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Articles

Ligand-Induced Variations in the Reactivity of Thio Groups of the α-Subunit of the Acetylcholine Receptor from *Torpedo californica*†

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ABSTRACT: We have studied alkylation of the acetylcholine receptor by *N*-[^{3}H]ethylmaleimide ([^{3}H]NEM) under various conditions. The radiolabeled preparations were submitted to sodium dodecyl sulfate–polyacrylamide gel electrophoresis to separate the receptor complex into subunits, and the incorporation of ^{3}H into each type of chain was determined. We found the following: (i) When cysteines of native receptor in intact membranes were reacted with [^{3}H]NEM, only the β-subunit was labeled; the extent of alkylation did not change significantly if cholinergic effectors were present during this reaction. (ii) When the disulfide bonds of the receptor were reduced with dithiothreitol (DTT), the α- and β-chains were labeled with [^{3}H]NEM. The presence of receptor agonists and competitive antagonists during alkylation significantly altered the labeling patterns. Gallamine and hexamethonium markedly enhanced, while carbamylcholine and decamethonium markedly lessened, labeling of the α-subunit. Choline, *d*-tubocurarine, and α-neurotoxin induced small, but significant decreases in alkylation of the α-subunit, while procaine had no effect. (iii) When the same ligands were present during the reduction step, subsequent labeling with [^{3}H]NEM produced patterns similar to those described in (ii). We also investigated the effects of gallamine and hexamethonium on reduction of the disulfide bond located near the acetylcholine binding site by using the affinity alkylating reagent (bromoacetyl)choline (BAC). Gallamine (0.1 mM) was able to increase the rate of reduction of this particular disulfide bond 3-fold in comparison to the control. In these experiments, alkylation by BAC blocked 50% of the toxin binding sites. Hexamethonium (1 mM) had a similar effect. The results suggest that the effector-induced changes in reactivity of thio groups arise from stabilization of different states of the receptor by the ligands tested. These changes are similar both in reduced and in native receptor.

The nicotinic acetylcholine receptor (AChR)[1] is an integral postsynaptic membrane protein of the vertebrate neuromuscular junction and electric tissue of *Torpedo*, *Narcine*, and *Electrophorus*. Upon binding of agonists such as acetylcholine, the receptor mediates ion flux through a cation conductive channel.

The AChR isolated from *Torpedo californica* is composed of four different polypeptide chains with apparent molecular weights of 39 000 (α), 48 000 (β), 58 000 (γ), and 64 000 (δ) (Weill et al., 1974). In the receptor complex, these subunits are present with a stoichiometry of $\alpha_2\beta\gamma\delta$ (Reynolds & Karlin, 1978; Lindstrom et al., 1979; Raftery et al., 1980). The four chains show considerable sequence homology (Noda et al., 1982; Claudio et al., 1982; Noda et al., 1983a,b).

Chemical reduction of the acetylcholine receptor changes a number of its properties. There is an increase in the response to decamethonium in eel electroplax (Karlin, 1969; Podleski et al., 1969). Hexamethonium, a competitive antagonist, is converted into an activator in eel electroplax (Karlin & Winnik, 1968) and chicken muscle (Rang & Ritter, 1971) but remains an antagonist with *Torpedo* membranes (Walker et al., 1981). The response to monoquaternary ammonium agonists is decreased (Karlin & Bartels, 1966), and the Hill coefficient of the response to carbamylcholine is decreased

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[1] Abbreviations: AChR, nicotinic acetylcholine receptor; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; MBTA, [4-(*N*-maleimido)-benzyl]trimethylammonium iodide; BAC, (bromoacetyl)choline; [^{3}H]-BAC, (bromoacetyl)[*methyl*-^{3}H]choline; MOPS, 4-morpholinepropanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; α-toxin, α-neurotoxin 3 of *Naja naja siamensis* venom; [^{3}H]toxin, [^{3}H]methyl-α-neurotoxin 3; $NaDodSO_4$, sodium dodecyl sulfate.

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from 2 to 1 in reduced electroplax (Karlin, 1967). Treatment with dithiothreitol (DTT) causes a decrease in the mean open time and conductance of the AChR channel (Landau & Ben Haim, 1974; Ben Haim et al., 1975; Terrar, 1978; Cox et al., 1979a,b).

Alkylation of reduced receptor-rich membranes from *T. californica* (Walker et al., 1981; Blanchard et al., 1982) and of reduced eel electroplax (Karlin & Bartels, 1966) with *N*-ethylmaleimide (NEM) completely inhibits receptor function, possibly by interfering sterically with the binding of agonists or by reacting directly with a sulfhydryl in the ion channel. In addition, the existence of a free sulfhydryl located in a hydrophobic pocket of the receptor complex and essential for receptor activity has recently been proposed (Huganir & Racker, 1982).

Because of the diverse effects of reducing and alkylating reagents on receptor function, the disulfides and sulfhydryls on the receptor have received considerable attention. After reduction of a disulfide in the vicinity of the acetylcholine binding site, the α-chain of *Torpedo* receptor can be affinity labeled with [4-(*N*-maleimido)benzyl]trimethylammonium iodide, MBTA (Weill et al., 1974), or (bromoacetyl)choline, BAC (Chang et al., 1977; Damle & Karlin, 1978; Moore & Raftery, 1979a). This disulfide can be protected from reduction when the site is occupied by agonists and to a small extent by competitive antagonists (Bregestovski et al., 1977; Damle & Karlin, 1980). Other subunits also have sulfhydryls or disulfides that have been studied. The δ-chain of the receptor is disulfide bonded to a second δ-chain to produce receptor dimer, and the β-chain has a free sulfhydryl, which upon oxidation can lead to the appearance of receptor oligomers (Hamilton et al., 1979). The accessibility of free sulfhydryls on the receptor to alkylation by NEM permits their classification into three groups (Chang & Bock, 1981): (1) those accessible in membrane-bound receptor, (2) those accessible after Triton X-100 solubilization of membranes, and (3) those accessible only after protein denaturation. On receptor dimers, these sulfhydryls have been found in a ratio of 2:12:8, respectively. The sulfhydryls accessible to NEM in intact membranes appear to be exclusively associated with the β-subunit.

In this paper, we report the effects of various cholinergic ligands on chemical modification of cysteine and cystine residues of the AChR subunits. Our results show that both agonists and antagonists can modify the reactivity of disulfide bonds and of the sulfhydryls generated by reduction of these groups. These changes, as assessed by variations in [^{3}H]NEM labeling, are restricted to the α-subunit of the AChR and may arise from stabilization of distinct receptor conformations by certain ligands.

Materials and Methods

Materials

Torpedo californica were obtained live from Pacific Biomarine Laboratories (Venice, CA). The electric organs were immediately dissected and stored in liquid nitrogen.

