Structural Stabilization of Isolated Acetylcholine Receptor: Specific Interaction with Phospholipids†

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ABSTRACT: Purification of acetylcholine receptor from *Torpedo californica* by affinity chromatography is known to result in the conversion of a majority of the high affinity acetylcholine binding sites, as determined by equilibrium binding assays, to a state of lower affinity. In this report we demonstrate that specific interaction between phospholipids and acetylcholine receptor appears to exist even in 1% nonionic detergent solutions, and we find a definite relationship between the amount of endogenous phospholipid remaining in the purified receptor and its retention of high affinity acetylcholine sites. Phospholipase A_2 treatment or delipidation of the receptor resulted in the loss of 69% and over 80% of the high affinity sites, respectively. The addition of phosphatidylinositol and, to a lesser extent, phosphatidylcholine to the delipidated receptor results in a partial recovery of the high affinity state. By limiting the extent of delipidation with minimum volumes and concentrations of detergent during purification, the original high affinity sites for acetylcholine in the crude extract could be preserved. The dissociation constant associated with the high affinity sites of the H-form (13S) receptor in such preparations was $4 \pm 1 \times 10^{-9}$ M. Positive cooperativity was indicated in acetylcholine binding and was characterized by a Hill coefficient of about 2.0 for the crude receptor extract, and this coefficient decreased to 1.7 for the purified receptor, suggesting that some structural alteration is still occurring on purification. The amount of phospholipid tightly associated with this purified receptor was 20 ± 2 relative to one α-bungarotoxin site. Receptor purified under these limited delipidation conditions contained 4 to 5 sulfhydryl groups per α-bungarotoxin site. These groups comprised two classes that could be distinguished by their rate of reaction with 5,5′-dithiobis(2-nitrobenzoic acid) in the presence of sodium dodecyl sulfate. The first class displayed a pseudo-first-order rate constant $k_1 = 2.0$ min^{-1} associated with 2–3 sulfhydryls, while the second class showed a rate constant $k_2 = 0.52$ min^{-1} associated with 2 sulfhydryls. Delipidated receptor contained only 2 sulfhydryls per α-bungarotoxin site, one from each of the two classes. In the presence of these endogenous phospholipids, receptor sulfhydryl groups appear to be partially protected from intramolecular oxidation. The protection of these sulfhydryl groups presumably minimizes modification of receptor structure that leads to the irreversible loss of the high acetylcholine affinity state. These results suggest that acetylcholine receptor protein requires a highly specific hydrophobic environment, perhaps defined by certain phospholipids, in order to retain native structure.

A major objective of studies on isolated acetylcholine receptor is an understanding of the molecular mechanism responsible for its control of ionic membrane permeabilities. Such studies require that the purified receptor protein be obtained in the *native functional state*. In spite of several recent advances, a large diversity in the reported properties of the isolated receptor still exists, both among various laboratories and within different preparations from the same laboratory (see Neumann & Bernhardt, 1977; Heidmann & Changeux, 1978). These variable properties include the affinity for acetylcholine, the ratio of acetylcholine to α-neurotoxin binding sites, and the extent of cooperativity in the binding. Recent observations of acetylcholine binding to receptor-rich *fresh Torpedo* membrane fragments vs. the 1% Triton extract of these fragments indicate that nonionic detergent solubilization does not alter the high affinity dissociation constant ($K_d \sim 10^{-8}$ or 10^{-9} M) for this activator as determined by equilibrium binding (Sugiyama & Changeux, 1975; O'Brien & Gibson, 1975).

We recently showed that heterogeneity in the molecular forms of acetylcholine receptor from *Torpedo californica* results from a variable amount of chemical modification that occurs during the conventional *homogenization* of electric tissue even prior to its detergent extraction. We have further established that the heavy (H) form (13S) is a dimer of the light (L) form (9S) linked by an intermolecular disulfide bond involving the ~67000 dalton subunits of the receptor and have proposed that the apparently *native dimer*, H form, is split by endogenous reactive protein sulfhydryl groups during homogenization (Chang & Bock, 1977). This monomer–dimer relationship in acetylcholine receptor from *Torpedo californica* has also been reported by other investigators (Hamilton et al., 1977; Witzemann & Raftery, 1978), and the presence of a disulfide-bridged 140000-dalton dimer of 68000 dalton subunits, in acetylcholine receptor rich *Torpedo* membrane fragments, has been unequivocally demonstrated (Suárez-Isla & Hucho, 1977). In crude detergent extract, acetylcholine is bound more tightly to the receptor H form than to the L form; the respective overall equilibrium dissociation constants are $3 \pm 1 \times 10^{-9}$ and $2 \pm 1 \times 10^{-8}$ M (see Figure 6 in Chang & Bock, 1977).

