DAP release was monitored by the fluorescence intensity increase at 403 nm. (■) Counts per minute of [^{125}I]-α-BuTx–AcChR complex formed; (●) increase in fluorescence as a function of added α-BuTx.

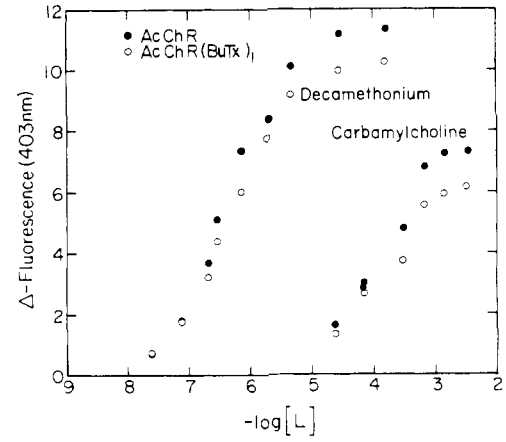

FIGURE 4: Comparison of DAP displacement by decamethonium and carbamylcholine from AcChR and AcChR half-saturated with α-BuTx. AcChR (6.9×10^{-7} M in α-BuTx binding sites) was first saturated with DAP (6×10^{-6} M) and then half the total α-BuTx necessary to achieve full saturation was added to one sample (2 mL of this solution). This sample and an equivalent one to which no α-BuTx had been added were dialyzed in separate dialysis bags against the same buffer to yield identical free DAP concentrations; $[DAP]_{free} = 8 \times 10^{-8}$ M. Both samples were then titrated with decamethonium and carbamylcholine and the release of bound DAP was monitored as a function of added ligand.

approximately half the α-BuTx necessary for displacement had been added. Use of [^{125}I]-α-BuTx allowed direct demonstration of linear [^{125}I]-α-BuTx–AcChR complex formation, according to the assay method of Schmidt & Raftery (1973a), following all additions of the radiolabeled toxin. Extrapolation of observed DAP release (Figure 3) indicated that, of the total toxin binding sites present in *T. californica* AcChR, half can be occupied by DAP, even in the presence of α-BuTx binding to the other half of the sites.

Bungarotoxin and Ligand Site Interactions. It was possible to investigate whether saturation of half of its binding by α-BuTx affected the association of cholinergic ligands. First, an AcChR preparation was saturated with DAP and then with half the amount of α-BuTx necessary for saturation of its binding sites. A second identical receptor solution was saturated with DAP, but no α-BuTx was added. Displacement of DAP from these two preparations by Deca or Carb is illustrated in Figure 4. Bound DAP was displaced, within the error of the method, in an identical manner from both preparations by the two ligands, thereby indicating that the presence of α-BuTx at half of its sites did not cause significant modification of ligand binding sites under the experimental conditions used.

DAP Displacement by Cholinergic Ligands. Such displacement was initially studied by virtue of regeneration of DAP fluorescence upon dissociation from the AcChR. DAP "displacement" by Hexa, Deca, *d*-Tc, and Carb is illustrated in Figure 5 and compared to the amount of DAP maximally displaced by saturating concentrations of α-BuTx. Addition of Hexa and Deca resulted in smooth titration of DAP from its bound environment with complete displacement relative to that removed by α-BuTx. The midpoints of these titration curves agreed well with known K_d values for these two ligands (Moody et al., 1973).

Carb, even at concentrations 10^2 times its K_d value, did not totally displace the bound DAP. *d*-Tc displaced DAP completely but appeared to do so in stepwise fashion, the midpoint of the lower titration curve corresponding to the known K_d value for this ligand binding to *T. californica* AcChR (Moody et al., 1973). Results similar to those depicted for Carb were obtained for AcCh. Such results for *d*-Tc, Carb, and AcCh raised the possibility that ternary complexes are formed between AcChR, DAP, and certain other ligands.

HexaDAP Displacement by Cholinergic Ligands. The percent AcChR-bound HexaDAP released vs. log free inhibitor concentration is shown in Figure 6. Deca inhibited HexaDAP binding more efficiently than did Carb. Further, the data in Figure 6 show that Deca inhibited HexaDAP binding more efficiently in buffer A (K_i = 0.4 μM) than in buffer B (K_i = 2.2 μM), whereas Carb inhibited HexaDAP binding with the same apparent affinity in buffers A and B. These data indicate that high concentrations of Na^+ inhibit Deca binding, whereas Carb binding was unaffected. In addition, the Deca inhibition curves could be fit by assuming competitive inhibition while Carb curves were shallower in slope and could not be well fit by assuming competitive displacement. It seemed possible that ternary complexes could be formed between the AcChR, HexaDAP, and Carb.

