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Effects of Thio-Group Modifications on the Ion Permeability Control and Ligand Binding Properties of *Torpedo californica* Acetylcholine Receptor†

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ABSTRACT: Chemical modification of membrane-bound *Torpedo californica* acetylcholine receptor by the disulfide reducing agent dithiothreitol has two major effects on receptor function: (1) it shifts the dose–response curve for agonist-induced increases in $^{22}Na^+$ permeability to 10-fold higher concentrations, and (2) it decreases the binding affinity of the receptor for the same agonist about 6-fold. In the experiments reported here, the agonist used was carbamoylcholine. Despite the quantitative changes in agonist binding and flux response, dithiothreitol-treated membranes display all other functional properties expected of the receptor. The flux response is blocked by preincubation of the membranes with carbamoylcholine, a phenomenon known as desensitization. In parallel, the receptor undergoes a carbamoylcholine-induced shift from a low-affinity to a high-affinity binding state for the same agonist. All of the effects of dithiothreitol are reversed by the oxidizing agent 5,5′-dithiobis(2-nitrobenzoic acid). Alkylation of the membranes with *N*-ethylmaleimide after dithiothreitol reduction results in complete inhibition of the flux response, and the effect is not reversed by the reoxidation treatment. The *N*-ethylmaleimide also shifts the receptor into a very low-affinity binding state for carbamylcholine that is shifted to only a slightly higher affinity by preincubation with carbamoylcholine. Prior to reduction, *N*-ethylmaleimide has no effect on receptor binding or flux properties. Detailed binding studies on affinity-alkylated receptor membranes indicate that the α-neurotoxin binding site not occupied by the affinity label displays all the same properties as unlabeled membranes, including the dithiothreitol and *N*-ethylmaleimide effects. The results are discussed in the context of several hypotheses previously proposed to account for the diverse effects of thio-group modifications on the acetylcholine receptor.

Understanding the relationship between the ligand binding and the ion permeability control properties of the nicotinic acetylcholine receptor (AcChR)[1] at postsynaptic membranes remains a major goal of current receptor research. One approach has been to characterize the effects of specific chemical modifications on the binding site and/or on the presumed ion channel. In one of the first chemical modification studies of a receptor, Karlin & Bartels (1966) showed that dithiothreitol (DTT) dramatically decreased the response of isolated electric eel electroplax to applied acetylcholine (AcCh). This result suggested the involvement of disulfides in normal AcChR function, since DTT was known to reduce disulfides to free sulfhydryls. The DTT effect was completely reversed by the oxidizing agent 5,5′-dithiobis(2-nitrobenzoic acid) [$(Nbs)_2$]. Alkylation of reduced electroplax with *N*-ethylmaleimide (MalNEt) prior to $(Nbs)_2$ treatment prevented reversal of the DDT effect (Karlin & Bartels, 1966).

DTT also altered the response of electroplax to other pharmacologic agents. For example, decamethonium, a bis-quaternary partial agonist, became a more potent agonist, and hexamethonium, normally an antagonist, became an activator of the reduced receptor (Karlin & Winnik, 1968). In addition, dose–response curves for carbamylcholine (Carb) showed a decreased affinity and a decreased slope of Hill plot (from 1.8 to 1.1), indicating a decrease in the apparent cooperativity of the response (Karlin, 1969).

Similar effects of sulfhydryl and disulfide modifications on physiologic responses have been measured on several vertebrate muscle preparations (Albuquerque et al., 1968; Mittag & Tormay, 1970; Rang & Ritter, 1971; Lindstrom et al., 1973; Ben Haim et al., 1973, 1975; Terrar, 1978) and on isolated neurones from the mollusc *Limnaea stagnalis* (Bregestovski et al., 1977). At the frog neuromuscular junction, DTT was shown to decrease both the lifetime and the conductance of single channels without any decrease in the number of functional channels (Ben Haim et al., 1975). In a recent study, bisulfite was found to enhance the depolarization response of the frog neuromuscular junction to AcCH (Steinacker, 1979). Unlike DTT, bisulfite heterolytically cleaves the disulfide to give a thiosulfate.

Karlin and co-workers also discovered [for reviews, see Karlin (1974, 1980) and Barrantes (1979)] that compounds containing both binding affinity for the AcChR and a sulfhydryl reactive group acted as affinity alkylating agents after

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[1] Abbreviations used: DTT, dithiothreitol; AcCh, acetylcholine; $(Nbs)_2$, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB in figures); AcChR, acetylcholine receptor; MalNEt, *N*-ethylmaleimide (NEM in figures); Carb, carbamoylcholine; BAC, bromoacetylcholine; MBTA, maleimidobenzyltrimethylammonium iodide; α-[^{125}I]BgTx, α-[^{125}I]bungarotoxin; PCMB, *p*-(chloromercuri)benzoate; Mops, 3-(*N*-morpholino)propanesulfonic acid.

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reaction of eel electroplax with DTT. Affinity compounds were synthesized that acted as either activators or inhibitors of AcChR function. Radioactive affinity labels have served as convenient and specific covalent reagents for assaying the AcChR binding site during solubilization and purification of the AcChR. An interesting property of the affinity labels is an apparent half-of-the-sites reactivity. Bromoacetylcholine (BAC, an agonist) and maleimidobenzyltrimethylammonium ion (MBTA, an antagonist) each react with only one of the two available α-neurotoxin binding sites (Damle & Karlin, 1978; Damle et al., 1978). There is now convincing evidence that reversible ligands, such as AcCh and Carb, bind to both of the α-neurotoxin sites [Neubig & Cohen, 1979; Delegeane & McNamee, 1980; see also Karlin (1980)].

Recently, most biochemical and biophysical studies of AcChR function have focused on the AcChR from the electroplax of marine rays, such as *Torpedo californica* and other *Torpedo* species. The *Torpedo* electroplax is the richest source of AcChR, and large quantities of pure AcChR protein can be obtained. In addition, membrane vesicles enriched in the AcChR can be isolated, and the highly purified vesicles retain all the ligand binding and ion permeability control properties expected of native AcChR [for a review, see Karlin (1980)]. For example, membrane fragments containing over 50% of the total protein as the AcChR have been obtained, and these membranes respond to activators, such as Carb, with a large selective increase in cation permeability that is specifically blocked by AcChR antagonists, such as α-bungarotoxin. The membranes also show a "desensitization" phenomenon, in that prolonged exposure of the membranes to an activator blocks the permeability increase. The functional desensitization has been correlated with a shift in activator binding from a "low"-affinity state to a "high"-affinity state (Weiland & Taylor, 1979; Quast et al., 1978; Weber & Changeux, 1974). Recently, solubilized AcChR has been reincorporated into lipid vesicles or into black films and been shown to recover ion permeability control properties (Huganir et al., 1979; Nelson et al., 1980; Gonzalez-Ros et al., 1980; Epstein & Racker, 1978; Wu & Raftery, 1979; Changeux et al., 1979; Schindler & Quast, 1980).