Carbamylcholine chloride, choline bromide, decamethonium bromide, procaine hydrochloride, *d*-tubocurarine chloride, gallamine triethiodide (lots 11F-0276 and 82F-0302), hexamethonium bromide (lots 44C-1746 and 22F-0293), neostigmine bromide, and *N*-ethylmaleimide (NEM) were from Sigma, and dithiothreitol (DTT) was from BRL. *N*-[2-^{3}H]-Ethylmaleimide ([^{3}H]NEM; 55.8 Ci/mmol or 1.1 Ci/mmol) was purchased from New England Nuclear, diluted with 1 volume of pentane, and stored at −20 °C. The higher specific activity NEM was used in all experiments except those described in Figure 3.

Methods

Purification and Tritiation of Naja naja siamensis α-Neurotoxin. α-Neurotoxin 3 (α-toxin) was purified from *Naja naja siamensis* crude venom according to Karlsson et al. (1971) and radiolabeled as described by Damle & Karlin (1978). The [^{3}H]methyl-α-neurotoxin ([^{3}H]-α-toxin) preparations used in these experiments had specific activities of 4.1 and 11 mCi/mmol.

Synthesis of (Bromoacetyl)choline (BAC). (Bromoacetyl)choline bromide (BAC) and (bromoacetyl)[*methyl*-^{3}H]choline bromide ([^{3}H]BAC) were synthesized by the procedures published by Damle et al. (1978).

Preparation and Assay of Receptor-Rich Membranes. Membranes were prepared by the procedure of Hamilton et al. (1979), with the following modifications: (1) The buffer (10 mM MOPS, 1 mM EDTA, 3 mM NaN_3, pH 7.4) used throughout the procedure contained 32% (w/w) sucrose. (2) Protease inhibitors (leupeptin, 5 μg/mL; pepstatin, 1 μg/mL; aprotinin, 5 μg/mL; phenylmethanesulfonyl fluoride, 0.1 mM; *p*-aminobenzamidine, 0.1 mM) were included in all solutions; crude membranes from the high-speed pellet were layered over step gradients containing 10 mL of 34% (w/w) sucrose, 10 mL of 36% (w/w) sucrose, and 5 mL of 39% (w/w) sucrose and centrifuged in a SW28 rotor for 15 h at 28 000 rpm. The receptor-rich membranes were isolated in two discrete bands, one on top of the 36% layer and one on top of the 39% layer. After resuspension in buffer I (50 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, and 3 mM NaN_3, pH 7.0), these preparations were stored in liquid nitrogen, at a protein concentration of 2–4 mg/mL and specific activities of 1.2–1.9 nmol of toxin binding sites/mg of protein. The [^{3}H]-α-toxin binding assay was carried out as described by Damle & Karlin (1978). Protein concentrations were estimated by the Lowry method (Lowry et al., 1951), with bovine serum albumin as standard.

Alkaline Treatment of Membranes. Membranes were alkaline extracted (Neubig et al., 1979) by dilution with 6–10 volumes of NaOH, pH 11.0. The pH was adjusted to 11, and the suspension was stirred gently for 1 h at 23 °C. After centrifugation in a 70 TI rotor for 30 min at 30 000 rpm, the pellets were resuspended in buffer I and frozen in liquid nitrogen. The [^{3}H]-α-toxin binding activities of the base-treated membranes used in the experiments with [^{3}H]NEM varied from 2.0 to 3.5 nmol of toxin binding sites/mg of protein.

[^{3}H]NEM Labeling. See figure legends.

Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed according to Laemmli (1970), with 10% resolving gels and 3% stacking gels. Just before electrophoresis, samples in $NaDodSO_4$–sample buffer [2% (w/v) $NaDodSO_4$, 0.0624 M Tris-HCl, 10% glycerol, and 0.001% (w/v) bromophenol blue, pH 6.8] were reduced with 5 mM DTT for 1 h at 50 °C, followed by alkylation with 20 mM NEM for 15 min at room temperature. Gels were stained and fixed with 0.025% (w/v) Coomassie Brilliant Blue R-250 in 2-propanol–acetic acid–water (25:10:65), destained in 10% acetic acid, and photographed. The bands corresponding to the receptor subunits were identified by comparison to a purified receptor standard electrophoresed in the same gel. Apparently molecular weights were estimated relative to standards (Bio-Rad high molecular weight calibration kit). The radiolabeled bands were localized by fluorography: gels were treated with Enhance (New England Nuclear), vacuum dried, and exposed to Kodak X-Omat AR-5 film for 24 h at

–70 °C. Quantitative results were obtained by excising the receptor bands (as well as nonreceptor bands when these were labeled) from the gels; these gel fragments were digested with 0.6 mL of NCS tissue solubilizer (Amersham) for 24 h at 50 °C and counted in 10 mL of toluene-based scintillant.

Affinity Alkylation with BAC. The effects of ligands on reduction of the disulfide bond located near the acetylcholine binding site were determined by a modification of a procedure described by Damle & Karlin (1980). Membranes (100 pmol of toxin binding sites in 40 μL of buffer I) were mixed with 10 μL of ligand stock solution in buffer I (or 10 μL of buffer I, in the control). After 30 min at room temperature, 50 μL of 0.4 mM DTT in buffer II (20 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, 0.02% NaN_3, pH 8.3) was added, and reduction was allowed to proceed for the times specified under Results. The reaction was quenched by dilution with 100 μL of ice-cold buffer I, immediately followed by centrifugation in a Beckman Airfuge at 197000*g* for 2 min. The pellets were resuspended in 200 μL of ice-cold buffer I and centrifuged again. Final resuspension of the membranes was in 2 mL of buffer I containing 10 μM neostigmine bromide. This buffer was used throughout the alkylation procedure. Aliquots (containing 1.5–2 pmol of toxin binding sites) were taken from each sample; of which, half was treated with excess unlabeled α-toxin, while buffer was added to the other half. After 30 min at room temperature, duplicate mixtures from each set (treated or not with α-toxin) were mixed with a BAC solution in buffer (final concentration was 1 μM) or with buffer alone. Alkylation was allowed to proceed for 20 min and was stopped by the addition of NEM to a concentration of 2.5 mM. In experiments with unlabeled BAC, the number of toxin binding sites in all aliquots was determined in a final step with [^{3}H]-α-toxin by the method described by Damle & Karlin (1978). When alkylation was performed with [^{3}H]BAC, the isolation and quantitation of radiolabeled receptor was carried out as described by Damle et al. (1978), while the concentration of toxin binding sites in each sample was determined with [^{3}H]-α-toxin in a parallel experiment.