Ternary Complex Formation of AcCh and DAP. Purified AcChR in Triton X-100 solution can be pelleted by centrifugation at 100000*g* for 5 h (T. Lee and M. A. Raftery, unpublished experiments). To ascertain whether DAP fluorescence generated by addition of a ligand was due to DAP released into solution or to unquenched AcChR-bound DAP, a comparison was made of fluorescence before and after centrifugation (or after centrifugation with and without mixing). The data in Figure 7 illustrate that DAP fluorescence generated by Deca was the same before and after centrifugation, and this result was interpreted to mean that Deca displaced DAP from the AcChR into solution. With AcCh as a displacing ligand, the DAP fluorescence generated remained associated with the AcChR, most likely as a ternary complex.

AcChR-Enriched Membranes. The binding of DAP to AcChR-enriched membranes is shown in Figure 8 with the Scatchard profile indicating more than one class of binding site. Of these, the strongest class corresponds to about half the total number of α-BuTx sites and has an estimated K_d = 1.3×10^{-8} M. The data can be extrapolated to indicate a total number of DAP sites equal to the number for α-BuTx.

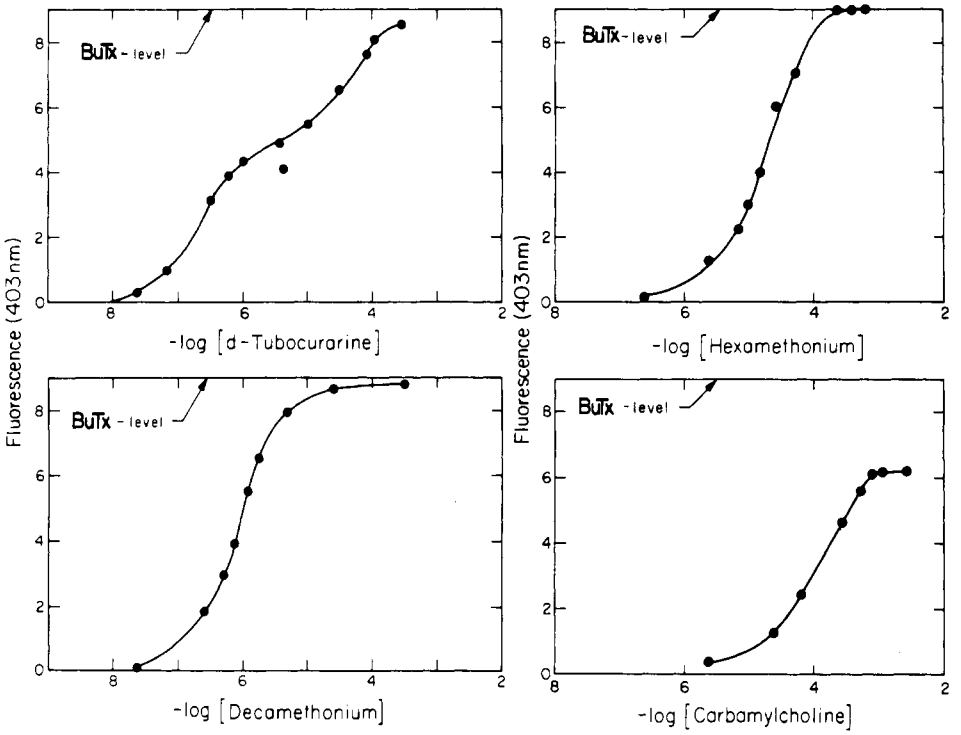

FIGURE 5: Displacement of DAP from AcChR by *d*-tubocurarine, hexamethonium, decamethonium, and carbamylcholine compared with the amount of DAP released by α-BuTx (shown as BuTx level). [AcChR] = 5.8×10^{-7} M; [DAP] = 1.25×10^{-7} M.

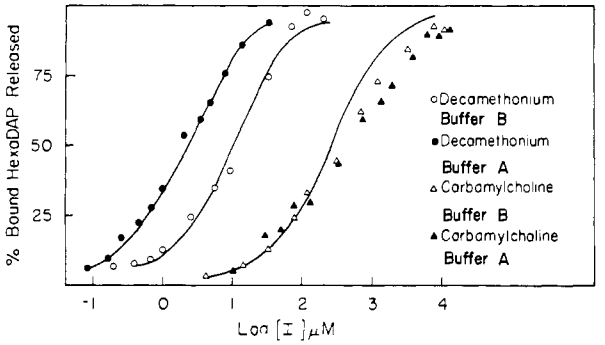

FIGURE 6: Plot of the percent of bound HexaDAP released vs. log free inhibitor concentration (I_f). When buffer A was used, the total concentration of HexaDAP was 0.125 μM and the receptor concentration was 0.17 μM in α-BuTx binding sites. When buffer B was used, the total HexaDAP concentration was 0.36 μM and the receptor concentration in α-BuTx binding sites was 0.52 μM. The lines drawn represent the best fit by assuming competitive inhibition.