The effects of disulfide modifications or affinity labeling on the ligand binding or ion permeability properties of *Torpedo* membranes have been studied by several laboratories (Schiebler et al., 1977; Moore & Raftery, 1979a,b; Miller et al., 1979; Delegeane & McNamee, 1980). Schiebler et al. (1977) first reported that disulfide reduction decreased the ion permeability response of the AcChR in membranes and that the decrease was correlated with decreased binding affinity of the AcChR for activators. One group has attempted to establish a quantitative correspondence between the specific effects of disulfide modifications on the α-neurotoxin and ligand binding properties of the *Torpedo* AcChR and the α-bungarotoxin binding component from rat brain (Lukas et al., 1979; Miller et al., 1979).

The binding studies of Moore & Raftery (1979b) and Lukas et al. (1979) have resulted in specific proposals about the functional consequences of disulfide modifications. In this paper, the effects of DTT, $(Nbs)_2$, and MalNEt treatment on both the ligand binding *and* the ion permeability control properties of isolated *Torpedo* membranes are examined together in detail. Some of the results were discussed in an earlier paper concerned with activation of affinity-labeled AcChR (Delegeane & McNamee, 1980). A new observation is that DTT alters the binding affinity of the AcChR for Carb in both the low-affinity (activatable) and high-affinity (desensitized) binding states but does not interfere with the interconversion between low- and high-affinity binding states. MalNEt added after DTT reduction completely blocks the ion permeability response but does not completely block Carb binding. The results are discussed in the context of models of AcChR structure and function. Aspects of the sulfhydryl chemistry of the AcChR not directly related to ion permeability control are not discussed. For example, the disulfide linkage that results in AcChR dimer formation has not been shown to play any functional role [see Karlin (1980)]. Since completion of this work, a report has appeared containing some binding data and conclusions similar to those presented here (Barrantes, 1980).

Materials and Methods

Torpedo Membranes. Acetylcholine receptor rich membrane fragments were isolated as described (Delegeane & McNamee, 1980) from *Torpedo californica* electroplax that had been stored in liquid N_2. Typical preparations gave binding activities for α-[^{125}I]bungarotoxin (α-[^{125}I]BgTx) of 500–1500 pmol/mg of protein. Equilibrium binding of α-[^{125}I]BgTx was measured in 0.1% Triton X-100 by filtration on DE-81 disks (Damle & Karlin, 1978). The toxin was a gift from E. Bennett (Chemical Biodynamics Laboratory, University of California, Berkeley) and was iodinated by the method of Lukasiewicz et al. (1978). Membrane protein concentration was determined by the Lowry method (Lowry et al., 1951).

Chemical Modification. To 1.0 mL of *Torpedo* membranes ($\sim$10 mg/mL) in vesicle dilution buffer (VDB: 250 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 0.02% NaN_3, and 10 mM Tris-HCl, pH 7.6) was added 10 μL of 100 mM DTT, and the mixture was kept at room temperature for 20 min. Reduced membranes were then treated with 10 μL of one of the following solutions and incubated for an additional 20 min at room temperature: 300 mM $(Nbs)_2$, or 300 mM MalNEt, or VDB only. Labeling with MBTA was done slightly differently to minimize the amount of MBTA that had to be used. A 1-mL sample of DTT-treated membranes was diluted in VDB and centrifuged at 150000g for 30 min to remove DTT. The pellet was suspended in $\sim$1 mL of VDB and immediately treated with 10 μL of 10 mM MBTA for 20 min at room temperature. Membranes were washed by dilution in VDB and centrifuged to remove unreacted MBTA. All modified membranes were then kept at 4 °C or stored in liquid N_2. Solutions of DTT and $(Nbs)_2$ were prepared in VDB. MalNEt was dissolved in distilled water, and MBTA was in 10^{-4} M HCl. MBTA was prepared by the method of Karlin (1977), and DTT, $(Nbs)_2$, and MalNEt were obtained from Sigma Chemical Co.

$^{22}Na^+$ Flux Assays. $^{22}Na^+$ influx and $^{22}Na^+$ efflux were measured by using the Millipore (0.45 μm) filtration technique with 30 s or 1 min of flux at 0 °C as described previously (Delegeane & McNamee, 1980). Initial membrane concentrations were $\sim$10 mg/mL to maximize $^{22}Na^+$ flux amplitudes. In most cases, $^{22}Na^+$ influx was most convenient, not requiring overnight equilibration of membranes with $^{22}NaCl$.

Toxin Rate Binding. The initial rate of α-[^{125}I]BgTx binding was measured under conditions that make the reaction first order in receptor concentration. Membranes (5 nM in toxin sites) were reacted with 30 nM α-[^{125}I]BgTx in 700 μL of incubation buffer (100 mM NaCl, 10 mM sodium phosphate, pH 7.0, and 0.5 mg/mL bovine serum albumin) at room temperature. At specific time intervals, 100-μL aliquots were removed, filtered through two DE-81 disks under low pressure, and washed with 10 mL of wash buffer (10 mM NaCl, 10 mM

Mops, pH 7.4, and 0.1% Triton X-100). Filters were dried and counted in a Packard γ scintillation spectrometer.

(*1*) *Qualitative Evaluation of Affinity States.* Three toxin binding reactions were carried out as just described for each membrane preparation. Aliquots (100 μL) were filtered at 1-min intervals for 5 min. The control reaction had 30 nM α-[^{125}I]BgTx but no Carb in the mixture, and the reaction was started by the addition of 10 μL of membranes at a toxin site concentration of 350 nM. Coincubation mixtures contained α-[^{125}I]BgTx and an appropriate concentration of Carb (usually 2 μM) in the mixture, and the reaction was started by adding 10 μL of membranes as above. Preincubation curves were obtained by premixing 10 μL of membranes (350 nM in toxin sites) with 10 μL of 4 μM Carb for 30 min at 0 °C and then adding 20 μL to a reaction mixture containing the α-[^{125}I]BgTx and 2 μM Carb.