Results

[^{3}H]NEM Labeling of Receptor-Rich Membranes. (A) Unreduced Membranes. When alkaline-extracted membranes were reacted with [^{3}H]NEM and the labeled polypeptides were characterized by $NaDodSO_4$–polyacrylamide gel electrophoresis and fluorography, three major radioactive bands were observed (Figure 1, lane 1). The high molecular weight band (95 000) is a nonreceptor protein present in variable amounts in alkaline-extracted membrane (Elliott et al., 1980; Lindstrom et al., 1980; Conti-Tronconi et al., 1982).

At $M_r \simeq 50\,000$, a doublet was seen; the upper band comigrated with the β-subunit of the acetylcholine receptor. The other polypeptide is probably a degradation product of the δ-chain (Wennogle et al., 1981); proteolysis of membrane-bound receptor has been reported to occur at increased rates after alkaline extraction (Verdenhalven et al., 1982). No significant incorporation of ^{3}H occurred into the α-, γ-, or δ-chains of the receptor. Incubation of these membranes with saturating concentrations of cholinergic ligands before addition of [^{3}H]NEM did not alter the labeling patterns (Figure 1, lanes 2–5).

The amount of radiolabel in individual bands was determined by cutting out the bands from the gels and counting the digested fragments by liquid scintillation (see Methods). No changes in ^{3}H incorporation into the β-subunit were observed in three independent experiments with different membrane preparations. When nonalkaline-extracted membranes were used, many peripheral proteins were labeled, the most prominent of these being the 43 000-dalton protein (Sobel et al., 1978). None of the ligands tested changed the amount of radioactivity associated with these bands (data not shown), or with the 95 000-dalton band. This was also true in the experiments described below.

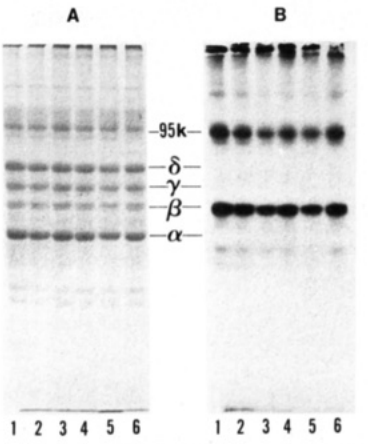

FIGURE 1: Incorporation of [^{3}H]NEM into polypeptides of unreduced membranes alkylated in the presence of ligands. Membranes (100 μg) in 90 μL of buffer I were incubated with 10 μL of freshly prepared ligand stock solutions in buffer I or 10 μL of buffer I in the control. After 30 min at room temperature, the samples were transferred to ice, and 5 μL of [^{3}H]NEM was added. Alkylation was stopped after 5 min by dilution with 100 μL of 20 mM NEM. The mixtures were centrifuged in a Beckman airfuge at 197000*g* for 2 min, the supernatants were discarded, and the pellets were resuspended in 200 μL of 10 mM NEM. The centrifugation was repeated, and the pellets were solubilized in 50 μL of $NaDodSO_4$–sample buffer. (A) $NaDodSO_4$–polyacrylamide gel stained with Coomassie Brilliant Blue; (B) fluorogram (lanes) (1) control (no ligand added), (2) 0.1 mM *d*-tubocurarine, (3) 1 mM hexamethonium, (4) 0.1 mM gallamine, (5) 50 μM α-toxin, (6) 0.1 mM carbamylcholine (concentrations of ligands during alkylation with [^{3}H]NEM).

(B) Reduced Membranes. Reduction by dithiothreitol increases the sulfhydryl content of all the acetylcholine receptor subunits, as assessed by reaction with [^{3}H]NEM (Hamilton et al., 1979). The following experiments were designed to test the effects of cholinergic ligands on the alkylation of reduced membranes by [^{3}H]NEM. Alkaline-extracted membranes were reduced with 1 mM DTT for 30 min after which the excess DTT was removed by repeated centrifugations. The washed membranes (0.6–1.2 μM sites) were incubated with ligands (or buffer, as a control), and after 30 min, [^{3}H]NEM was added to a final concentration of 0.4 μM and allowed to react for 5 min at 4 °C. The results of a typical experiment are shown in Figure 2. In the control (lane 8), a band of $M_r \simeq 40\,000$ corresponding to the α-subunit of the receptor became heavily labeled. Increases (as compared to unreduced membranes) in ^{3}H incorporation into the β-, γ-, and δ-chains were also observed, as well as in the 95 000-dalton band.

The radioactivity associated with the α-chain changed markedly when cholinergic ligands were added to the reaction mixture, whereas labeling of the other bands remained unaltered (Figure 2). Two nicotinic antagonists, gallamine and hexamethonium, increased alkylation of the α-subunit by [^{3}H]NEM, in contrast with the action of agonists (carbamylcholine, decamethonium, choline) and two other antagonists (*d*-tubocurarine, α-toxin), which decreased the labeling of the α-chain. Procaine did not significantly affect the labeling patterns even at a relatively high concentration (5 mM).

When membranes were reduced for 5 min with 0.2 mM DTT (pH 8.0, room temperature), the labeling patterns were similar to those presented in Figure 2. The labeling of the

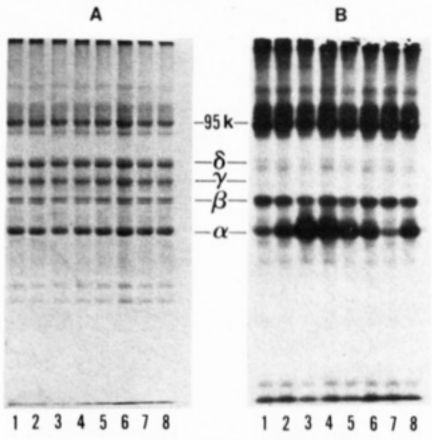

FIGURE 2: Incorporation of [^{3}H]NEM into polypeptides of reduced membranes alkylated in the presence of ligands. Membranes (100 μg) in 50 μL of buffer I were mixed with 50 μL of 2 mM DTT in buffer II (final pH 8.0) and incubated for 30 min at room temperature. Reduction was quenched with 100 μL of ice-cold buffer I, and centrifugation was in an airfuge for 2 min at 197000g. The pellets were washed twice by resuspension in 200 μL of ice-cold buffer I and centrifugation. Reduced membranes were resuspended in 90 μL of buffer I; incubation with ligands and [^{3}H]NEM labeling were performed as described in the legend of Figure 1. (A) $NaDodSO_4$–polyacrylamide gel stained with Coomassie Brilliant Blue; (B) fluorogram; (lanes) (1) 0.1 mM carbamylcholine, (2) 0.1 mM d-tubocurarine, (3) 1 mM gallamine, (4) 0.1 mM hexamethonium, (5) 5 mM procaine, (6) 50 μM α-toxin, (7) 0.1 mM decamethonium, (8) control (concentrations during alkylation with [^{3}H]NEM).