In addition to the heterogeneity in acetylcholine binding constants arising from different molecular forms in the crude extract from fresh tissue, chemical modification of the receptor occurs during affinity chromatography purification. After purification, the affinity of more than 80% of the receptor acetylcholine binding sites is reduced by some two orders of magnitude (Sugiyama & Changeux, 1975; Moody et al., 1974; O'Brien & Gibson, 1975; Eldefrawi et al., 1975; Chang & Neumann, 1976). Similar decreases in acetylcholine binding affinities have been reported for membrane fragments from *Torpedo* electric organs upon prolonged storage (O'Brien & Gibson, 1975). It has been suggested that the appearance of such low affinity binding components is attributable to the oxidation of sulfhydryl groups (O'Brien & Gibson, 1975; Chang & Neumann, 1976).

A general feature of biomembrane structure is the interaction of proteins with membrane phospholipids. In this report

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we demonstrate that specific phospholipid–acetylcholine receptor interactions exist even in 1% nonionic detergent solutions, and we present evidence that the loss of endogenous phospholipids in the receptor leads to conversion of high acetylcholine affinity sites of the receptor to a state of lower affinity. By limiting delipidation, we have found a means to minimize the conversion of high affinity sites. Furthermore, a correlation between the degree of delipidation and the number of free sulfhydryl groups associated with the receptor is demonstrated. This correlation suggests that sulfhydryl oxidation may be responsible for the irreversible loss of the high affinity state.

Materials and Methods

Materials. Unless otherwise indicated the acetylcholine receptor preparations used were purified from electric organs of *Torpedo californica* obtained live from Pacific Biomarine Supply Co., Venice, Calif., frozen and stored in liquid nitrogen. The nonionic detergent, Renex 30 (polyoxyethylene (12) tridecyl ether) was a gift of ICI United States, Inc. [*acetyl*-^{3}H]Acetylcholine, 250 mCi/mM, and *N*-ethyl[2,3-^{14}C]-maleimide, 5 mCi/mM, were obtained from Amersham/Searle. Lubrol WX (polyoxyethylene(17) cetyl and stearyl ether) was purchased from Sigma Chemical several years ago. The precoated TLC plates were obtained from New England Nuclear, and Phospray (Zinzadze reagent) was a product of Supelco Inc. α-L-Phosphatidylcholine (from egg yolk), α-L-phosphatidylinositol (from soybean), α-L-phosphatidylserine (from bovine brain), α-L-phosphatidylethanolamine (from egg yolk), and phospholipase A_2 (EC 3.1.1.4) (from bee venom) were purchased from Sigma Chemical. Gallamine triethiodide (Flaxedil) was a product of K & K Laboratory. Tetram (*O,O*-diethyl *S*-(β-diethylaminoethyl) phosphorothiolate) was synthesized as described previously (Calderbank & Ghosh, 1955). All other materials were reagent grade and obtained from commercial sources. Detergent solutions were prepared from 10% aqueous stock solutions filtered through Millipore membrane (HAWP 0.45 μm). Ten percent solutions of recently obtained batches of Lubrol WX from Sigma Chemical required centrifugation at 4 °C for 20 min at 48 000*g* to remove insoluble material before Millipore filtration. Unless otherwise noted, "stock buffer" denotes a 20 mM Hepes,[1] 3 mM sodium azide (pH 7.0) solution, Millipore filtered and deaerated just before use.

Extraction of Acetylcholine Receptor as H Form. Liquid nitrogen stored tissue (400 g) was pulverized, quickly thawed by homogenization with 1200 mL of 25 °C "stock buffer" containing 5 mM *N*-ethylmaleimide (NEM), 50 μM phenylmethanesulfonyl fluoride ($PhCH_2SO_2F$) in a Waring Blendor for 60 s, and centrifuged 50 min at 25 000*g* at 4 °C (step 1). The pellet was rehomogenized 60 s with 650 mL of the step 1 buffer containing 1 M NaCl to extract high salt soluble proteins (step 2). The homogenate was then centrifuged as before and the resulting pellet was resuspended in 650 mL of fresh step 2 buffer without NEM (step 3). Subsequently the pellet was twice washed and centrifuged by suspension in 650 mL of stock buffer (steps 4 and 5). The resulting pellet was suspended in 200 mL of stock buffer containing 1.2% Lubrol WX, 50 μM $PhCH_2SO_2F$ and shaken for 4 h at 4 °C. Three milliliters of 50 mM $CaCl_2$ solution was added dropwise with mixing to make a final Ca^{2+} concentration of 1 mM. After centrifugation at 46 000*g* for 1 h the supernatant, "crude acetylcholine receptor extract", was immediately applied to the affinity column.

