Studies of Reversible and Irreversible Interactions of an Alkylating Agonist with *Torpedo californica* Acetylcholine Receptor in Membrane-Bound and Purified States†

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**Abstract:** The interaction of a cholinergic depolarizing agent, bromoacetylcholine, with acetylcholine receptor (AcChR) enriched membrane fragments and Triton-solubilized, purified AcChR from *Torpedo californica* has been studied. The reagent bound to membrane-bound AcChR reversibly with an apparent dissociation constant of 16 ± 1 nM at equilibrium. This 600-fold higher affinity for the receptor than found from physiological studies \(K_{act} \approx 10 \mu M\) (Karlin, A. (1973) *Fed. Proc., Fed. Am. Soc. Exp. Biol.* 32, 1847-1853) can be attributed to a ligand-induced affinity change of the membrane-bound receptor upon preincubation with bromoacetylcholine. At equilibrium \[^{3}H\]bromoacetylcholine, like acetylcholine, bound to half the number of \(\alpha\)-bungarotoxin sites present in the preparation without apparent positive cooperativity, and this binding was competitively inhibited by acetylcholine. In the presence of dithiothreitol, \[^{3}H\]bromoacetylcholine irreversibly alkylated both membrane-bound and solubilized, purified acetylcholine receptor, with a stoichiometry identical with that for reversible binding. NaDODSO₄-polyacrylamide gel electrophoresis of the labeled acetylcholine receptor showed that only the 40,000-dalton subunit contained the label. From these results it is concluded that the 40,000-dalton subunit represents a major component of the agonist binding site of the receptor.

The acetylcholine receptor (AcChR) has been isolated from the electric organs of *Torpedo californica* both in a membrane-bound state (Duguid & Raftery, 1973) and in Triton-solubilized, highly purified form (Schmidt & Raftery, 1973b). These preparations provide a convenient source for in vitro studies of the AcChR at the molecular level, which facilitate an understanding of synaptic transmission at nicotinic synapses.

The receptor of the electroplax of *Electrophorus electricus* was found to contain a readily reducible disulfide bond believed to reside in the vicinity of a cholinergic ligand binding site (Karlin, 1969). Reduction of this bond altered the physiological response of the receptor to cholinergic ligands, and the effect could be reversed by reoxidation with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). Similar results were found with the AcChR at the frog neuromuscular junction (Ben-haim et al., 1973). The reduced single-cell AcChR preparation could be alkylated by various active site directing reagents; some of these resulted in irreversibly activated receptors and others in irreversibly inhibited receptors (Silman & Karlin, 1969; Kalderon & Silman, 1971; Bartels-Bernal et al., 1976). One of these, 4-(N-maleimido)benzyltrimethylammonium iodide (MBTA), has been used extensively as an affinity label for the subcellular characterization of AcChR.

The reagent was found to bind to a single subunit of approximately 40,000 daltons in both *Electrophorus* and *Torpedo* receptors (Karlin & Cowburn, 1973; Weill et al., 1974). Moreover, both fish receptors bound twice as much \(\alpha\)-neurotoxin as they did MBTA. With denervated rat skeletal muscle, however, \[^{3}H\]MBTA labeled two different subunits of the reduced AcChR, and the amount of specific labeling per toxin site was also different from that for the fish receptors (Froehner et al., 1977).

In this report, we describe the interaction of an affinity label, bromoacetylcholine (BrAcCh), with the membrane-bound and solubilized AcChR from *T. californica*. BrAcCh differs from MBTA in two respects in its physiological effects: first, in the absence of DTT, it acts as a reversible depolarizing agent, whereas MBTA acts as an inhibitor of cholinergic ligand induced depolarization; secondly, in the presence of DTT, it irreversibly activates, while MBTA irreversibly inhibits, the receptor (Karlin, 1969).