(*2*) *Low- and High-Affinity K_D.* Quantitation of Carb dissociation constants was achieved by varying the final concentration of Carb in the 700-μL reaction mixture from 0.01 μM to 3 mM. For measurement of the low-affinity K_D, coincubation curves were generated for each Carb concentration. Aliquots (100 μL) were removed and filtered every 30 s for 2 min. Rate constants of toxin binding were determined by linear least-squares analysis of a plot of ln $([AcChR]_{free}(t)/[AcChR)_{total}]$ essentially as described by Quast et al. (1978). The ratio of the observed pseudo-first-order rate constant in the presence of Carb (k) to the rate constant with no Carb (k_{max}) was plotted vs. the log of the Carb concentration to determine K_D (see Results). In all cases, the values for k/k_{max} were identical with the ratios of the relative initial rates measured directly from the slope of toxin binding curves such as those shown under Results (Figure 5). High-affinity dissociation constants (K_D') were determined by preincubation of concentrated membrane samples (5 μM toxin sites) with enough Carb (10 μM) to completely desensitize the receptor. Membranes were then diluted 1:15 in VDB, and immediately 10 μL was added to 700 μL of reaction mixture containing α-[^{125}I]BgTx and various Carb concentrations. This technique was used to obtain the maximum dilution (~1:1000) of the Carb used during preincubation. The final concentration of toxin sites was 5 nM, and the lowest possible concentration of Carb used with desensitized membranes during the toxin binding reaction was 10 nM. Initial rates were determined by linear least-squares analysis, and the ratios of the initial rates in the presence and absence of Carb were plotted as for the low-affinity K_D.

The time course of the transition from the low-affinity to the high-affinity state was measured as follows. To 600 μL of incubation buffer containing a specific concentration of Carb (e.g., 2 μM) was added 10 μL of membranes (final concentration 5 nM in toxin sites) at zero time. At various times after the addition of membranes, 100 μL of α-[^{125}I]BgTx was added, and the initial rate of toxin binding was measured for 2 min. The coincubation curve for the same concentration of Carb served as the control (desensitization time equals zero). The ratios of rate constants were plotted vs. the time between addition of membranes and the addition of α-[^{125}I]BgTx. All toxin rate binding reactions were done in duplicate at room temperature.

Results

$^{22}Na^+$ Flux Response. AcChR-rich membranes from *Torpedo californica* responded to the receptor activator carbamylcholine (Carb) with a large increase in $^{22}Na^+$ flux over that observed in the absence of activator (Figure 1) as observed previously (Andreasen & McNamee, 1977). In these experiments, the magnitude of the flux response was determined by the difference in vesicle-entrapped $^{22}Na^+$ counts per minute after 30 s or 1 min of flux in the presence and absence of Carb. It has been shown by rapid mixing and quench techniques that desensitization of the flux response occurs very rapidly (within 1 s, and the Carb-related differences in counts per minute are most probably generated within the first 10 ms of flux at high Carb concentrations (Hess et al., 1979; Neubig & Cohen, 1980). The responses measured by the "slow" filtration technique used here are thus integrated responses and are used as an empirical measure of changes in receptor flux properties. For native AcChR-rich membranes, a maximum $^{22}Na^+$ flux response occurred at a concentration of 10^{-3} M Carb, while the concentration that gave a 50% response (C_{50}) was 2×10^{-5} M Carb (Figure 2). A consistent observation was that the response decreased at very high Carb concentrations (10^{-2} M), suggesting extremely rapid desensitization. The same C_{50} value was obtained for both influx (Figure 2A) and efflux measurements (Figure 2B).

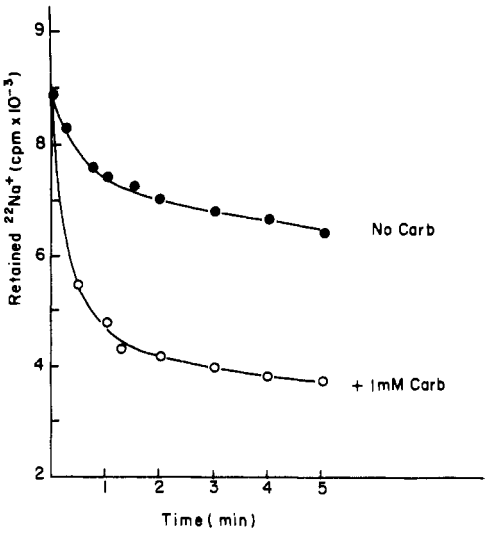

FIGURE 1: Time course of $^{22}Na^+$ efflux. *Torpedo* membranes preloaded with ^{22}NaCl were diluted 100-fold into VDB at 0 °C containing 1 mM Carb (○) or no Carb (●). At the indicated times, 500-μL aliquots were filtered on Millipore filters as described under Materials and Methods. Each data point represents the average of duplicate samples.

Treatment of AcChR-rich membranes with 1 mM DTT resulted in a marked shift in the dose response for $^{22}Na^+$ flux to higher Carb concentrations (Figure 2). The C_{50} for reduced membranes was 2×10^{-4} M, 10-fold greater than native AcChR-rich membranes. Treatment of reduced AcChR with the oxidizing agent $(Nbs)_2$ reversed the shift in the dose response induced by DTT and appeared to restore the reduced AcChR to the native state [Figure 2; see also Delegeane & McNamee (1980)].

After DTT treatment, MalNEt inhibited the Carb-stimulated flux response completely (Figure 2). If there was any response at all, the C_{50} would have to exceed 10^{-2} M, the largest concentration tested. Prior to DTT treatment, MalNEt had no effect on $^{22}Na^+$ flux properties (data not shown). Addition of $(Nbs)_2$ after the sequential DTT and MalNEt treatment did not restore the permeability response.

In trial experiments, the effects of several other agents were examined. *p*-(Chloromecuri)benzoate (PCMB) appeared to destroy the integrity of the membranes at a final concentration of 1 mM as judged by a complete loss of trapped $^{22}Na^+$ counts upon dilution. Bisulfite at 1 mM had no effect on the flux properties compared to native membranes. In addition, 1 mM hexamethonium had no effect on $^{22}Na^+$ efflux either before