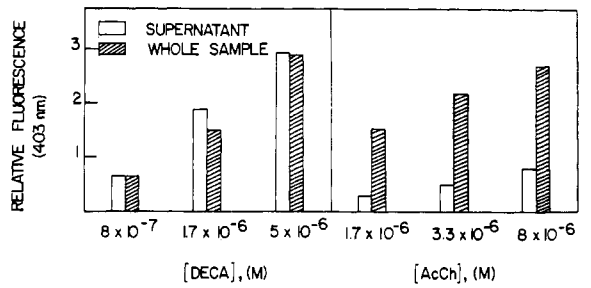

FIGURE 7: Comparison of the displacement of DAP from its bound state with AcChR by decamethonium and acetylcholine to determine if DAP was completely freed from AcChR. [AcChR] = 3.5×10^{-7} M in α-BuTx sites; $[DAP]_0 = 1.3 \times 10^{-7}$ M. These solutions were incubated with concentrations of decamethonium (8×10^{-7}, 1.7×10^{-6}, and 5×10^{-6} M) or with concentrations of AcCh (1.7×10^{-6}, 3.3×10^{-6}, and 8×10^{-6} M) and centrifuged at 100000*g* for 5 h. Fluorescence intensity was monitored in the supernatants and after remixing.

However, such a conclusion must be tempered based on the data shown in Figure 9. Here a comparison is made between

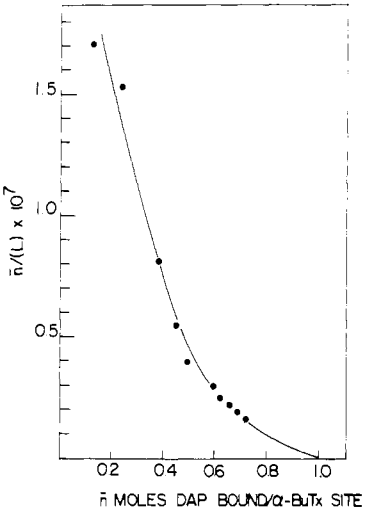

FIGURE 8: Scatchard plot of DAP binding to AcChR-enriched *T. californica* membrane fragments. [AcChR] = 5×10^{-7} M in [^{125}I]-α-BuTx sites. The curve drawn through the data points is not theoretically derived.

the release of bound DAP from membrane preparations by addition of saturating amounts of α-BuTx and by titration with hexamethonium (Figure 9A). Hexa displaced DAP, with generation of fluorescence to the level displaced by α-BuTx and with a midpoint corresponding to the known value for its dissociation constant. However, upon further addition of Hexa still more DAP was released. This result was corroborated by first removing specifically bound DAP with saturating amounts of α-BuTx, followed by aliquots of Hexa when further DAP was again displaced. Such results indicate that at the DAP concentrations used ([AcChR] = 5×10^{-7} M in α-BuTx binding sites and [DAP] = 2×10^{-7} M) considerable nonspecific binding occurred. It should be noted, however, that this concentration is an order of magnitude above the K_d for strong DAP binding to half the toxin sites (Figure 8). It seems that DAP (and other bisquaternary ligands that displace it)