In the work reported here, it was found that BrAcCh binds specifically and reversibly to the (nonreduced) membrane receptor preparation with this binding being competitive with AcCh. Following reduction with DTT, the reagent irreversibly alkylates the AcChR. Using \[^{3}H\]BrAcCh we found that only the 40,000-dalton subunit was covalently labeled. This result strongly suggests that the agonist binding site(s) resides on this polypeptide component of the AcChR protein complex. In addition, the number of sites alkylated corresponded to half the number of \[^{125}I\]-%BuTx sites present in the preparation, a result that agrees with previous determinations of the number of binding sites for AcCh and Carb (Moody et al., 1973; Raftery et al., 1975).

**Experimental Section**

**Materials**

*T. californica* was obtained locally. Lyophilized venom of *Bungarus multicinctus* was obtained from Sigma Chemical Co. Na\(^{125}\)I was purchased from New England Nuclear Co. DE-81 DEAE discs were from Whatman, Ltd. S-Acetylthiocholine, AcCh, DNPP, bromoacetyl bromide, and choline chloride were obtained from Aldrich Chemical Co., Inc. Carb, d-Tc, DTT, and eserin were from Sigma Chemical Co., and \[^{acetyl-1}H\]AcCh and \[^{3}H\]choline chloride were from New England Nuclear Co. Reagents for gel electrophoresis were prepared from Aldrich Chemical Co.

† Abbreviations used: AcChR, acetylcholine receptor; MBTA, 4-(N-maleimido)benzyltrimethylammonium iodide; BrAcCh, 2-bromoacetylcholine; DTT, dithiothreitol; %BuTx, \(\alpha\)-bungarotoxin; AcCh, acetylcholine; DNPP, diethylthioctyl phosphosphate; Carb, carbamylocholine; d-Tc, d-tubocurarine; NaDODSO₄, sodium dodecyl sulfate; AcChE, acetylcholinesterase; POPP, 1,4-bis[2-methyl[5-phenyloxazolyl)]benzene; PPO, 2,5-diphenyloxazole; CBB, Coomassie Brilliant Blue; DAPA, 1,10-bis(3-azidopyridinio)decanium perchlorate.

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obtained from Pierce Chemical Co. All other chemicals were of the highest purity commercially available.

Methods

AcChR-enriched membrane fragments were prepared as previously described (Duguid & Raftery, 1973; Reed et al., 1975) from fresh or frozen (−80°C) electric organs of T. californica. The buffer used throughout the preparation was 10 mM sodium phosphate, pH 7.4, 0.4 M NaCl, 1–5 mM EDTA, and 0.02% NaN₃. Highly purified AcChR was prepared by affinity chromatography after Triton solubilization as described previously (Schmidt & Raftery, 1972, 1973b; Vandlen et al., 1976). α-BuTx was purified from lyophilized venom according to the method of Clark et al. (1972) and was labeled with [125I]-α-BuTx binding to AcChR was carried out by the DEAE filter disc assay method of Schmidt & Raftery (1973a).

Bromoacetylcholine perchlorate was prepared from bromoacetyl bromide and choline chloride according to the method of Chiou & Sastry (1968). The reaction product was recrystallized from 1:6 ace tone–ethyl acetate. The resulting colorless needles had a melting point of 103–103.5°C and showed one NH₂0H-FeC1₃ positive spot on thin-layer chromatography (n-BuOH-EtOH-H₂O-HOAc, 4:2:3:1, as solvent). [3H]Bromoacetylcholine perchlorate was synthesized from [3H]choline by the same procedure. The radioactive product cochromatographed with the unlabeled BrAcCh (Rᵢ = 0.74) in the TLC system described above. Specific activities of [3H]BrAcCh were 42 and 70 mCi/mmol for the two batches obtained.