Purification by Affinity Chromatography with Limited Delipidation. The affinity ligand, methyl[*N*-(6-aminocaproyl-6′-aminocaproyl)-3-amino]pyridinium bromide hydrobromide (Dicaproyl-MP) was synthesized as previously described (Rosenberry et al., 1972; Chang, 1974) except that a water-soluble carbodiimide, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (Aldrich Chemical) was used in place of dicyclohexylcarbodiimide for the amine and carboxy group condensation steps. This method made it easier to crystallize intermediate products. The 1% Lubrol WX crude receptor extract was applied to a column (2.4 × 22 cm) containing 100 mL of affinity resin at a concentration of 1 μM Dicaproyl-MP per mL of packed Sepharose 4B. The column was washed first with 170 mL of 0.3% Lubrol WX, 1 mM $CaCl_2$ in stock buffer, then with 150 mL of 0.2% Lubrol WX, 1 mM $CaCl_2$, 30 mM NaCl in stock buffer, and finally with 150 mL of 0.01% Lubrol WX, 1 mM $CaCl_2$, 25 mM NaCl in stock buffer. The acetylcholine receptor was eluted with 70 μM Flaxedil in the buffer solution used for the last wash. Fractions with OD_{280} greater than 1.0 were pooled (pool 1) and dialyzed against three changes of 1 L each of deaerated dialysis solution (stock buffer with 1 mM $CaCl_2$ and 0.1 M NaCl) at 4 °C for a total of 17 h. The protein fractions with OD_{280} between >0.1 and <1.0 (pool 2) were concentrated under argon by vacuum dialysis using a collodion membrane bag (Schleicher & Schuell, Inc.) against three changes of the above dialysis solution. The dialyzed receptor pools (~1 mg/mL) were centrifuged at 35 000*g* for 15 min to remove particulate matter.

Since Hepes buffer interferes with Lowry protein determination, protein was determined by the absorbance at 280 nm using the extinction coefficient, $\epsilon_{280nm}^{1\%} = 18.5 \pm 5$, determined recently in our laboratory for *Torpedo* receptor, predominantly in the H form, in phosphate buffer containing 0.05% Renex 30 using the Lowry method (Lowry et al., 1951).

α-[^{125}I]Bungarotoxin binding assays using DE-81 filter discs were performed as previously described (Chang, 1974) except that only a small excess (less than 1.5 times the estimated number of toxin binding sites) of α-[^{125}I]bungarotoxin was used to minimize background counts and that the wash solution consisted of 0.1% Renex 30 and 30 mM NaCl in 10 mM sodium phosphate buffer (pH 7.4). Since maximum counting efficiency was not achieved until several hours after addition of the scintillation cocktail (Scintisol, Isolab), the samples were counted only after such a time lag. Standards were prepared by pipetting 60 μL of wash solution and known amounts of α-[^{125}I]bungarotoxin onto filter discs for concomitant scintillation counting.

Sucrose Gradient Centrifugation. The conditions used for the analysis of α-[^{125}I]bungarotoxin labeled receptor on linear 5–20% sucrose gradients were as previously described (Chang & Bock, 1977).

Determination of Sulfhydryl Groups. The sulfhydryl group concentrations in purified and crude detergent extracts of acetylcholine receptor were determined spectrophotometrically with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) using a previously described method (Chang & Bock, 1977). The concentration of sulfhydryl groups was calculated using an extinction coefficient of 13 600 M^{-1} cm^{-1} for the reduced DTNB (Ellman, 1959) and expressed in terms of nmol relative

[1] Abbreviations used: NEM, *N*-ethylmaleimide; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetate; $NaDodSO_4$, sodium dodecyl sulfate; Dicaproyl-MP, methyl[*N*-(6-aminocaproyl-6′-aminocaproyl)-3-amino]pyridinium bromide hydrobromide.

to nmol of α-bungarotoxin sites. The change in absorbance at 412 nm with time was analyzed according to the method described by Murphy (1976).