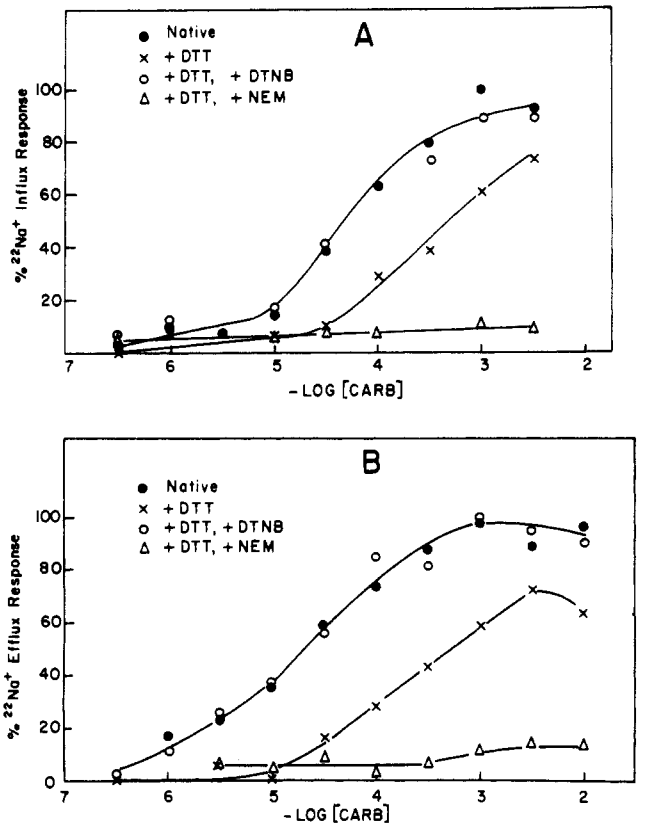

FIGURE 2: Effect of Carb concentration (M) on $^{22}Na^+$ influx (A) and $^{22}Na^+$ efflux (B) in chemically modified membranes. Flux response is the difference in $^{22}Na^+$ cpm in the presence and absence of Carb after 1 min of flux at 0 °C. 100% response is defined as the response of native membranes to 10^{-3} M Carb. Native membranes (●), DTT membranes (×), DTT + $(Nbs)_2$ membranes (○), DTT + MalNEt membranes (△).

or after DTT reduction, and hexamethonium also blocked the response to Carb.

Flux Desensitization. Pharmacological desensitization, the decrease in ion permeability of AcChR membranes due to prolonged exposure to cholinergic agonists, can be measured in vitro by using the $^{22}Na^+$ flux assay. Incubation of *Torpedo* vesicles with 1 mM carbamoylcholine for 10 min prior to the start of a flux measurment blocked Carb-stimulated increases in $^{22}Na^+$ flux (Table I). DTT-treated membranes and DTT–$(Nbs)_2$ membranes also showed the densensitization phenomenon (Table I). Since DTT–MalNEt membranes showed no $^{22}Na^+$ flux response, functional desensitization could not be measured after DTT–MalNEt treatment.

The *rates* of desensitization of the flux response were measured at several Carb concentrations for native and DTT-treated membranes by preincubating membranes with Carb for various times before dilution into Carb-containing buffer to start the efflux measurements. At 0.1 and 1 mM Carb, all membranes were completely desensitized within 1 min, and differences in the rates between native and DTT-treated membranes could not be reproducibly measured. At low Carb concentrations (e.g., 5 μM), the native and DTT-treated membranes were not completely desensitized even after 15 min of preincubation although the native membranes were more desensitized (50%) than the DTT-treated membranes (20%). The high concentrations of membranes required for flux studies made it difficult to obtain preincubation conditions suited to measure slow but complete desensitization. Although preliminary, the results are consistent with a decreased sensitivity of DTT-treated membranes to Carb-induced desensitization.

Table I: Effects of Chemical Modifications on Desensitization of $^{22}Na^+$ Flux[a]

chemical modification	preincubation with 1 mM Carb	relative flux response[b] (%)
none (native)	–	100
	+	8
DTT only	–	84
	+	4
DTT and $(Nbs)_2$	–	88
	+	0
DTT and MalNEt	–	0
	+	0
MalNEt only	–	(100)[c]
	+	(0)[c]
DTT, MalNEt, and $(Nbs)_2$	–	(0)[c]

[a] *Torpedo* membranes (~10 mg of protein/mL) were incubated overnight with $^{22}Na^+$ in preparation for efflux experiments. After treatment with the various chemicals at 25 °C (see Materials and Methods), 100-μL aliquots were pretreated with 5 μL of 20 mM Carb or with 5 μL of VDB for 10 min. Aliquots (20 μL) were then added to 1 mL of VDB or to 1 mL of VDB containing 1 mM Carb at 0 °C, and efflux was allowed to occur for 30 s at 0 °C. The entire mixture was then filtered, washed, and counted [see Delegeane & McNamee (1980)]. [b] The relative flux response is calculated from the difference in counts per minute between the samples diluted into no Carb and those diluted into the 1 mM Carb and compared to the response for the native membranes. All measurements were made in duplicate on the same membrane preparation. The actual counts per minute for the native membranes were 4288 (– Carb) and 1563 (+ 1 mM Carb). [c] The "MalNEt only" and DTT–MalNEt–$(Nbs)_2$ data were obtained on a different membrane preparation and compared to native membranes for the same membrane preparation.

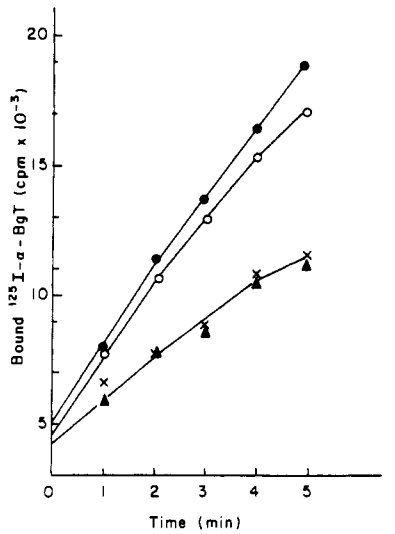

FIGURE 3: Initial rates of α-[^{125}I]BgTx binding to modified *Torpedo* membranes in the absence of Carb. (●) Native membranes, (○) MBTA affinity alkylated membranes adjusted to contain an equal number of *free* α-toxin sites (5 nM), (▲) native membranes reduced with DTT and treated with MalNEt, (×) membranes treated with 3 mM MalNEt following the incubation of DTT-reduced membranes with 0.1 mM MBTA (see Materials and Methods). Higher concentrations of MalNEt (6 or 12 mM) did not further decrease the rate of α-[^{125}I]BgTx binding.

Toxin Binding Properties of Chemically Modified Membranes. Binding studies were carried out on chemically modified membranes in an attempt to correlate changes in C_{50} values with changes in Carb binding affinities. Modified membranes were characterized by the specific activity of α-[^{125}I]BgTx binding at equilibrium and by the initial rate of α-[^{125}I]BgTx binding (Figure 3). Reduction of the AcChR with DTT did not change the number of α-bungarotoxin sites

Table II: Summary of Binding Properties of Chemically Modified *Torpedo* Membranes[a]

modification	no. of α-toxin sites (nmol/mg of protein)	rate constant for α-toxin binding,[b] k_T (M^{-1} s^{-1})	Carb binding[c] K_D (μM)	Carb binding[c] K_D' (μM)	flux response C_{50} (Carb)[d] (μM)
native	1.19	1.45×10^5	8	0.02	20
DTT	1.12	1.45×10^5	50	0.50	200
DTT + $(Nbs)_2$	1.17	1.38×10^5	8	0.02	20
DTT + MBTA	0.61	1.40×10^5	5	0.04	200[e]
DTT + MalNEt	1.09	0.80×10^5	250	120	
MalNEt	1.15	1.45×10^5	6	0.03	20