Recrystallization of 250 pM (for Triton-solubilized, purified receptors) or 40 pM (for membrane-bound receptors). Reduction was performed at room temperature for 1 h, various concentrations of [3H]BrAcCh were then added, and the reaction was allowed to continue for another hour at 4°C. Two 100-μL aliquots of reaction mixtures were pipetted onto DE-81 discs to adsorb the AcChR and, therefore, the covalently attached [3H]BrAcCh moieties. Unreacted [3H]BrAcCh was then washed away with 10 mM sodium phosphate, pH 7.4, 0.1% Triton X-100, and 50 mM NaCl for 10 min. This washing procedure was then repeated twice more. The discs were soaked in 800 μL of H₂O for 1 h, shaken, and counted in 10 mL of toluene-based scintillation fluid containing 25% Triton X-100 and 0.55% Permablend III. For NaDodSO₄-polyacrylamide gel electrophoresis, the labeled product was dialyzed against the reaction buffer for 4 h at 4°C. This dialysis was then repeated twice more. NaDodSO₄ gel electrophoresis with 12.5% acrylamide and 0.1% bis(acrylamide) was carried out according to Laemmli (1970). Gels were stained with CBB and scanned at 550 nm in a Gilford spectrometer. Slices (1 mm) of the gels were sliced on a Mickel gel slicer, dissolved in 0.5 mL of H₂O₂ at 60–80°C for 5–6 h, and counted in 10 mL of the tolune-based scintillation cocktail described above.

Stability of BrAcCh was determined under the experimental conditions used for binding assays by determination of the ester content (Hestrin, 1949). No significant hydrolysis was observed for periods up to 4 h at room temperature. Similar stability was found with the covalently attached [3H]AcCh moieties on AcChR. No loss of radioactivity was detected after dialysis against Torpedo Ringer's solution, pH 7.4, at 4°C for over 16 h.

Results

(A) Reversible Binding of Bromoacetylcholine to Membrane-Bound AcChR. Induction of Change in AcChR Affinity State(s) by BrAcCh. In the absence of DTT, AcChR-enriched membrane fragments from T. californica underwent a change from low to high affinity state(s) upon incubation with BrAcCh. As shown in Figure 1A, the initial rate of [125I]-α-BuTx binding to the AcChR was measured in the absence of agonist and in the presence of 1 μM Carb, added either 30 min prior to or at the same time as [125I]-α-BuTx, under conditions in which the toxin was in large excess over AcChR toxin binding sites. Only preparations with equal initial toxin binding rates observed in the absence of Carb and when Carb and toxin were added simultaneously were used for the studies presented here, i.e., preparations in the low affinity state. Since BrAcCh is a substrate for acetylcholinesterase (AcChE) hydrolysis (Chiou & Sastry, 1968), eserin or diethylnitrophenyl phosphate (DNPP) was routinely added to the AcChR preparations to inhibit the AcChE activity, which was assayed by the method of Ellman et al. (1961) with S-acetyltiocholine as substrate. Alternatively, the method of Johnson and Russel (unpublished experiments) was employed to detect low levels of AcChE activity; to 100 μL of [acetyl-3H]AcCh (1–1000 μM) in 25 mM potassium phosphate, pH 7.0, was added 10 μL of the sample whose AcChE activity was to be determined, and the mixture was incubated at room temperature for 20 min. The reaction was stopped by adding 100 μL of a solution of 0.1 M in chloroacetic acid, 2 M in NaCl, and 0.5 M in NaOH. Esterase activity was determined by assaying the amount of [3H]acetic acid selectively extracted into 3 mL of scintillation cocktail (10% isoamyl alcohol in toluene, containing 1.14 mg of POPOP and 18.95 mg of PPO).