Table I: Effect of Cholinergic Ligands on Alkylation of the α-Subunit of Reduced Receptor[a]

ligand	(mM)[b]	relative labeling of α-subunit[c] 1	2	3
carbamylcholine	0.1	0.45	0.27	0.32
decamethonium	1.0	0.33		
	0.1		0.34	
choline	1.0	0.51	0.52	0.43
d-tubocurarine	0.1	0.61	0.69	0.76
α-toxin	0.05	0.85	0.79	0.70
gallamine	0.1	2.56	2.61	
	0.01		2.10	
hexamethonium	1.0	1.51	1.70	
procaine	1.0	1.24	1.15	
	5.0			0.86

[a] The values were obtained with three different membrane preparations; the experiments were performed as described in the legend of Figure 2, except for the reduction step, where membranes were allowed to react with 0.2 mM DTT for 5 min, at room temperature (pH 8.0). [b] Concentration of ligand during alkylation by [^{3}H]NEM. [c] Ratio between cpm in the α-subunit of samples in which ligand was present during alkylation and cpm in the α-subunit of the control sample.

receptor subunits was quantitated as described previously, and the results (for the α-subunit) of three such experiments are presented in Table I. The data are reported as relative labeling, that is to say, the ratio between cpm in the presence of ligand and cpm in the control. This form of presentation was chosen to normalize for different specific activities of various membrane preparations and [^{3}H]NEM batches. In most of the experiments to be described, the concentration of [^{3}H]NEM was 0.4 μM and was lower than the concentration of membrane toxin sites (0.6–1.2 μM). Under these conditions, approximately 25–30% of the radiolabel was incorporated into proteins. Total incorporation of radiolabel requires about 20 min and reaches the same equilibrium level whether or not gallamine is present (data not shown).

The values in Table I show that the increases and decreases

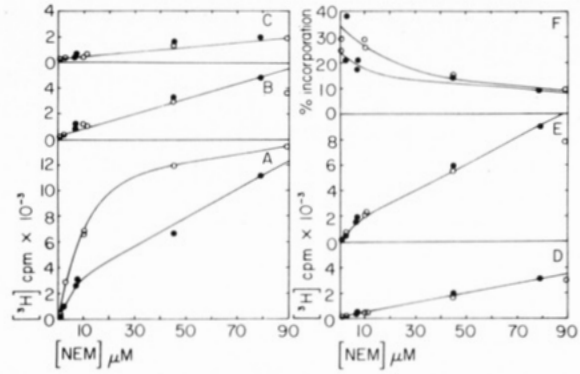

FIGURE 3: Effect of NEM concentration on total incorporation and incorporation into individual polypeptides in the presence and absence of gallamine. Membranes were reduced and washed as described in Figure 2. Reduced membranes were resuspended in 90 μL of buffer I and incubated with gallamine at 0.1 mM as described, and 5 μL of [^{3}H]NEM (1.1 mCi/mol) in pentane was added. As pentane solutions are difficult to add accurately, the concentration of NEM in each sample was determined by counting aliquots of each sample. Following a 5-min incubation at 4 °C, the membranes were washed by pelleting in a Beckman airfuge and prepared by resuspending in $NaDodSO_4$–sample buffer. Aliquots were counted to determine the amount of radiolabel incorporated. Following $NaDodSO_4$ gel electrophoresis, the Coomassie Brilliant Blue R-250 stained bands were excised, digested with NCS, and counted as described under Methods. (A) Incorporation into α-, (B) β-, (C) γ-, and (D) δ-subunit; (E) incorporation into ν or 43K; (F) % total incorporation. Open circles correspond to labeling in the presence of gallamine while closed circles represent labeling in its absence.

in radioactivity associated with the α-subunit are reproducible and significant. The antagonists gallamine and hexamethonium were able to increase ^{3}H incorporation up to 2.6- and 1.6-fold, respectively. Of the ligands able to protect the α-chain against alkylation, carbamyl choline and decamethonium were the most potent, displaying similar efficacies (70% protection), while choline had a smaller effect. A moderate protection (20–30%) was afforded by d-tubocurarine and α-toxin.

The effect of varying the [^{3}H]NEM to sites ratio on total and subunit incorporation in the presence and absence of 0.1 mM gallamine is shown in Figure 3. At all concentrations of NEM tested, the total incorporation was less than the NEM added (Figure 3F), and gallamine enhanced the labeling of the α-subunit (Figure 3A) with little or no effect on the labeling of β- (Figure 3B), γ- (Figure 3C), or δ-subunit (Figure 3D) or the 43K protein (Figure 3E), which is often seen to copurify with receptor-rich membrane (Sobel et al., 1978; Neubig et al., 1979). These results suggest that the incorporation of [^{3}H]NEM under the experimental conditions described in this paper relates to the relative reactivities of the sulfhydryl groups.

The low amounts of ^{3}H incorporated into the γ- and δ-subunits did not vary significantly. Analysis of data on the β-subunit was hindered by the proximity of another polypeptide, as these bands could not be excised separately from the gels. However, any major changes in labeling would certainly be evident in the fluorograms, and that was not the case.

Reduced and Solubilized Membranes. AChR-rich membrane preparations consist of sealed right-side out vesicles and open membrane fragments. Under conditions where the membrane permeability is altered, groups present at the internal surface of the vesicles are more accessible to externally added reagents. Thus, the effects of gallamine and hexamethonium might be the result of perturbation of the lipid bilayer that surrounds the receptor, exposing the cytoplasmic

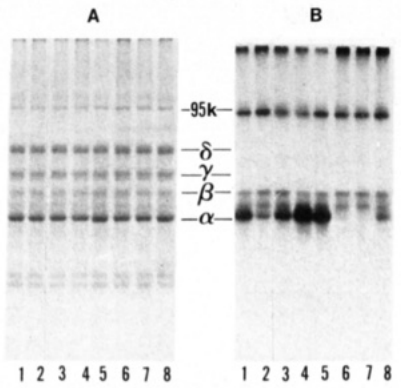

FIGURE 4: Incorporation of [^{3}H]NEM into polypeptides of membranes in the presence of cholinergic ligands. Membrane suspensions (100 μg in 40 μL of buffer I) were mixed with 10 μL of ligand solution in buffer I or with 10 μL of buffer I. After 30 min at room temperature, reduction was started by adding 50 μL of 0.4 mM DTT in buffer II. The reaction was quenched after 5 min by addition of 100 μL of ice-cold buffer I and by centrifugation in an airfuge for 2 min at 197000g. Pellets were washed twice by resuspension in 200 μL of ice-cold buffer I and centrifugation as above. Reduced membranes were resuspended in 100 μL of buffer I and alkylated with 5 μL of [^{3}H]NEM for 10 min on ice. Reaction was stopped by dilution with 100 μL of 20 mM NEM, and the labeled membranes were washed and dissolved in sample buffer as described in the legend of Figure 1. (A) NaDodSO$_4$–polyacrylamide gel stained with Coomassie Brilliant Blue; (B) fluorogram; (lanes) (1) 5 mM procaine, (2) 0.1 mM *d*-tubocurarine, (3) control, (4) 0.1 mM gallamine, (5) 1 mM hexamethonium, (6) 0.1 mM decamethonium, (7) 0.1 mM carbamylcholine, and (8) 1 mM choline (concentrations during the reduction stage).