Alternatively, sulfhydryl groups in the purified receptor were determined by means of [^{14}C]NEM labeling. The concentration of a [^{14}C]NEM (5 mCi/mmol) stock solution in water was determined spectrophotometrically (ϵ_{300nm} = 620 M^{-1} cm^{-1}; Gorin et al., 1966). For the determination of accessible sulfhydryl groups, 220 μg of freshly purified acetylcholine receptor in 180 μL of 0.02% Lubrol, 1 mM $CaCl_2$, 100 mM NaCl in stock buffer was reacted with 90 μL of 16.4 mM [^{14}C]NEM for 40 min at 0 °C, and the reaction mixture was dialyzed against three changes of 500 mL of 5 mM sodium phosphate, 1 mM $CaCl_2$, 3 mM NaN_3 (pH 7.0). The radioactivity in 100 μL from the inside and outside of the dialysis bag was determined, and the difference in cpm was expressed in terms of nmol of [^{14}C]NEM-labeled sulfhydryl groups per nmol of α-bungarotoxin sites in the receptor. To label sulfhydryl groups titratable only under denaturing conditions, purified receptor (150 μL, 1.47 mg/mL) was first incubated with 30 μL of 20 mM cold NEM for 40 min at 0 °C and dialyzed as above. To 160 μL of the dialysate were added 25 μL of 20% $NaDodSO_4$, 5 μL of 100 mM EDTA, and 90 μL of the 16.4 mM [^{14}C]NEM stock solution. After 30 min at room temperature, the reaction mixture was again dialyzed, and the [^{14}C]NEM-labeled sulfhydryl concentration was determined as before.

Phospholipid Analysis. Quantitative organic phosphate analysis was carried out on crude acetylcholine receptor extracts (30 μL) and on lyophilized purified receptor samples (300 μg) by the method of Ames (1966) with the following small variation: the detergent solution gave a brown residue during the 10% $Mg(NO_3)_2$ digestion; therefore, after addition of the molybdate reagent mix and color development, the solid residue was removed by centrifugation before reading the absorbance.

Individual phospholipids in lyophilized receptor samples as well as phospholipid standard were identified by TLC using a chloroform–methanol–acetic acid–water (25:15:4:2 by volume) solvent system (Skipski et al., 1964). Either Phospray (Zinzadze, 1935) or iodine vapor was used for the visualization of spots.

Acetylcholine Equilibrium Binding Assays. The exact concentration of a putative 1 mM aqueous solution of [^{3}H]acetylcholine was determined by the following modification of the method of Hestrin (1949). To 250 μL of the [^{3}H]acetylcholine solution and the same volume of a set of aqueous acetylcholine standard solutions (prepared from recrystallized dried acetylcholine) in the range of 0.4–1.5 mM was added 500 μL of a solution consisting of equal volumes of 2 M hydroxylamine hydrochloride and 3.5 N NaOH with mixing. A few minutes thereafter, 250 μL of approximately 6 N HCl, whose concentration had been previously adjusted such that the final pH of the assay mix would be 1.2 ± 0.2, was added. Finally 250 μL of 10% $FeCl_3 \cdot 6H_2O$ in 0.1 N HCl (Millipore –0.45 μm filtered) was added with mixing. After 10 min the optical density of the solution at 540 nm vs. a blank was determined using a pair of semimicrocuvettes. (Care was taken to avoid interference from CO_2 bubbles that tend to form when the solutions are placed into the cuvettes.)

Aliquots of 0.15 mL, containing 25–40 μg of acetylcholine receptor preincubated for 1 h with 80 μM Tetram, were placed into 1-cm diameter dialysis bags and dialyzed at 4 °C against 50 mL of various concentrations of [^{3}H]acetylcholine (1.5 nM to 5 μM) in stock buffer with 0.1% Lubrol, 1 mM $CaCl_2$, 100 mM NaCl, 5 μM Tetram. After 16–18 h of gentle shaking, 0.1 mL aliquots of the dialysates and the dialysis solutions were transferred to 10 mL of Scintisol for scintillation counting.