To study reversible binding of [3H]BrAcCh to membrane-bound AcChR, we incubated various concentrations of [3H]BrAcCh with AcChR-enriched membrane fragments in Torpedo Ringer's solution (0.25 M NaCl, 5 mM KCl, 2 mM MgCl₂, 4 mM CaCl₂, and 5 mM Tris-HCl, pH 7.4) for 20 min at 4°C. Membrane fragments were then pelleted by centrifugation in a Beckman Type 65 rotor at 40000 rpm for 1 h. Triplicate 100-μL aliquots of samples were withdrawn before and after centrifugation, and these were counted in a Packard TriCarb liquid scintillation spectrometer (Model 3375), with 10 μL of Aquasol as scintillator, to determine the total and free concentrations of [3H]BrAcCh. [3H]H₂O was used as external and internal standard to determine counting efficiency.

Covalent labeling of reduced AcChR with [3H]BrAcCh was carried out as follows. DTT was added to the AcChR preparations (0.5–1 μM in α-BuTx sites) to a final concentration of 10 μM (for Triton-solubilized, purified receptors) or 40 μM (for membrane-bound receptors). Reduction was performed at room temperature for 1 h, various concentrations of [3H]BrAcCh were then added, and the reaction was allowed to continue for another hour at 4°C. Two 100-μL aliquots of reaction mixtures were pipetted onto DE-81 discs to adsorb the AcChR and, therefore, the covalently attached [3H]BrAcCh moieties. Unreacted [3H]BrAcCh was then washed away with 10 mM sodium phosphate, pH 7.4, 0.1% Triton X-100, and 50 mM NaCl for 10 min. This washing procedure was then repeated twice more. The discs were soaked in 800 μL of H₂O for 1 h, shaken, and counted in 10 mL of toluene-based scintillation fluid containing 25% Triton X-100 and 0.55% Permablend III. For NaDodSO₄-polyacrylamide gel electrophoresis, the labeled product was dialyzed against the reaction buffer for 4 h at 4°C. This dialysis was then repeated twice more. NaDodSO₄ gel electrophoresis with 12.5% acrylamide and 0.1% bis(acrylamide) was carried out according to Laemmli (1970). Gels were stained with CBB and scanned at 550 nm in a Gilford spectrometer. Slices (1 mm) of the gels were sliced on a Mickel gel slicer, dissolved in 0.5 mL of H₂O₂ at 60–80°C for 5–6 h, and counted in 10 mL of the tolune-based scintillation cocktail described above.

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Preincubation of the membrane-bound receptor with BrAcCh preparations under conditions in which the toxin was in large excess over the AcChR binding sites for the toxin followed inhibited the initial toxin binding rate, but the kinetics remained pseudo first order during the time period the reaction was followed. Figure 1B illustrates the observed pseudo-first-order rate constant, $k_{\text{obsd}}$, as a function of free BrAcCh concentration after preincubation. The data fit the equation (Quast et al., 1978)

$$k_{\text{obsd}} = \frac{kT_0}{1 + [L]/K_{\text{pp}}}$$

where $T_0$ is the total toxin concentration ($T_0 \gg [\text{AcChR}]$), $k$ is the bimolecular rate constant of toxin binding to AcChR, $[L]$ is the free BrAcCh concentration, and $K_{\text{pp}}$ is the apparent dissociation constant for BrAcCh. The apparent dissociation constant for BrAcCh was found to be $17 \pm 4 \text{ nM}$, and the bimolecular rate constant ($A$) of toxin binding to this membrane preparation was $2.0 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$, in excellent agreement with the data of Quast et al. (1978) who determined a value of $(2.0 \pm 0.2) \times 10^6 \text{ M}^{-1} \text{s}^{-1}$.