domain of the α-subunit to [^{3}H]NEM. We tested this possibility by performing experiments with reduced membranes solubilized with 0.7% Triton X-100 prior to ligand and [^{3}H]NEM addition. In this solubilized preparation, gallamine and hexamethonium had effects similar to those observed with membrane-bound receptor, increasing the labeling of the α-chain 2.7- and 2.1-fold, respectively, in comparison with control (data not shown). As expected (Raftery et al., 1975; Damle & Karlin, 1980), solubilization interfered markedly with the protective action of the agonists decamethonium (see labeling as control) and carbamylcholine (21% of protection), while *d*-tubocurarine was still able to decrease the radiolabeling of the α-subunit to 50% of the control value. When the concentration of carbamylcholine was raised to 1 mM, this ligand was again able to protect the α-subunit of solubilized receptor against [^{3}H]NEM labeling, in agreement with results from Damle & Karlin (1980).

Membranes Reduced in the Presence of Ligands. The results above indicate that gallamine and hexamethonium enhance the reactivity of sulfhydryls in reduced receptor. This raised the possibility that the reactivity of the disulfide bonds on the α-subunit of native receptor might also be increased in the presence of these antagonists. In order to test this hypothesis, membranes were incubated with ligands, reduced for 5 min with 0.2 mM DTT (pH 8, room temperature), and washed before reaction with [^{3}H]NEM. The labeling patterns observed[2] (Figure 4) were similar to those shown in Figure 2. Total incorporation of [^{3}H]NEM ranged from 20 to 35% of the added label. Thus, gallamine and hexamethonium increased the reactivity of both sulfhydryls on reduced receptor and disulfide bonds of unreduced receptor.

Quantitation of these experiments generated the values shown in Table II, which are similar to those in Table I, again

[2] In these experiments, the α-toxin was not tested, as it has disulfide bonds that would also react with dithiothreitol.

Table II: Effect of Cholinergic Ligands on Reduction of the α-Subunit of the Acetylcholine Receptor[a]

ligand	concn (mM)[b]	relative labeling of α-subunit[c] 1	2	3
carbamylcholine	0.1	0.24	0.24	0.28
	0.01	0.27		
	0.001	0.90		
decamethonium	1.0	0.31	0.26	
	0.1			0.38
choline	1.0	0.51	0.62	0.60
d-tubocurarine	0.1	0.55	0.62	0.61
gallamine	0.1	2.09	2.07	2.10
hexamethonium	1.0	1.75	1.26	1.63
procaine	1.0	1.17	1.03	
	5.0			1.03

[a] The values were obtained with three different membrane preparations; the experiments were performed as described in the legend of Figure 4. [b] Concentration of ligand during reduction by DTT. [c] Ratio between cpm in the α-subunit of samples where ligands were present during reduction and cpm in the α-subunit of the control sample.

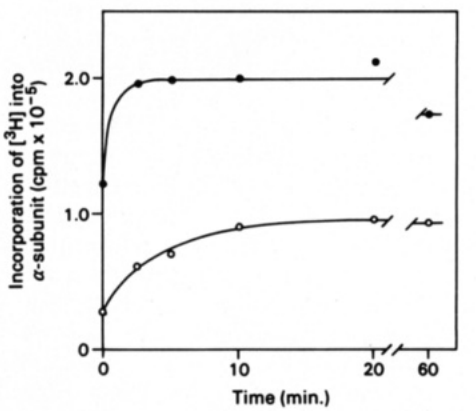

FIGURE 5: Effect of gallamine on the time course of reduction of the disulfide bonds in the α-subunit. Membranes were incubated with 0.1 mM gallamine for 30 min and reduced with 0.2 mM DTT as in Figure 3, except for the variable reaction times. Washing and [^{3}H]NEM labeling were as in Figure 3. The abscissa shows the time elapsed between addition of DTT and dilution with ice-cold buffer I. Closed symbols (●), 0.1 mM gallamine during reduction; open symbols (○), control.

supporting the conclusion that the differences in incorporation reflect differences in reactivity of sulfhydryls. This also suggests that the same basic structural changes that occur upon ligand binding to native or reduced receptor are responsible for the difference in thio group reactivity.

Time Course of Reduction. The incorporation of radioactivity into the α-subunit, when reduction was performed in the presence and absence of gallamine, was investigated as a function of time of exposure to 0.2 mM DTT (Figure 5). The incubation with [^{3}H]NEM was again for 5 min at 4 °C. When 0.1 mM gallamine was present, the α-chain was reduced at a higher rate than that of the control. The labeling in the presence of gallamine reached a final level that was twice that of the control. This suggests that binding of gallamine exposes groups that would not be reduced in the control even after prolonged incubation with 0.2 mM DTT.

Note that the values of ^{3}H incorporation into the α-chain at zero time indicate that the procedure used here to stop the reduction is not instantaneously effective; thus, the times plotted in the abscissa of Figure 5 are only approximate.

BAC Labeling of Receptor-Rich Membranes. The extent of reduction of a disulfide located near the acetylcholine binding site in one of the α-subunits of *Torpedo* receptor can

Table III: Effect of Cholinergic Ligands on Reduction of the ACh Binding Site Disulfide[a]

ligand	concn (mM)[b]	% of total toxin sites labeled by BAC[c]
carbamylcholine	0.1	3.4, 4.6
d-tubocurarine	0.1	10.7, 10.1[c]
hexamethonium	0.1	35.4[c]
	1	44.0, 47.7
gallamine	1	49.4 ± 6.0[d]
	0.1	44.9
	0.01	37.5
	0.001	30.9
procaine	5.0	33.2, 26.1
none		29.0 ± 6.2[d]

[a] Experiments performed as described under Methods. [b] Concentration of ligand during reduction. [c] Values obtained by the direct method (sites with bound [^{3}H]BAC per total toxin sites). All other results are from experiments where blockage of [^{3}H]-α-toxin binding by bound BAC was determined in duplicate aliquots. [d] Values are averages from seven experiments, with the corresponding standard deviations.

be determined through the use of affinity alkylating agents like BAC (Damle et al., 1978; Moore & Raftery, 1979) or MBTA (Weill et al., 1974). Damle & Karlin (1980) reported that agonists were able to inhibit the reaction of DTT with that particular, disulfide, resulting in decreased MBTA labeling; antagonists were found not to affect reduction. However, those experiments could only determine the number of disulfides *not* reduced by DTT. Increased reactivity of disulfides would not be detected by a protocol where NEM is used to stop an initial reduction step, since all accessible sulfhydryls are alkylated before the final reduction and assay with MBTA. In our experiments, membranes were reduced just once, and reaction with DTT was stopped by dilution with ice-cold buffer, followed immediately by centrifugation in an airfuge.