[a] Chemical modifications, binding assays, and the flux assay were carried out as described under Materials and Methods and Results. [b] The second-order rate constant (k_T) was calculated from the pseudo-first-order rate constant (k) measured in the presence of excess toxin ($k_T = k/[\text{toxin}]$). [c] K_D and K_D' correspond to the measurement of Carb dissociation constants by the toxin competition assay either without (K_D) or with (K_D') preincubation with Carb. [d] C_{50} values correspond to the concentration of Carb giving a flux response equal to 50% of the maximum response measured for a particle membrane sample. [e] Data from Delegeane & McNamee (1980).

or the initial rate of toxin binding to the AcChR (Table II). $(Nbs)_2$ oxidation of reduced membranes also had no effect on these toxin binding properties. In contrast, DTT–MalNEt treatment significantly decreased the initial rate of toxin binding (Figure 3). However, the number of α-[^{125}I]BgTx molecules bound at equilibrium was unchanged (Table II). Treatment with higher concentrations of MalNEt (6 and 12 mM) after DTT reduction did not further decrease the initial rate of toxin binding (data not shown).

Toxin binding properties were also measured for MBTA affinity labeled membranes since the flux properties of the MBTA membranes were examined in detail previously (Delegeane & McNamee, 1980). The results were consistent with the assumption that MBTA reacted with and blocked one-half of the toxin sites. MBTA membranes showed a decreased initial *rate* of toxin binding and one-half of the specific activity of α-[^{125}I]BgTx binding at equilibrium [Table II; see also Delegeane & McNamee (1980)]. Damle & Karlin (1978) have shown that this half-of-the-sites reactivity represented MBTA labeling one and only one of the two α-toxin sites per receptor monomer (M_r = 250 000). The remaining α-toxin site appeared to have unaltered α-[^{125}I]BgTx binding kinetics since the second-order *rate constants* for α-[^{125}I]BgTx binding to AcChR membranes were the same for native and MBTA membranes (Table II). Under the conditions of the binding assay, the unlabeled site would have become reoxidized even if it has originally been reduced by DTT. Treatment of MBTA-labeled membranes with DTT followed by MalNEt caused a further decrease in the initial rate of α-[^{125}I]BgTx binding (Figure 3). This result suggests that DTT effectively reduced disulfide bonds in both α subunits and that both were susceptible to alkylation by MalNEt, but that only one in each receptor monomer was affinity-labeled with MBTA.

Affinity States of Chemically Modified Membranes. Affinity state changes of the AcChR for Carb were investigated by an α-[^{125}I]BgTx competition assay. Two states, one of low affinity and one of higher affinity, have been reported to exist for native *Torpedo* membranes (Weiland & Taylor, 1979; Weber & Changeux, 1974; Quast et al., 1978). Preincubation of AcChR membranes with Carb induces a slow (seconds to minutes) transition to the high-affinity state, which appears to be characteristic of the desensitized receptor (Weiland & Taylor, 1979). Figure 4 demonstrates the existence of two affinity states for Carb in various chemically modified membranes. Reduction with DTT (Figure 4B) did *not* prevent Carb from inducing a transition in the AcChR from a low- to a high-affinity state. This observation is apparently contradictory to data presented by Moore & Raftery (1979b) in which they showed that DTT membranes did not undergo the shift to high affinity following preincubation with 1 μM Carb.

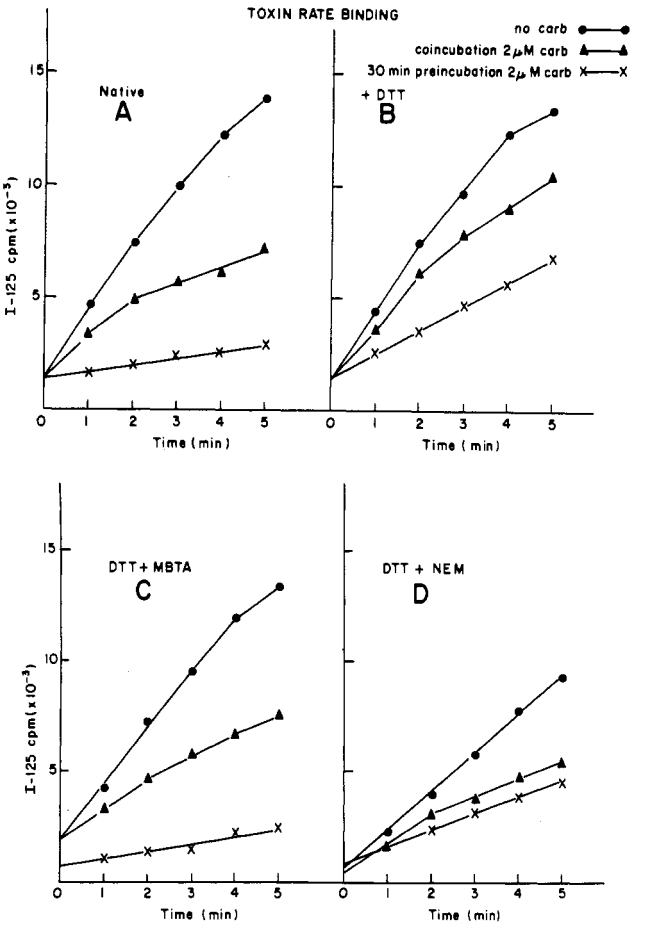

FIGURE 4: Effects of chemical modification on Carb affinity states. Rates of α-[^{125}I]BgTx binding to *Torpedo* membranes were measured in the absence of Carb (●), in the presence of 2 μM Carb (▲, coincubation), and after preincubation with 2 μM Carb for 30 min and then dilution into a reaction mixture containing 2 μM Carb (×). (A) Native membranes; (B) DTT-treated membranes; (C) MBTA-modified membranes; (D) DTT + MalNEt membranes. All reaction mixtures contained 30 nM α-[^{125}I]BgTx and 5 nM α-toxin sites. For DTT–MalNEt membranes (D), the concentration of Carb used was 20 μM.

The affinity shift of the reduced AcChR requires a 10-fold higher Carb concentration than native AcChR membranes (see Discussion).