**Direct Binding of $[^3\text{H}]\text{BrAcCh}$**

The binding of $[^3\text{H}]\text{BrAcCh}$ to membrane-bound AcChR was studied directly by centrifugation assay, and, as can be seen in Figure 2A, the binding curve was composed of two components: a saturable, specific binding hyperbola superimposed on a linear, nonspecific binding component. Subtraction of the nonspecific binding component from the total binding curve yielded the specific binding hyperbola. The ratio of the number of specifically bound $[^3\text{H}]\text{BrAcCh}$ molecules to the number of $\alpha$-BuTx sites was 0.51 ± 0.03. A Scatchard plot of data from similar experiments conducted with low receptor concentrations is shown in Figure 2B; a straight line was obtained with an $x$ intercept corresponding to $0.50 \pm 0.02 \text{ nM}[^3\text{H}]\text{BrAcCh}$ sites/$\alpha$-BuTx site. From the slope, the apparent dissociation constant of $[^3\text{H}]\text{BrAcCh}$ to membrane-bound AcChR was found to be $16 \pm 1 \text{ nM}$, in excellent agreement with the value obtained from the toxin binding inhibition technique (Figure 1B). In addition, no cooperativity was detected in the binding; a Hill plot of the binding data gave a straight line with slope $0.98 \pm 0.02$ (Figure 2C).

**Inhibition of $[^3\text{H}]\text{BrAcCh}$ Binding by AcCh.**

Binding of $[^3\text{H}]\text{BrAcCh}$ to the receptor membrane fragments as detailed in Figure 2 was inhibited by the presence of AcCh. The data in Figure 3 represent binding studies conducted in the presence of several fixed concentrations of AcCh. Since the receptor concentration in these experiments was relatively large compared to the AcCh concentration, correction was made for the bound AcCh by an iterative nonlinear least-squares fit. The parameters obtained from such a fit were 15 ± 1 nM for the dissociation constant of BrAcCh and 8 ± 1 nM for the dissociation constant of AcCh; this value for the $K_i$ of AcCh agrees very well with previously published values of direct measurements of the binding of the ligand to membrane preparations (Weber & Changeux, 1974).

**B** Covalent Labeling of Reduced AcChR by Bromoacetylcholine. Stoichiometry. After reduction of the AcChR with dithiothreitol, BrAcCh was found to covalently modify...
concentrations of AcCh (40, 80, and 120 nM). For a fixed concentration of AcCh, a series of [3H]BrAcCh binding studies, as described in Figure 2, were carried out. The reciprocal of bound [3H]BrAcCh is plotted vs. the reciprocal of free [3H]BrAcCh. (■) Observed data points. (●) Nonlinear least-squares fit of the data to a competitive model, with correction for free [AcCh] by an iterative method. The fit gives $K_{app}$ for BrAcCh = 15 ± 1 nM and $K_f$ for AcCh = 8 ± 1 nM.

**FIGURE 3:** Inhibition of [3H]BrAcCh binding to receptor membrane preparation by AcCh. To membrane fragments (2.29 × 10⁻⁷ M in α-BuTx sites) in Tris to Ringer's solution were added various concentrations of AcCh (40, 80, and 120 nM). For a fixed concentration of AcCh, a series of [3H]BrAcCh binding studies, as described in Figure 2, were carried out. The reciprocal of bound [3H]BrAcCh is plotted vs. the reciprocal of free [3H]BrAcCh. (■) Observed data points. (●) Nonlinear least-squares fit of the data to a competitive model, with correction for free [AcCh] by an iterative method. The fit gives $K_{app}$ for BrAcCh = 15 ± 1 nM and $K_f$ for AcCh = 8 ± 1 nM.

the receptor. The amount of irreversibly bound [3H]BrAcCh was determined from the radioactivity associated with an extensively dialyzed reaction mixture or by the DEAE disc method described under Experimental Section. Curve B: AcChR was preincubated with excess unlabeled α-BuTx for 3–5 h before reduction and alkylation. Curve C: same as in curve A, except that DTT was replaced by buffer. (---) Subtraction of curve B from curve A to obtain the specific binding component.

**FIGURE 4:** Covalent labeling of reduced AcChR by [3H]bromoaceticholine. (A) Triton-solubilized, purified AcChR. (B) Membrane-bound AcChR. Curve A: AcChR (5.7 × 10⁻⁷ M in α-BuTx sites) was first reduced by DTT. Various concentrations of [3H]BrAcCh were then added to start the labeling reaction. The amount of [3H]BrAcCh covalently attached to the receptor was determined by DEAE disc assay as described under Experimental Section. Curve B: AcChR was preincubated with excess unlabeled α-BuTx for 3–5 h before reduction and alkylation. Curve C: same as in curve A, except that DTT was replaced by buffer. (---) Subtraction of curve B from curve A to obtain the specific binding component.