Phospholipase A_2 (EC 3.1.1.4) Digestion. Purified acetylcholine receptor (0.57 mg, 0.85 mL) in 0.2 M Hepes (pH 7.4), 0.05% Lubrol WX, 2 mM $CaCl_2$ was incubated with 50 μL (160 μg, 208 units) of phospholipase A_2 for 3 h at 4 °C with shaking. The reaction mixture was diluted with 1.9 mL of 0.05% Lubrol WX in stock buffer, and equilibrium dialysis measurements of acetylcholine binding were carried out as before. Samples of the reaction mixture along with samples that were not treated with phospholipase A_2 were subjected to polyacrylamide gel electrophoresis in 1% $NaDodSO_4$ (Fairbanks et al., 1971; Chang & Bock, 1977) to investigate the presence of protease contaminants in phospholipase A_2. The extent of phospholipid hydrolysis by phospholipase A_2 under the above mild conditions was investigated in the following way: sonicated solutions of phosphatidylcholine and phosphatidylinositol (0.2 mg in 0.27 mL of 0.2 M Hepes, 0.07% Lubrol WX, 2 mM $CaCl_2$ (pH 7.4)) were incubated with 33 μg (43 units) of phospholipase A_2 at 4 °C for 3 h with shaking. The samples were lyophilized and analyzed by TLC for the appearance of the corresponding lysophospholipids.

Delipidation and Relipidation. Purified acetylcholine receptor (5 mg) was adsorbed onto a column of 4 mL of DEAE-cellulose (Whatman DE-32) which had been preequilibrated with stock buffer containing 1 mM $CaCl_2$ and 0.5% Renex 30. After washing the column with 5 column volumes of the same buffer, the receptor was eluted with 0.05% Lubrol WX, 1 mM $CaCl_2$, 0.35 M NaCl in stock buffer. Fractions with OD_{280} greater than 0.5 and OD_{280}/OD_{254} = >2 were pooled and diluted to about 0.5 mg of receptor/mL with 0.05% Lubrol WX, 1 mM $CaCl_2$ in stock buffer. Phospholipid stock solutions were prepared by sonicating 2 mg of phospholipid/mL in stock buffer containing 0.1% Lubrol WX and 1 mM $CaCl_2$ until the solutions were optically clear, followed by Millipore (0.45 μm) filtration.

In a typical relipidation experiment, 50-μL (25–30 μg) aliquots of freshly delipidated receptor were incubated with various amounts of phospholipid stock solutions, 7 μL of 1 mM Tetram, and sufficient 0.1% Lubrol WX, 1 mM $CaCl_2$ in stock buffer to bring the total volume to 150 μL. The mixture was thoroughly vortex mixed and, after the specified time, subjected to equilibrium dialysis against 40 nM [^{3}H]acetylcholine.

Results

Receptor Extraction and Purification. Incorporation of the sulfhydryl alkylating agent NEM (5 mM) into the buffers used both for the initial homogenization of electric tissue and for the first of the two salt extraction steps is sufficient to maintain over 90% of the receptor in the H form (13S) during subsequent detergent extraction and purification steps. This is in agreement with earlier observations (Chang & Bock, 1977); thus the presence of NEM in these subsequent steps is not required. We have proposed that the observed conversion to the L form (9S) which occurs in the absence of added NEM is mediated by an endogenous reactive protein sulfhydryl group (Chang & Bock, 1977). In the initial extraction steps 1 and 2, this sulfhydryl group presumably reduces an intersubunit disulfide bond in the H form to generate two L forms. The total receptor extracted by 1% Lubrol WX for 4 h in the absence of added $CaCl_2$ had an average of 1.8 nmol of α-toxin binding sites per gram of tissue. Although the presence of calcium is desirable because it appears to help protect sulfhydryl groups from oxidation, its addition to the detergent extraction buffers significantly lowers the yield of receptor.

Table I: Characterization of Various Acetylcholine Receptor Preparations

receptor preparations	α-toxin[a] (nmol/mg of protein)	phospholipids[b] (nmol/nmol of α-toxin)[c]	-SH groups[d] (nmol/nmol of α-toxin)[c] exposed	-SH groups[d] (nmol/nmol of α-toxin)[c] total	AcCh binding[e] K_d (nM)	AcCh binding[e] B_{max} (nmol/nmol of α-toxin)[c]	AcCh binding[e] Hill coeff
crude extract 1[f]	0.8 ± 0.06	1024	23.8	35.7	4.2 ± 0.8	0.34	2.0
purified (prep 1)[g]	11.0 ± 0.7	18.6	1.8	3.7	4.0 ± 0.8	0.34	1.7
prep 1 + phospholipase A_2[h]	11.0 ± 0.7				3 ± 1	0.11	~1
prep 1 + delipidated[i]	9.9 ± 0.6	<1[l]	1.2	2.2	4.5 ± 0.9	0.037	~1
crude extract 2[j]	1.1 ± 0.06				3 ± 1	0.4	1.7
purified (prep 2)[k]	12.2 ± 0.7	8	1.1	2.2	3 ± 1	0.14	1.1