For further investigation of the chemically modified membranes, Carb binding constants were quantitated by using the α-[^{125}I]BgTx competition assay. The apparent Carb dissociation constant (K_D) was defined as the concentration of Carb required to decrease the initial relative rate of iodinated bungarotoxin binding by 50%. If AcChR membranes are

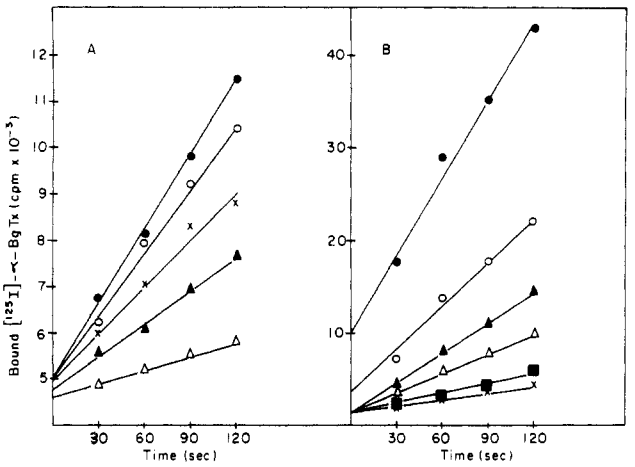

FIGURE 5: Inhibition of the initial rate of α-[^{125}I]BgTx binding by increasing concentrations of Carb for DTT–$(Nbs)_2$ membranes in the (A) low-affinity and (B) high-affinity states (5 μM membrane preincubated with 10 μM Carb for 30 min at 0 °C). The final concentration of toxin sites was 5 nM, and α-[^{125}I]BgTx concentration was 30 nM. Initial rates were measured in 2 min to minimize desensitization or recovery from desensitization. All data points represent the mean of duplicate samples. (A) Low affinity: no Carb (●); 10^{-7} M (○); 10^{-6} M (×); 10^{-5} M (▲); 3×10^{-5} M (△). (B) High affinity: no Carb (●); 10^{-8} M (○); 3×10^{-8} M (▲); 10^{-7} M (△); 3×10^{-7} M (■); 10^{-6} M (×).

exposed to Carb and α-[^{125}I]BgTx simultaneously, the receptor should be predominantly in the low-affinity state, and the low-affinity K_D can be measured. If AcChR membranes are preincubated with Carb for 30 min and then diluted into assay buffer containing Carb and α-[^{125}I]BgTx, the high-affinity K_D can be determined. For the simplest case of competitive binding, semilogarithm plots of the inhibition of α-[^{125}I]BgTx binding by increasing concentrations of Carb can be fitted to theoretical curves for competitive ligand binding to identical noninteracting sites obtained from eq 1. k is the observed

$$\frac{k}{k_{max}} = \frac{1}{1 + [Carb]/K_D} \quad (1)$$

pseudo-first-order rate constant derived from measurements of the initial rate of formation of toxin–receptor complexes in the presence of excess toxin, and k_{max} is the value for k measured in the absence of Carb. The validity of the above treatment for Carb and toxin binding has been demonstrated by Quast et al. (1978), and the treatment is also compatible with a more complex reaction scheme proposed by Bulger et al. (1977) since the toxin concentrations used here are relatively low. Typical plots of the initial rate of toxin binding for various concentrations of Carb are shown in Figure 5 for DTT–$(Nbs)_2$ membranes in both the low- and high-affinity states. Low concentrations of Carb were found to inhibit α-[^{125}I]BgTx binding better than predicted by eq 1 and from the concentration that gave 50% inhibition. For an explanation of this, an equilibrium between low- and high-affinity states was assumed to preexist in the membrane-bound receptor as demonstrated by others (Cohen & Boyd, 1979; Heidmann & Changeux, 1979). The observed data fit a theoretical curve obtained from eq 2, where a is the fraction of the AcChR in

$$\frac{k}{k_{max}} = \frac{a}{1 + [Carb]/K_D} + \frac{b}{1 + [Carb]/K_D'} \quad (2)$$

the low-affinity state and b the fraction in the high-affinity state. For typical membranes, a was 0.85, and b was 0.15. Interestingly, the small percentage of high-affinity receptor was observed in both native and modified membranes. The k/k_{max} plots are shown in Figure 6 for native, DTT, and

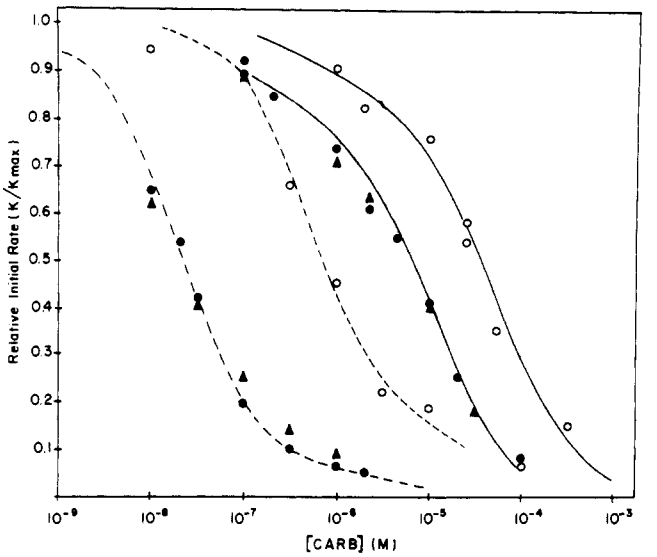

FIGURE 6: Effect of Carb concentration on the initial rate of α-[^{125}I]BgTx binding in native (●), DTT-treated (○), and DTT + $(Nbs)_2$ membranes (▲). For each sample, semilogarithm plots were made for membranes not preincubated (low affinity, solid lines) and membranes preincubated with 10 μM Carb for 30 min (high affinity, dashed lines). The ratio of the initial rate of toxin binding in the presence of Carb (k) to the initial rate with no Carb (k_{max}) was obtained from data such as that shown in Figure 5. Curves that gave the best fit to the data were obtained from the equation $k/k_{max} = a/(1 + [Carb]K_D) + b/(1 + [Carb]/K'_D)$ where a and b are the fraction of receptor in the low- and high-affinity states, respectively.

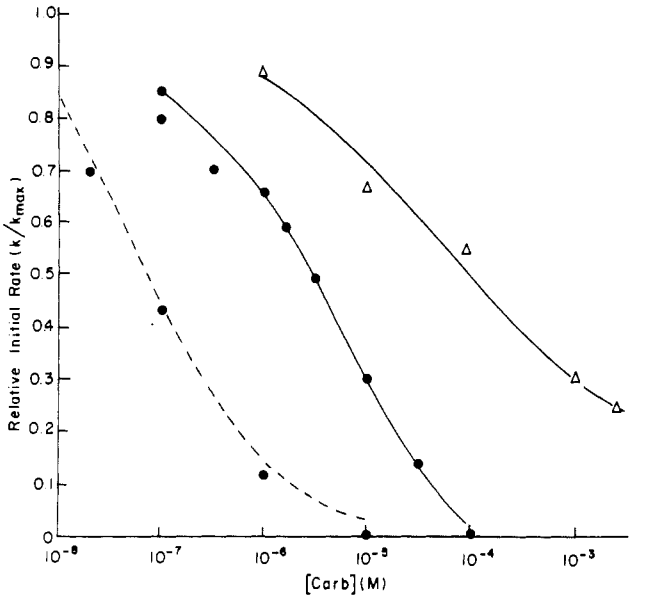

FIGURE 7: Effect of Carb concentration on the initial rate of α-[^{125}I]BgTx binding in MBTA-labeled membranes. Low-affinity binding was measured without preincubation with Carb (●—●), and high-affinity binding was measured following a 30-min preincubation with 10 μM Carb. The effect of DTT–MalNEt treatment was determined without Carb preincubation (△).