**FIGURE 5:** Protection of membrane-bound AcChR from covalent [3H]BrAcCh labeling. The labeling reaction was carried out with a receptor membrane preparation as in Figure 4B, curve C, except that 8 × 10⁻⁵ M AcCh (○), 5 × 10⁻⁵ M d-Tc (●), and a 10-fold excess of unlabeled BrAcCh (□) were added immediately before [3H]BrAcCh addition.

**FIGURE 6:** NaDodSO₄-polyacrylamide gel electrophoresis of [3H]BrAcCh-labeled AcChR. The radioactivity incorporated is plotted as a function of gel slice number. (■) Labeled, Triton-solubilized, purified AcChR. (○) Membrane-bound AcChR labeled in the presence of 8 × 10⁻⁴ M d-Tc; (□) in the presence of 5 × 10⁻⁵ M d-Tc; and (△) in the absence of DTT or in the presence of a large excess of unlabeled BrAcCh or α-BuTx.

[3H]BrAcCh sites/α-BuTx site was obtained for both membrane-bound and solubilized, purified AcChR. In the absence of DTT, no specific irreversible labeling could be detected (Figure 4, curve C).

Protection Studies. When the reduction and alkylation of membrane-bound AcChR by [3H]BrAcCh was carried out in the presence of 8 × 10⁻⁴ M AcCh, the naturally occurring agonist for the AcChR, or in the presence of 5 × 10⁻⁵ M d-Tc, a potent antagonist, the extent of labeling was appreciably reduced (Figure 5). Exposure of AcChR-containing membranes to unlabeled BrAcCh prior to introduction of [3H]BrAcCh also inhibited the incorporation of radioactivity. Similar results were obtained with Triton-solubilized, purified AcChR (data not shown).

NaDodSO₄-Polyacrylamide Gel Electrophoresis and the Site of [3H]BrAcCh Labeling. The AcChR from T. californica has been shown to contain 40,000-, 60,000-, and 65,000-dalton subunits (Raftrey et al., 1974; Weil et al., 1974; Raftrey et al., 1975; Karlin et al., 1975; Vandlen et al., 1976; Witzemann & Raftrey, 1978). NaDodSO₄ gel electrophoresis of membrane-bound and purified AcChR which had been covalently labeled with [3H]BrAcCh revealed a prominent, single peak of radioactivity associated with the
BrAcCh for its binding site. (4) determination of whether AcCh was competitive for affinity upon preincubation, (3) localization of the binding sites to the 40 000-dalton reversibly depolarizes the nonreduced electroplax of the electric for BrAcCh by covalent incorporation of a 3H-labeled derivative. A Triton-solubilized AcChR, [(125I)-α-BuTx] to AcChR (3.1 × 10⁻⁶ M) in Torpedo Ringer's solution was added [(125I)-α-BuTx], and the formation of receptor-toxin complex was followed by DEAE disc assay. (D) Same as in (A) for AcChR alkylated with 80 μM BrAcCh or 200 μM (α), 40 μM (β), and 300 μM (γ) BrAcCh. (A) Triton-solubilized AcChR, [(125I)-α-BuTx]}_\text{final} = 2.5 × 10^{-7} M. (B) Membrane-bound AcChR, [(125I)-α-BuTx]_\text{final} = 6.25 × 10^{-7} M.

40 000-dalton polypeptide. The protective effect exerted by cholinergic ligands and α-BuTx was evident from the decreased incorporation of radioactivity into this receptor subunit (Figure 6).