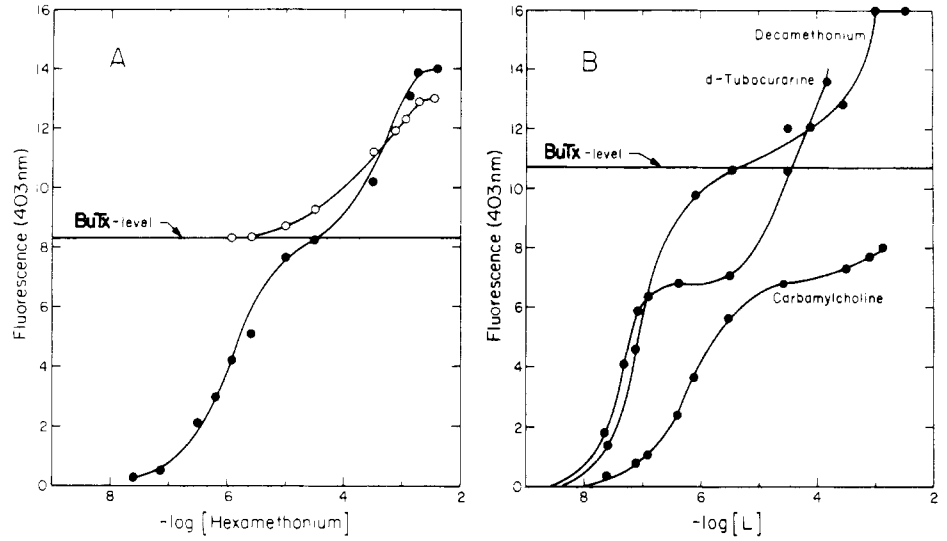

FIGURE 9: Displacement of DAP from AcChR-enriched membranes by cholinergic ligands. BuTx level refers to the amount of DAP released by saturating amounts of α-BuTx. [AcChR] = 5×10^{-7} M; [DAP] = 2×10^{-7} M.

at higher concentrations can associate with sites other than the specific cholinergic ligand binding sites affected by α-BuTx.

Additional evidence for such nonspecific binding of DAP (at similarly high concentrations) is presented in Figure 9B where Deca or *d*-Tc displaced DAP in excess of the amount displaced by α-BuTx. The midpoints of the titration curves for Deca and *d*-Tc displacement of DAP, however, correspond well with independently determined values for their K_d values. An especially interesting aspect is displayed by *d*-Tc and also by Carb (Figure 9B)—these ligands appeared to titrate only approximately 65% of the specifically bound DAP, as judged by generation of fluorescence. At *d*-Tc concentrations 3 orders of magnitude greater essentially all DAP appeared to be displaced. However, in the case of Carb this was not observed; some additional displacement appeared at concentrations greater than 10^{-3} M, where the charged ligand probably acted merely as a cation, displacing DAP by a different mechanism (Martinez-Carrion & Raftery, 1973). These results strongly suggest that a ternary complex was formed between the AcChR and the ligands DAP (an antagonist) and Carb (an agonist). A further feature worth noting is that the midpoint of the Carb titration (Figure 9B) occurs at least 1 order of magnitude higher in concentration than the known K_d value for Carb binding to *T. californica* AcChR enriched membranes [K_d = 50–120 nM; Quast et al. (1978)].

Discussion

The results presented in this communication clearly show (see Figure 1) that isolated purified AcChR contains half the number of binding sites for the series of fluorescent ligands studied as it does binding sites for α-BuTx. This agrees with previous observations of Moody et al. (1973) for binding of AcCh, *d*-Tc, and Deca and extends the observations of Martinez-Carrion & Raftery (1973) for DAP. It is also interesting to note (Figure 4) that under the conditions used the binding of DAP appeared to be impaired little or not at all by the presence of half the total possible number of α-BuTx molecules associated with the AcChR. The half-of-the-sites phenomenon observed in the binding experiments could be explained by ligand-induced negative cooperativity (Conway & Koshland, 1968) or by preexistent nonequivalence in binding sites in ligand and/or toxin. It is not possible at the present time to distinguish between these mechanisms.

The second finding of interest deals with characterization of binding subsites for DAP on the purified AcChR. The results presented in Figures 5–7 show that bisquaternary ligands such as Hexa or Deca displaced DAP completely from its binding sites to the same level as did α-BuTx. On the other hand, monoquaternary ligands such as Carb or AcCh appeared to form ternary complexes in the presence of DAP. Additionally, previous results (Martinez-Carrion & Raftery, 1973) have clearly shown that inorganic cations are competitive with DAP binding, while it has also recently been demonstrated (Moody, 1977) that inorganic cations have little or no effect on the binding of acetylcholine (Moody, 1977) or Carb (D. Vandlen and M. A. Raftery, unpublished experiments). Preferential chemical modification (Chao & Raftery, 1974) by reaction of isolated AcChR with trimethyloxonium fluoroborate abolished DAP binding, drastically reduced the number of Deca binding sites without affecting the Deca binding constant, but had little effect on the binding of AcCh. The simplest interpretation of these results is that the specific acetylcholine binding site was unaffected by reaction with trimethyloxonium fluoroborate, whereas cation and DAP binding sites were chemically modified.

This series of results points to marked differences in the properties of the AcChR binding sites for DAP and inorganic cations on the one hand and AcCh and Carb on the other. The simplest explanation of these findings, on a physical or structural basis, is that the ligands that form ternary complexes do so by binding to distinct sites. The alternative model which cannot at present be excluded is that one of the quaternary pyridinium moieties of DAP and the other strongly binding fluorescent probes associate with the AcCh binding site while the other pyridinium ring binds to another subsite with which cations associate. Ternary complex formation would then be explained by AcCh mediated removal of one aminopyridinium terminal of DAP which could then be followed by its association with some other negatively charged area of the AcChR, which did not, however, result in fluorescence quenching.

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