[a] Nanomoles of α-[^{125}I]bungarotoxin binding sites, determined by DEAE filter disc assay, per mg of total protein (see Materials and Methods). [b] Total phospholipids, expressed as organic phosphate, determined as described in Materials and Methods. [c] Phospholipid and -SH group concentrations as well as B_{max} were normalized with respect to α-bungarotoxin binding sites (see *a*). [d] -SH groups titrable with DTNB under nondenaturing conditions (exposed) and in the presence of 1.7% $NaDodSO_4$ (total). [e] [^{3}H]Acetylcholine equilibrium dialysis binding parameters associated with high affinity binding sites determined from double-reciprocal and Hill plots. [f] 1% Lubrol WX extract from frozen *Torpedo* electric organ in stock buffer with 1 mM $CaCl_2$, 50 μM $PhCH_2SO_2F$, that had been treated with 5 mM NEM during steps 1 and 2 (see Materials and Methods). [g] Affinity chromatography purified crude extract 1 using 0.3-0.02% Lubrol WX in the wash and elution buffers. [h] Phospholipase A_2 incubated preparation 1 (see Materials and Methods). [i] Preparation 1 delipidated by washing with 0.5% Renex 30 (see Materials and Methods). [j] Fresh *Torpedo* electric organ extract similar to crude extract 1 except 1% Renex was used for extraction. [k] Affinity chromatography purified crude extract 2 using 0.5-0.1% Renex 30 in the wash and elution buffers. [l] Our assays did not detect any phospholipids (see Materials and Methods).

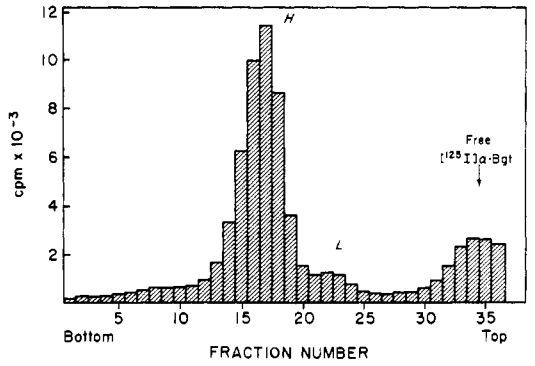

FIGURE 1: Sucrose density gradient sedimentation profile of α-[^{125}I]bungarotoxin-labeled acetylcholine receptor purified from *Torpedo* electric organ that was treated with 5 mM NEM in the first two tissue preparation steps.

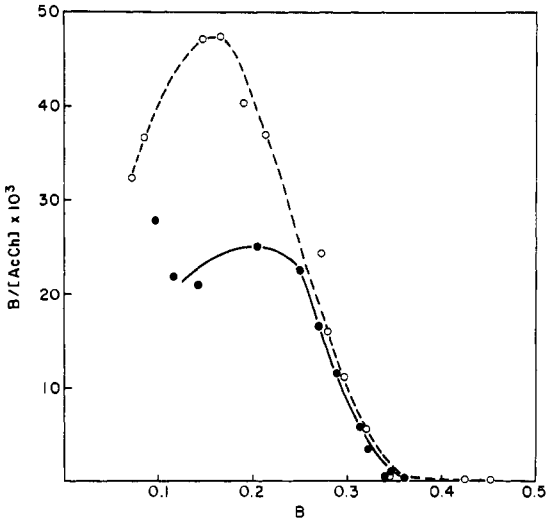

FIGURE 2: Scatchard plots of [^{3}H]acetylcholine equilibrium binding data. The procedure is described in Materials and Methods. (●—●) Crude 1% Lubrol WX extract of acetylcholine receptor. (○---○) Acetylcholine receptor purified from the above crude detergent extract. *B*, nmol of ligand bound per nmol of α-[^{125}I]bungarotoxin binding sites. [AcCh], molar concentration of free [^{3}H]acetylcholine after equilibration.

Therefore, $CaCl_2$ to a final concentration of 1 mM was added *after* detergent extraction.

About 10% of the receptor in crude extract was not retained by the affinity column, about 15% was desorbed by the 30 mM NaCl containing buffer wash, and about 50% (35 mg from 400 g of tissue) was eluted by 70 μM Flaxedil. Purifications using affinity gel regenerated with 6 M guanidine hydrochloride normally increased the yield up to 70% of the output. Although much lower detergent concentrations were used both in the column wash and for receptor elution than those in our previous purification method, the specific activity of the purified receptor was just as high: approximately 10 nmol of α-[^{125}I]bungarotoxin sites per mg of protein. Sucrose density gradient centrifugation of both crude and purified receptor indicated that there was no change in the H/L ratio during purification. However, the L form did increase slowly upon storage at 0 °C. A sedimentation profile of α-[^{125}I]bungarotoxin labeled purified receptor is presented in Figure 1.