DTT–$(Nbs)_2$ membranes. Similar measurements were made for MBTA-labeled membranes (Table II and Figure 7) to examine the binding properties of the site *not* labeled by MBTA. The second site showed binding properties similar to the native AcChR (Figure 7; see also Table II) both before and after treatment with DTT, DTT–$(Nbs)_2$, or DTT–MalNEt.

The data in Tables I and II and Figures 4 and 6 clearly demonstrate that DTT alters the binding affinity of the AcChR for Carb but does not prevent a Carb-induced shift from a low-affinity to a high-affinity binding state. The effect

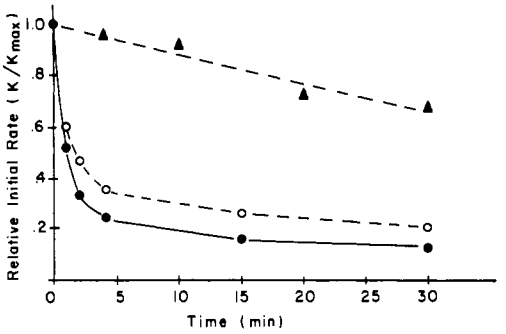

FIGURE 8: Time course of desensitization for native (●) and DTT-treated (○, ▲) membranes. Preincubation was initiated by adding 10 μL of membranes to 600 μL of incubation buffer containing Carb. At indicated times, 100 μL of α-[^{125}I]BgTx was added, and the initial rate of toxin binding was measured as in Figure 5. The maximum initial rate was for coincubation with the same Carb concentration. Data points represent averages of duplicate samples. Native membranes + 2 μM Carb (●); DTT membranes + 2 μM Carb (▲); DTT membranes + 20 μM Carb (○).

of DTT reduction on the *rate* of the Carb-induced transition from low- to high-affinity binding was examined. For native membranes, the affinity shift in the presence of 2 μM Carb was nearly complete within 10 min. By contrast, DTT-treated membranes underwent the shift very slowly at 2 μM Carb (Figure 8). If the Carb concentration was increased to 20 μM, however, the rate of desensitization was about the same as that observed for native membranes at 2 μM. This 10-fold increase necessary to achieve the same rate of desensitization correlates well with the 6–10-fold differences in binding affinities and C_{50} values between native and DTT-treated membranes.

Discussion

The goal of these experiments was to establish the correlation between ligand binding to the AcChR and ion permeability control by using disulfide and sulfhydryl reagents as selective modifiers of the receptor protein. The effects of DTT, $(Nbs)_2$, and various alkylating agents on the AcChR have been well documented by electrophysiologic or binding studies [see Karlin (1980)]. The purified AcChR-rich *Torpedo californica* membranes used here made it possible to characterize both the binding and ion permeability changes under similar reaction conditions, as originally done on a limited scale by Schiebler et al. (1977).

Reaction of *Torpedo* membranes with the disulfide reducing agent DTT had two main effects on the function of the AcChR: (1) it shifted the dose–response curve for the Carb-induced increase in $^{22}Na^+$ permeability to higher Carb concentrations, and (2) it decreased the binding affinity of the AcChR for Carb. Specifically, the C_{50} value for flux, which is a measure of the Carb concentration necessary to give 50% of the maximum flux response, was increased from 20 μM for native membranes to 200 μM for DTT-treated membranes. The dissociation constant for Carb (K_D), measured by an α-bungarotoxin competition assay without preexposure of the membranes to Carb, was increased from 8 μM for native membranes to 50 μM for DTT-treated membranes. The initial rate of binding of iodinated α-bungarotoxin in the absence of Carb was not affected by DTT treatment. Qualitatively, the results are consistent with a hypothesis that the decreased response is a direct result of the decreased binding affinity. At concentrations of Carb that gave the same occupation of binding sites, the flux responses were similar.

It is clear from the data, however, that the K_D and C_{50} values are not identical for either native or DTT-treated membranes. Although the dissociation constants provide a quantitative measure of Carb affinity, the C_{50} values measured here provide only a qualitative measure of Carb-induced ion permeability changes since the measured fluxes are a time-averaged response that reflects both channel activation and desensitization. Quantitative analysis is also hindered by the presence of spare receptors (Neubig & Cohen, 1980). Recent rapid flux measurements in native *Torpedo* membranes indicate that the actual C_{50} value for the initial flux response to Carb is about 600 μM (Neubig & Cohen, 1980). High C_{50} values have also been measured in *Electrophorus* vesicles by rapid flux techniques (Aoshima et al., 1980). Since the rapid flux measurements suggest an even greater discrepancy between K_D and C_{50} values, it has been suggested that a third ultralow-affinity binding state might exist for the native AcChR (Neubig & Cohen, 1980). However, a functionally relevant ultralow-affinity state has not yet been detected.

When the native or DTT-treated membranes were incubated with Carb prior to the start of a $^{22}Na^+$ flux assay, the Carb-induced increase in $^{22}Na^+$ flux was completely blocked. Thus, the desensitization phenomenon characteristic of the AcChR both in vivo and in vitro was not altered by reduction. In parallel, the DTT-treated membranes underwent a shift in the binding affinity for Carb from a K_D of 50 μM to a K'_D of 0.50 μM following preincubation with Carb. This 100-fold increase in binding affinity was comparable to the 400-fold increase observed for native membranes in this study. The shift in the binding affinity of the AcChR for agonists appears to be a characteristic feature of functional AcChR desensitization. Barrantes (1980) also detected a shift in the binding affinity for DTT-treated membranes although the change was much smaller than the one reported here (70 → 50 μM). Miller et al. (1979) measured a high-affinity dissociation constant of 5 μM for DTT-treated membranes.

The *rate* of the low- to high-affinity binding shift was slower for DTT-treated membranes than for native membranes. However, if the concentration of Carb was adjusted to give the same predicted site occupancy, the rate was identical. At low Carb concentrations (e.g., 2 μM), native membranes were readily shifted to the high-affinity form, but DTT-treated membranes were not. Thus, under some conditions, it can appear that DTT-treated membranes are protected from desensitization. Such an observation led to a suggestion that intact disulfides were essential for the "low"- to "high"-affinity change (Moore & Raftery, 1979b).