**Toxin Binding Rate.** The effect of BrAcCh alkylation on the binding of [125I]-α-BuTx to the AcChR was investigated, and, as shown in Figure 7, the initial rate of [125I]-α-BuTx binding to both purified and membrane-bound AcChR was greatly suppressed after alkylation with BrAcCh. The total extent of receptor-toxin complex formed 15-20 h after toxin addition was 45-60% (for purified AcChR) and 12-26% (for membrane-bound AcChR) of that found for nonalkylated receptor.

**Discussion**

Electrophysiological studies have shown that BrAcCh reversibly depolarizes the nonreduced electroplax of the electric eel, *E. electricus*; reduction of the electroplax, followed by BrAcCh addition, resulted in an irreversible activation of the cell, presumably due to the covalent alkylation of the AcChR by BrAcCh. However, the covalently attached activator could be "competed away from its binding site by high concentrations of d-Tc" (Silman & Karlin, 1969). These results suggested that BrAcCh acted as an irreversible affinity alkylating reagent with the exceptional property of being a receptor activator in addition. These properties make it an ideal reagent for identification of the ligand binding site(s) which, once occupied by an agonist, triggers the receptor system to depolarize the synaptic membrane. In this respect, it has an advantage over previously used affinity reagents, namely, MBTA (Well et al., 1974), DAPA (Witzemann & Raftery, 1977), or α-BuTx (Hucho, 1976; Witzemann & Raftery, 1977), all of which bind to the 40 000-dalton *T. californica* receptor subunit.

This study was conducted with the goal of characterizing the interaction of BrAcCh with the membrane-bound AcChR in terms of (1) the number of binding sites for the ligand compared with those for α-BuTx and whether or not there were interactions between the sites, (2) determination of whether BrAcCh caused conversion of the AcChR to a state of high affinity upon preincubation, (3) localization of the binding sites for BrAcCh by covalent incorporation of a 3H-labeled derivative into both membrane-bound and purified AcChR, and (4) determination of whether AcCh was competitive for BrAcCh for its binding site.

BrAcCh was found to bind to the unreduced membrane-bound AcChR reversibly because of the following. (1) The initial rate of [125I]-α-BuTx binding to the AcChR was inhibited by preincubating the receptor with BrAcCh (Figure 1B). The total number of receptor–toxin complexes formed 15–20 h after toxin addition was, however, unaffected by BrAcCh, the reaction being eventually driven to the receptor–toxin complex by the irreversible inhibitor α-BuTx. (2) Direct binding with [3H]BrAcCh revealed a specific, saturable uptake by the AcChR (Figure 2A). (c) In the absence of DTT, no irreversible labeling of the AcChR by [3H]BrAcCh was found after dialysis or by DEAE disc assay (Figure 4, curve C). These results suggest that [3H]BrAcCh binds reversibly to the nonreduced AcChR membrane preparation.

Figure 1A shows that the half-maximal initial rate of toxin binding was observed in the presence of 17 nM BrAcCh, indicating tight binding at equilibrium of BrAcCh to the receptor containing membranes. A similar dissociation constant was obtained by direct binding of the tritiated derivative (K_D = 16 ± 1 nM; Figure 2B). This affinity, measured at equilibrium, is 600-fold higher than that recorded for physiological response (K_ac ≈ 10 μM; Karlin, 1973). This difference may be explained by the finding that BrAcCh induced a slow transition of the membrane-bound AcChR from a low to a high affinity state(s) upon preincubation (Figure 1A), a process suggested to be a possible in vitro parallel of physiological desensitization (Weber et al., 1975; Weiland et al., 1976; Colquhoun & Rang, 1976; Lee et al., 1977; Quast et al., 1978).