Comparison of Acetylcholine Binding Properties of Crude and Purified Receptor. Results of [^{3}H]acetylcholine binding studies for preparation 1, representative of those obtained with several crude receptor preparations and receptor purified under conditions leading to only limited delipidation, are presented in Table I and Figure 2. Estimates of the apparent dissociation constants and the number of binding sites were obtained by analyzing the linear regions of Scatchard and double-reciprocal plots. As shown in the Scatchard plots in Figure 2, the number of high affinity acetylcholine binding sites in the crude extract, 0.34 nmol per nmol of α-toxin sites, that were associated with K_d (~4 nM) did not change appreciably during purification. However, additional medium affinity binding sites ($K_d \simeq 0.2$ μM) were seen in this purified receptor. The number of high affinity acetylcholine binding sites in the purified receptor varied in different preparations, ranging from 0.3 to 0.45 nmol per α-bungarotoxin site even though comparable conditions were used. Our estimates of total acetylcholine binding sites, including lower affinity ($K_d \sim 1$ μM) sites, in the purified receptor never exceeded 0.5 nmol per nmol of α-bungarotoxin site. Similar dissociation constants had been reported for certain detergent extracts from *Torpedo* (O'Brien & Gibson, 1975). Because the transition to lower affinity states is not well defined, our analyses will focus on the high affinity state. Scatchard plots from both crude and purified receptor show an upward curvature in the 3–7 nM acetyl-

choline concentration range, presumably due to positive cooperativity in acetylcholine binding (Eldefrawi & Eldefrawi, 1973; O'Brien & Gibson, 1975; Gibson, 1976). There appears to be a difference, however, in the degree of cooperativity in acetylcholine binding for the two preparations, as indicated by a difference in Hill coefficients of 2.0 and 1.7 for crude and purified receptor, respectively.

Phospholipid Analysis. All receptor preparations had been extensively salt extracted and washed using Hepes buffer solutions prior to detergent extraction. It is thus assumed that all phosphates found in detergent extracts were derived from phospholipids. Crude detergent extracts contained ~1 μmol of organic phosphate relative to 1 nmol of α-toxin sites, corresponding to about 2.3 mg of phospholipids per mL of crude extract, assuming an average molecular weight of 790 for the phospholipids (Table I). Since 1.9 g of tissue resulted in 1 mL of crude extract in this preparation, 1.6 μmol of phospholipid was extracted from 1 g of electric tissue. Phospholipids comprise 70–75% of the total lipids in electric tissue from *Torpedo californica* (Michaelson & Raftery, 1974; Schiebler & Hucho, 1978). The phospholipid content of purified receptor (preparation 1) was 18.6 nmol per nmol of α-toxin site. This corresponds to about 74 phospholipid molecules per molecule of purified H-form receptor, assuming 4 α-toxin sites for the H form. The amount of phospholipid found in six other similar purified receptor preparations was consistently 20 ± 2 nmol per nmol of α-bungarotoxin site. Qualitative TLC analysis did not reveal any preference by the receptor for particular phospholipids; crude and purified receptor preparations contained the same phospholipid distribution.

Neither the phosphate assay nor TLC analysis detected any phospholipid in delipidated receptor solutions containing amounts of receptor equal to that used in assaying preparation 1. Recently it was reported that approximately 5 phospholipid molecules per α-toxin binding site are tightly bound in purified receptor preparation in Triton X-100 (Reynolds & Karlin, 1978).

Effects of Phospholipase A_2 on High Affinity Binding. Very mild conditions were used for phospholipase A_2 digestion (see Materials and Methods) to minimize any modification of high affinity sites. Subsequent TLC analysis indicated that, while phosphatidylinositol (R_f = 0.59) was only partially converted to its lyso product (R_f = 0.44), the reaction of phosphatidylcholine (R_f = 0.42) to the corresponding lyso product (R_f = 0.26) was complete. Acetylcholine binding studies with phospholipase A_2 digested receptor (preparation 1) revealed a 69% loss of high affinity sites relative to the control (Table I) and showed an increase in medium affinity sites (K_d = 0.22 μM) at the expense of high affinity sites. Furthermore, the positive cooperativity in binding was lost as indicated by a Hill coefficient close to 1.