All of the effects of DTT treatment were reversed by the oxidizing agent $(Nbs)_2$. The C_{50} value for flux and the K_D and K'_D values for Carb binding were indistinguishable from those of the native membranes after the sequential DTT–$(Nbs)_2$ treatment. Such a result is consistent with the original electrophysiologic observations of Karlin & Bartels (1966) and reinforces the assumption that DTT is acting reversibly to reduce a disulfide(s) to sulfhydryl groups. The data here provide no support for the hypothesis of Lukas et al. (1979) that after DTT treatment $(Nbs)_2$ shifts the AcChR into a high-affinity binding state different from the native AcChR.

A striking feature of the binding data for native, DTT-treated, and DTT–$(Nbs)_2$-treated membranes is the excellent agreement between the observed initial rates for toxin binding and the theoretical curves generated by assuming competitive inhibition by Carb at independent sites (eq 2). The fraction of preexisting high-affinity binding sites for Carb in the absence of added ligand (typically 15% of the total sites) is preserved after both DTT and DTT–$(Nbs)_2$ treatment, consistent with reversible, cyclic equilibration of all four AcChR

states (high or low affinity, with or without ligand).

If the alkylating agent MalNEt was added to *Torpedo* membranes after DTT reaction, there was no measurable flux response at Carb concentrations as high as 10 mM. Prior to DTT treatment, MalNEt had no effect on the flux response. $(Nbs)_2$, added after DTT and MalNEt, did not restore the flux responses to Carb, an observation once again consistent with the electrophysiologic data (Karlin & Bartels, 1966). The K_D for Carb after DTT–MalNEt treatment was increased to 250 μM, a value ~30 times larger than the K_D for native membranes. Preincubation of DTT–MalNEt membranes with Carb caused a modest decrease in K_D to 120 μM, a value still much higher than the low-affinity K_D of either native or DTT-treated membranes. Since the DTT–MalNEt membranes showed no flux response, it could not be determined if the modest shift in binding was correlated with desensitization.

It has been suggested that DTT–MalNEt treatment "freezes" the AcChR in a very low-affinity form that could ultimately represent the functional ligand binding state of the receptor (Barrantes, 1980; Lukas et al., 1979; Miller et al., 1979). For example, the high K_D values after DTT–MalNEt treatment agree reasonably well with the C_{50} values measured for native membranes under rapid flux conditions (Neubig & Cohen, 1980). However, the DTT–MalNEt membranes showed a decreased rate constant for toxin binding, and none of the other treatments appeared to decrease the rate constant. More likely, MalNEt interferes sterically with the binding of both agonists (Carb) and antagonists (toxin). Since MalNEt completely blocked flux responses even though the Carb could bind (albeit weakly), it is also possible that MalNEt attacks other sulfhydryl groups after reduction that are directly associated with the ion channel. The decrease in single-channel conductance of AcCh receptors at the frog neuromuscular junction after DTT treatment (Ben Haim et al., 1975) is consistent with some channel-related DTT effects.

In contrast to the DTT–MalNEt treatment, affinity alkylation of DTT-treated membranes with MBTA resulted in membranes that still showed a flux response (Delegeane & McNamee, 1980). Similar results were recently obtained by Lindstrom et al. (1980) although the flux was attributed to a small fraction of unmodified receptors. The detailed binding studies reported here indicate, as expected, that MBTA labels only one of the two ligand binding sites. The second site appeared to retain the same ligand binding properties as the native AcChR, provided the membranes were allowed to oxidize in air following affinity alkylation. Rereduction of the unlabeled site with DTT caused a shift to a higher K_D, and this shift was increased further by subsequent MalNEt treatment. Thus, the ligand binding site *not* occupied by MBTA appeared to have a disulfide linkage that underwent DTT and MalNEt effects similar to the native membranes. On the basis of all of the binding data accumulated thus far [Ellena & McNamee, 1980; Delegeane & McNamee, 1980; see Karlin (1980)], the half-of-the-sites reactivity of MBTA and BAC labeling is not readily explained by differences between the two α subunits (M_r = 40 000), but could be explained by differences in subunit contacts.

The results discussed thus far provide a fairly simple picture of disulfide modifications in that alterations in binding affinity correlate reasonably well with flux changes. A direct effect of MalNEt on the ion channels of reduced membranes is one effect that will require additional study. One approach will be to determine if the DTT–MalNEt effect still presists even if the ligand binding sites are protected from DTT reduction by prior binding of agonists. Since completion of this work, Damle & Karlin (1980) have shown quantitatively that agonists can protect both native and desensitized *Torpedo* AcChR from DTT reduction. Interestingly, many antagonists did not provide protection even though the antagonists are believed to act as competitive inhibitors of agonist binding (Neubig & Cohen, 1979). Damle & Karlin (1980) suggest that local conformational changes accompanying agonist binding are more important than steric factors in providing protection against DTT reduction. Similar conclusions were reached by Bregestovski et al. (1977) from electrophysiologic measurements on mollusc neurones.

In preliminary experiments, we have examined the effects of bisquaternary compounds and bisulfite on flux responses since these compounds have been reported to affect *Electrophorus* electroplax or muscle synapses in qualitatively different ways before and after reduction (Karlin & Winnik, 1968; Rang & Ritter, 1971; Steinacker, 1979). Hexamethonium (1 mM) had no effect on flux either before or after reduction with DTT, and it blocked activation by Carb. Thus, it appeared to act as an antagonist both before reduction (as expected) and after reduction (a result different from *Electrophorus*). Bisulfite had no effect on the flux response to Carb at several different Carb concentrations. Thus, we have found no evidence in *Torpedo* membranes for some of the unusual properties observed electrophysiologically. The negative results for hexamethonium on *Torpedo* can be explained since bisquaternary compounds (such as decamethonium) can act as noncompetitive blockers of flux in a manner analogous to local anesthetics (Adams & Sakmann, 1978). The binding and fluorescence data of Barrantes (1980) for hexamethonium and decamethonium are also consistent with a dual action.

Experiments are now in progress to measure the initial flux responses of reduced and affinity-alkylated membranes on the millisecond time scale. As demonstrated here, reduction and alkylation provide a useful way to alter both the ligand binding and flux properties of the AcChR, and it should be possible to gain new insights into the coupling between ligand binding and the opening of the ion channel.

Added in Proof

Recently Wolosin et al. (1980) reported that bromoacetylcholine can react specifically with the same number of sites as α-neurotoxin under some conditions using reduced acetylcholine receptor from *Torpedo marmorata*. We have confirmed these results with *Torpedo californica*. The bromoacetylcholine results are consistent with our suggestion here that each reversible ligand binding site has an associated disulfide linkage.

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