The number of BrAcCh sites in the (unreduced) receptor membrane preparation, measured by direct binding, was found to be half of that for α-BuTx (Figure 2B). On the basis of our lowest estimate of the *T. californica* AcChR molecular weight of 270 000 (Martinez-Carrion et al., 1976) for the 9s form (Raftery et al., 1972) and the fact that the dimeric 13s form (Raftery et al., 1972) is the predominant form in the membrane preparation by virtue of S–S bonds formed between 65 000–67 000-dalton subunits (Suarez-Isla & Hucho, 1977; Chang & Bock, 1977; Hamilton et al., 1977; Witzemann & Raftery, 1978), we estimate that two BrAcCh molecules bind strongly to this receptor dimer. No cooperativity was detected for this binding (n_H = 0.98 ± 0.02; see Figure 2C). It is worthy of mention that the same stoichiometry of binding has been observed for binding of AcCh (Moody et al., 1973) and Carb (Raftery et al., 1975). In any case such cooperative binding, even if present, is unlikely to bear any relationship to cooperativity in conductance response since this event is a consequence of ligand binding of much lower affinity than found under equilibrium conditions. In any case, BrAcCh interacts in vitro with the unreduced AcChR in a manner very similar to that of the natural agonist AcCh, and its binding to the AcChR is competitively inhibited by the latter ligand (Figure 3).

Reduction of the AcChR with DTT prior to [3H]BrAcCh addition converted the reversible binding to an irreversible one, presumably by specific alkylation of a sulphydryl group(s) generated near the binding site (Figure 4). The incorporated radioactivity could not be dialyzed or washed away, suggesting a covalent linkage to the receptor. The amount of nonspecific labeling by [3H]BrAcCh, as measured with reduced toxin-blocked receptor, was higher than that measured with unreduced receptor (Figure 4, curves B and C). This is to be expected since reduction produced more available free sulphydryl groups for alkylation. When alkylation of the AcChR by [3H]BrAcCh was done in the presence of AcCh or d-Tc, the radioactivity incorporated after 1 h of labeling was appreciably decreased (Figure 5); it is possible that these ligands compete with BrAcCh for the anionic binding site for qua-
ternary ammonium groups and thus lower the rate of al-
kalyation and the extent of labeling after 1 h. Since AcCh
inhibited BrAcCh binding to both reduced and unreduced
AcChR, it is probable that BrAcCh binds to the same site in
both states of the receptor and that AcCh binds to this same
site.

The amount of specific labeling by \[^3H\]BrAcCh was
measured with \(\alpha\)-BuTx-labeled AcChR as a control. It was
found that BrAcCh irreversibly alkylated the reduced AcChR
with the same stoichiometry (BrAcCh site/\(\alpha\)-BuTx site = 0.5)
as found from direct binding measurements to nonreduced
receptor. This would imply that each ligand binding site has
one reactive disulfide in its vicinity and that alkylation by
BrAcCh occurs with one SH group of each of the reduced
disulfide bond(s). This is consistent with the assumption made
by Bartels-Bernal et al. (1976) to explain the effects of sulphhydryl reagents on the physiological response of affini-
ty-labeled AcChR.

No difference was found between the \[^3H\]BrAcCh al-
kylation of Triton-solubilized and membrane-bound AcChR;
both preparations were labeled by \[^3H\]BrAcCh in a single
subunit of molecular weight 40 000 (Figure 6), and this subunit
therefore contains all or part of the BrAcCh binding site(s).
A similar result was recently obtained for purified AcChR
from Narcine brazienssis (Chang et al., 1977). Taking into
account the protection afforded by AcCh against the incor-
poration of BrAcCh into both purified and membrane-bound
receptor in the experiments reported here in conjunction with
the apparent competitive binding between AcCh and BrAcCh
to membranes, we consider it most likely that AcCh and
BrAcCh bind to the same AcChR sites and with the same
stoichiometry. In addition, the results suggest that \(d\)-tubo-
curarine may bind to the specific agonist binding site(s) since
it also afforded protection against incorporation of BrAcCh
into DTT-reduced preparations. These results do not of course
preclude the binding of \(d\)-Tc to other postsynaptic sites.

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