Table III shows the levels of BAC labeling [using 1 μM (bromoacetyl)choline] observed when reduction was performed in the presence of nicotinic effectors. Note that in these experiments reduction was performed for just 5 min with 0.2 mM DTT, in order to achieve a relatively high extent of protection by ligands such as carbamylcholine and *d*-tubocurarine. Thus, the level of BAC labeling in the control were always less than 50% of the total toxin sites. The results are consistent with those obtained by [^{3}H]NEM labeling, indicating that at least part of the effects previously observed in the α-subunit arise from protection or exposure of the disulfide bond near the acetylcholine binding site. Carbamylcholine and *d*-tubocurarine decreased reduction of this particular disulfide bond, whereas gallamine and hexamethonium had the opposite effect. Procaine did not significantly affect the reaction. The effects of gallamine and hexamethonium were found to depend on their concentration during reduction. No BAC labeling was observed if incubation of membranes with gallamine was not followed by reduction. This observation, taken together with the experiment seen in Figure 1 (lane 4), implies that gallamine by itself does not interfere with the redox state of the receptor disulfides.

The time course of reduction of this disulfide bond was determined in the absence and in the presence of gallamine (Figure 6). As seen in this figure, gallamine apparently increases the rate of reaction of the active site disulfide with DTT. The rates calculated from these curves were 1300 L·mol^{-1}·min^{-1} with 0.1 mM gallamine and 420 mL·mol^{-1}·min^{-1} for control. These values are approximate, due to the uncertainty associated with the reduction times. Experiments

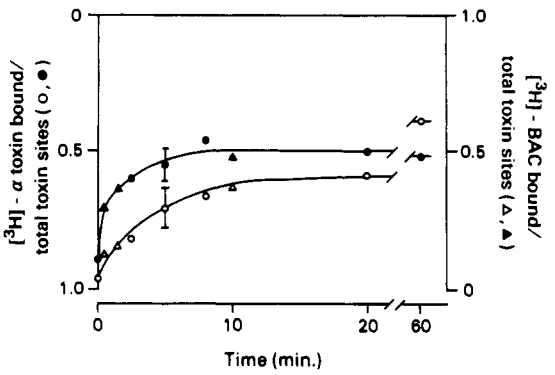

FIGURE 6: Effect of gallamine on the time course of reduction of the acetylcholine binding site disulfide. Membranes were incubated with 0.1 mM gallamine and reduced with 0.2 mM DTT as in Figure 5. Labeling with BAC was performed as under Methods. Data were obtained in five independent experiments, with two different membrane preparations. (Closed symbols) Reduction in the presence of gallamine; (open symbols) control, no ligand added. Experiments where [^{3}H]BAC was used are marked by (▲) and (△). All other points refer to measurements of [^{3}H]α-toxin binding in the presence or absence of BAC. The final BAC concentration was 1 μM for all experiments.

where BAC labeling was measured directly ([^{3}H]BAC bound) and indirectly (blockage of [^{3}H]-α-toxin binding by bound BAC) were in good agreement. Hexamethonium at a concentration of 1 mM was also able to increase the reaction with DTT (data not shown).

A comparison of Figure 5 and 6 shows that NEM labeling of the α-subunit after reduction in the presence of gallamine reaches a level equal to twice that of the control, whereas BAC labeling reaches a level approximately equal to that of the control. Only half of the toxin sites were labeled by BAC in both the control and gallamine-treated samples. Under our experimental conditions, there was no evidence that gallamine or hexamethonium was inducing affinity labeling of the second agonist binding site, which is known to react at much higher BAC concentrations, after extensive reduction with DTT (Wolosin et al., 1980). However, this site, when reduction is done in the presence of gallamine, could react with [^{3}H]NEM, a less specific reagent, leading to the doubling of the equilibrium level seen in Figure 5. Alternatively, the increased labeling could be due to the reduction of a disulfide not located in the active site region.

Discussion

The complete sequences for the precursors of the subunits of the nicotinic acetylcholine receptor of *T. californica* have been recently published (Noda et al., 1982, 1983a,b; Claudio et al., 1982), revealing a high degree of homology among the four polypeptides. The α-, β-, γ-, and δ-subunits contain seven, five, eight, and six cysteine residues, respectively. The arrangement of the disulfides formed from some of these cysteines is not known. Three cysteine residues are conserved in all four chains: one is located in a hydrophobic segment that may traverse the membrane, while the other two were proposed (Noda et al., 1982, 1983a,b) to form the disulfide bridge near the acetylcholine binding site on the α-subunit. The observed amino acid sequence homologies suggest that all subunits span the membrane in a similar fashion.

Our results show that not only agonists but also antagonists are able to affect the reactivity of cysteine and cystine residues of the acetylcholine receptor; furthermore, significant changes in [^{3}H]NEM labeling are restricted to the α-subunit. The variations in radioactivity content of the β-, γ-, and δ-subunits were found to lie within the limits of experimental error. This does not necessarily imply that only the α-chain is affected

by ligand binding. Nevertheless, if there are actual changes in reactivity of thio groups located in the other three chains, they are not detected by the technique described here. In addition, the labeling of nonreceptor bands was not changed by any of the ligands tested, indicating that the ligand effects observed are specific for the AChR.

The labeling experiments show that the incubation of membranes with specific ligands can lead to increases or decreases in the reactivity of disulfide bonds of unreduced receptor or sulfhydryl groups of reduced receptor, in comparison with the control. Moreover, each ligand tested alters reduction of native receptor and alkylation of reduced receptor essentially in the same way, suggesting that the nature of the ligand–α-subunit interaction is similar in native and reduced receptor. This is true even in the case of hexamethonium and decamethonium, confirming reports (Walker et al., 1981) that reduction of *Torpedo* actylcholine receptor does not lead to the altered response to bisquaternary compounds observed with eel electroplax (Karlin & Winnik, 1968) and chicken muscle (Rang & Ritter, 1971).

Ligand-induced protection of the disulfide located near the acetylcholine binding site against reduction by DTT has been reported, and the extent of protection was shown to be correlated with the potency of the ligand as an agonist (Bregestovski et al., 1977; Damle & Karlin, 1980). The decreases in reactivity observed were proposed, in both cases, to be the result of a conformational transition that occurs upon formation of the agonist–receptor complex. This conformational change was shown to be distinct from desensitization. The data reported here fully support this conclusion, as we do observe protection of the α-subunit disulfide bonds and sulfhydryl groups by carbamylcholine and decamethonium and, to a lesser extent, by choline. When reduced (1 mM DTT, 30 min) membranes were exposed to low concentrations of carbamylcholine ([carbamylcholine] = [toxin sites] = 2.5 μM) for 10 s at 4 °C, followed by alkylation with [^{3}H]NEM for 10 s, the labeling of the α-subunit was already decreased to 75% of the control (data not shown). As treatment with DTT decreases the rate of desensitization (Walker et al., 1981), this result suggests that the protective effect observed here is not entirely due to a slow stabilization of the desensitized state of the acetylcholine receptor by agonists.

Our results with gallamine and hexamethonium seem to be at variance with previous studies (Bregestovski et al., 1977; Damle & Karlin, 1980), which report that antagonists either do not affect the reactivity of disulfide bonds or decrease it by a small factor. However, in one case (Bregestovski et al., 1977), the experiments did not include gallamine and hexamethonium, and in the other (Damle & Karlin, 1980) the protocols followed were designed to exclusively detect protective effects. Under our experimental conditions, the presence of gallamine or hexamethonium during reduction or alkylation leads to considerable increases in the rates of both reactions. The maximum labeling by NEM when gallamine is present during reduction reaches a value equal to twice that of the control, suggesting the gallamine can expose disulfides to reduction that are not reduced in its absence. Wolosin et al. (1980) has demonstrated labeling of the second agonist binding site at high concentrations of BAC. We have recently confirmed this observation (S. L. Hamilton and D. R. Pratt, unpublished results). The additional disulfides reduced in the presence of gallamine do not give rise to BAC labeling of this second site, as BAC labeling reaches the same level whether membranes are reduced in the presence or absence of gallamine. Therefore, it appears that the lack of reactivity of the second site is not due to inaccessibility of the disulfide to reduction but rather to the inability of the sulfhydryls generated by reduction to react with BAC. The observed increases in reactivity could be explained by two basic alternatives: either disruptive interactions of gallamine or hexamethonium with the membrane lipids lead indirectly to exposure of groups, or the effect of these ligands is due to binding to the receptor protein itself. Experiments with solubilized membranes rule out the possibility that gallamine or hexamethonium are merely increasing the access of reagents to groups exposed on the cytoplasmic side of membrane vesicles through destabilization of the lipid matrix. Moreover, procaine a local anesthetic known to interact strongly with membrane phospholipids (Boulanger et al., 1979; Coster et al., 1981), does not affect reduction or alkylation under our experimental conditions.

Thus, we conclude that the effects of two of the cholinergic antagonists tested, gallamine and hexamethonium, are the result of reactivity changes on the respective antagonist–receptor complexes. Binding of these compounds to the AChR leads to a state that differs markedly (in terms of thio group reactivity) from the one evoked by agonist binding and from the resting, unliganded state, which is presumably present in the control incubations.

Gallamine has been shown to be both a competitive antagonist and an open channel blocker (Colquhoun & Sheridan, 1981) of the nicotinic acetylcholine receptor and may, therefore, have more than one type of binding site. Gallamine affects the binding of ligands to the muscarinic acetylcholine receptor (Riker & Wiscoe, 1971; Rathbun & Hamilton, 1970; Clark & Mitchelson, 1976; Stockton et al., 1983) and the catalytic activity of acetylcholinesterase (Changeux, 1966; Tomlenson et al., 1980). These effects are apparently via the binding of gallamine at a site distinct from the agonist binding site of the muscarinic receptor or the catalytic site of acetylcholinesterase. We cannot rule out the possibility that the change in the reactivity of the α-chain of the *Torpedo* nicotinic receptor is due to the binding of gallamine at a site distinct from the acetylcholine binding site. The effect, however, is not mimiced by the local anesthetic procaine, a well-known blocker of the open channel.

Two other antagonists, *d*-tubocurarine and α-toxin, were also tested for their effects on sulfhydryl reactivity. The decreases in [^{3}H]NEM labeling resulting from preincubation of reduced receptor with a bulky antagonist like the α-neurotoxin from *Naja naja siamensis* are small, supporting the conclusion that effector-induced conformational changes, and not steric hindrance of labeling, are responsible for protective effects. On the other hand, *d*-tubocurarine was able to decrease labeling of the α-subunit by [^{3}H]NEM to about 70% of the control value and also to inhibit the reduction of the active site disulfide bond (labeling is approximately 60% of the control value). For reasons already discussed, the protection afforded is not likely to be the result of steric effects; thus in our experiments, this antagonist seems to have a paradoxical "agonist-like" action. This might actually be the case, as *d*-tubocurarine has been shown to behave like a weak nicotinic agonist in embryonic rat myotubes (Ziskind & Dennis, 1978; Trautmann, 1982; Trautmann, 1983) and in adult human muscle cells (Jackson et al., 1982). This interpretation is tentative, as at present there is no direct evidence for activation of the ion channel of the AChR from *Torpedo* receptors by *d*-tubocurarine.

In conclusion, the labeling procedure reported here characterizes three different states of native and reduced AChR, in terms of reactivity of some disulfide bonds and sulfhydryl

groups in the α-subunit. In the absence of ligands, these groups are moderately accessible to DTT and NEM. Occupation of binding sites in the α-chain by agonists and possibly *d*-tubocurarine induces a conformational change that shields certain domains of this polypeptide from reagents. The increases in reactivity observed in the presence of gallamine and hexamethonium may also be due to another conformational transition, which leads to an enhancement in thio group reactivity.

Registry No. NEM, 128-53-0; BAC bromide, 22004-27-9; acetylcholine, 51-84-3; gallamine triethiodide, 65-29-2; hexamethonium bromide, 55-97-0; carbamylcholine chloride, 51-83-2; decamethonium bromide, 541-22-0; choline bromide, 1927-06-6; *d*-tubocurarine chloride, 57-94-3.

References

Ben Haim, D., Dreyer, F., & Peper, K. (1975) *Pflügers Arch. 335*, 19–26.

Blanchard, S. G., Dunn, S. M. J., & Raftery, M. A. (1982) *Biochemistry 21*, 6258–6264.

Boulanger, Y., Schreier, S., & Smith, I. C. P. (1979) *Biophys. J. 25*, 10a.

Bregestovski, P. D., Iljin, V. I., Jurchenko, O. P., Verprintsev, B. N., & Vulfius, C. A. (1977) *Nature (London) 270*, 71–73.

Chang, W. H., & Bock, E. (1981) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 40*, 1558.

Chang, R. S. L., Potter, L. T., & Smith, I. C. P. (1977) *Tissue Cell 9*, 623–644.

Clark, A. L., & Mitchelson, F. (1976) *Br. J. Pharmacol. 58*, 323–331.

Claudio, T., Ballivet, M., Patrick, J., & Heinemann, S. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1111–1115.

Colquhoun, D., & Sheridan, R. E. (1981) *Proc. R. Soc. London, Ser. B 211*, 181–203.

Conti-Tronconi, B. M., Dunn, S. M. J., & Raftery, M. A. (1982) *Biochemistry 21*, 893–899.

Coster, H. G. L., James, V. J., Berthet, C., & Miller, A. (1981) *Biochim. Biophys. Acta 641*, 281–285.

Cox, R. N., Karlin, A., & Brandt, P. W. (1979a) *J. Membr. Biol. 51*, 133–144.

Cox, R. N., Kawai, M., Karlin, A., & Brandt, P. W. (1979b) *J. Membr. Biol. 51*, 145–159.

Damle, V. N., & Karlin, A. (1978) *Biochemistry 17*, 2039–2045.

Damle, V. N., & Karlin, A. (1980) *Biochemistry 19*, 3924–3932.

Damle, V. N., McLaughlin, M., & Karlin, A. (1978) *Biochem. Biophys. Res. Commun. 84*, 845–851.

Elliott, J., Blanchard, S. G., Wu, W., Miller, J., Strader, C. D., Hartig, P., Moore, H.-P., Racs, J., & Raftery, M. A. (1980) *Biochem. J. 185*, 667–677.

Hamilton, S. L., McLaughlin, M., & Karlin, A. (1979) *Biochemistry 18*, 155–163.

Huganir, R. L., & Racker, E. (1982) *J. Biol. Chem. 257*, 9372–9378.

Karlin, A. (1967) *J. Theor. Biol. 16*, 306–320.

Karlin, A. (1969) *J. Gen. Physiol. 64*, 245s–264s.

Karlin, A., & Bartels, E. (1966) *Biochim. Biophys. Acta 126*, 525–535.

Karlin, A., & Winnik, M. (1968) *Proc. Natl. Acad. Sci. U.S.A. 60*, 668–674.

Karlsson, E., Arnberg, H., & Eaker, D. (1971) *Eur. J. Biochem. 21*, 1–16.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Landau, E. M., & Ben Haim, D. (1974) *Science (Washington, D.C.) 185*, 944–946.

Lindstrom, J., Merlie, J., & Yogeeswaran, G. (1979) *Biochemistry 18*, 4454–4470.

Lindstrom, J., Anholt, R., Einarson, B., Engel, A., Osame, M., & Montal, M. (1980) *J. Biol. Chem. 17*, 8340–8350.

Moore, H.-P., & Raftery, M. A. (1979) *Biochemistry 18*, 1862–1867.

Morris, C., Jackson, M., Lecar, H., & Wong, S. (1982) *Biophys. J. 37*, 19a.

Neubig, R. R., Krodel, E. K., Boyd, N. D., & Cohen, J. B. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 690–694.

Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Furutani, Y., Hirose, T., Asai, M., Inayama, S., Miyata, T., & Numa, S. (1982) *Nature (London) 299*, 793–797.

Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Kikyotani, S., Hirose, T., Asai, M., Takashima, H., Inayama, S., Miyata, T., & Numa, S. (1983a) *Nature (London) 302*, 251–255.

Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Kikyotani, S., Furutani, Y., Hirose, T., Takashima, H., Inayama, S., Miyata, T., & Numa, S. (1983b) *Nature (London) 302*, 528–532.

Podleski, T., Meunier, J.-C., & Changeux, J.-P. (1969) *Proc. Natl. Acad. Sci. U.S.A. 63*, 1239–1256.

Raftery, M. A., Bode, J., Vandlen, R., Michaelson, D., Deutsch, J., Moody, T., Ross, M. J., & Stroud, R. M. (1975) in *Protein-Ligand Interactions* (Sund, H., & Blauer, G., Eds.) pp 328–355, de Gruyter, Berlin.

Raftery, M. A., Hunkapiller, M. W., Strader, C. D., & Hood, L. E. (1980) *Science (Washington, D.C.) 208*, 1454–1457.

Rang, H. P., & Ritter, J. M. (1971) *Mol. Pharmacol. 7*, 620–631.

Rathbun, F. J., & Hamilton, J. T. (1970) *Can. Anaesth. Soc. J. 17*, 574–590.

Reynolds, J., & Karlin, A. (1978) *Biochemistry 17*, 2035–2038.

Rieker, W. F., & Wescoe, W. C. (1951) *Ann. N.Y. Acad. Sci. 64*, 373–394.

Sobel, A., Weber, M., & Changeux, J.-P. (1977) *Eur. J. Biochem. 80*, 215–225.

Sobel, A., Heidmann, T., Hofler, V., & Chaneux, J.-P. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 510–514.

Stockton, J. N., Birdsall, N. J. M., Burgen, A. S. V., & Hulme, E. C. (1983) *Mol. Pharmacol. 23*, 551–557.

Terrar, D. A. (1978) *J. Physiol. (London) 276*, 403–417.

Tomlinson, G., Mutus, B., & McLennan, I. (1980) *Mol. Pharmacol. 18*, 33–39.

Trautmann, A. (1982) *Nature (London) 298*, 272–275.

Trautmann, A. (1983) *J. Neural Transm. Suppl. 18*, 353–361.

Vandlen, R. L., Wilson, C.-S. W., Eisenach, J. C., & Raftery, M. A. (1979) *Biochemistry 18*, 1845–1854.

Verdenhalven, J., Bandini, G., & Hucho, F. (1982) *FEBS Lett 147*, 168–170.

Walker, J. W., Lukas, R. J., & McNamee, M. G. (1981) *Biochemistry 20*, 2191–2199.

Weill, C. L., McNamee, M. G., & Karlin, A. (1974) *Biochem. Biophys. Res. Commun. 61*, 997–1003.

Wennogle, L. P., Oswald, R., Saitoh, T., & Changeux, J.-P. (1981) *Biochemistry 20*, 2492–2497.

Wolosin, J. M., Lydiatt, A., Dolly, J. O., & Barnard, E. A. (1980) *Eur. J. Biochem. 109*, 495–505.

Zuskind, L., & Dennis, M. (1978) *Nature (London) 276*, 622–623.