To rule out the possibility that the loss of high affinity sites arises from proteolysis during phospholipase A_2 digestion, the digested receptor sample was subjected to polyacrylamide gel electrophoresis in $NaDodSO_4$ under disulfide reducing conditions. No additional protein staining bands were observed relative to the control, suggesting that proteolysis can be discarded as a reason.

Receptor Sulfhydryl Groups. In the presence of $NaDodSO_4$, the reaction of DTNB with crude acetylcholine receptor detergent extracts revealed high concentrations of sulfhydryl groups, despite the NEM treatment during the early stages of tissue extraction. The ratio of total sulfhydryl groups to α-toxin sites was about 36, of which 24 sulfhydryls were highly reactive. This total represents about 45% of the sulfhydryls found in similar extracts prepared without the use of NEM (Chang & Bock, 1977). The total sulfhydryl concentration of two purified receptor solutions prepared under limited delipidation conditions was 3.7 nmol (preparation A) and 4.5 nmol (preparation B) per nmol of toxin sites, equivalent to 41 nmol and 48 nmol of –SH per mg of protein. Even after 3–4 min in 1.7% $NaDodSO_4$, the sulfhydryl groups reacted with DTNB at two different rates, suggesting that the proteins are not completely unfolded under these conditions. A semilogarithmic plot of the absorbance change as a function of time for the reaction of DTNB with the purified receptor becomes linear only after 2.5 min, as shown in Figure 3 for preparation A. The analysis of the curve yields two pseudo-first-order rate constants, k_1 = 2.0 min^{-1} for the fast reaction associated with 1.8 sulfhydryls and k_2 = 0.52 min^{-1} for the slow reaction associated with 1.9 sulfhydryls per α-toxin site. In studies using preparation B, 2.5 sulfhydryls were found to be of the fast reacting type (presumably exposed), and 2 sulfhydryl groups reacted more slowly (presumably buried in the interior of the molecule). Sulfhydryl titrations with preparation B were also carried out with [^{14}C]NEM. This method indicated 3.2 nmol of –SH groups per nmol of α-toxin sites that reacted under nondenaturing conditions and an additional 2 nmol that were labeled after $NaDodSO_4$ incubation. This total is about 15% higher than that obtained using the DTNB method, probably indicating some nonspecific labeling.

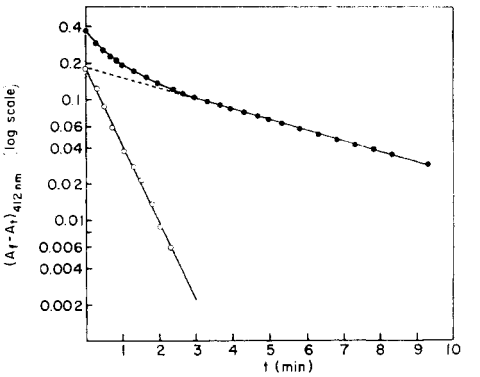

FIGURE 3: Titration of free sulfhydryl groups in purified acetylcholine receptor with DTNB. Semilogarithmic plots of the difference in final absorbance at 412 nm (A_f) and absorbance at time t (A_t) as a function of time are shown. A purified receptor sample (300 μL, 0.39 mg) was mixed with 250 μL of 0.1 M Tris-HCl (pH 8.0) containing 5 mM EDTA and 4% $NaDodSO_4$ in a 1-cm semimicrocuvette. Fifty microliters of 10 mM DTNB in 0.1 M Tris-HCl (pH 8.0) was added, and the absorbance at 412 nm vs. time was recorded against a blank without protein until there was no further increase (30 min). Curve 1 (●—●), observed absorbance difference; curve 2 (- - -), extrapolation of linear portion of curve 1; curve 3 (○—○), difference between curves 1 and 2.

In contrast to the above results, receptor delipidated by extensive detergent washing either during affinity chromatography or while adsorbed to DEAE-cellulose showed only 2 sulfhydryls per α-toxin site, one each of the exposed and nonexposed types (Table I; see Discussion).

Effect of Delipidation and Relipidation on the Receptor High Affinity State. Delipidation of receptor purified under conditions designed to minimize removal of phospholipids tightly bound to the receptor (preparation 1) resulted in an 80–90% loss of high affinity binding sites and loss of positive cooperativity relative to the purified receptor before delipidation (Figures 2 and 4). Addition of sonicated phospholipid solutions to the receptor immediately after delipidation resulted in an increase in acetylcholine binding at 40 nM [^{3}H]-

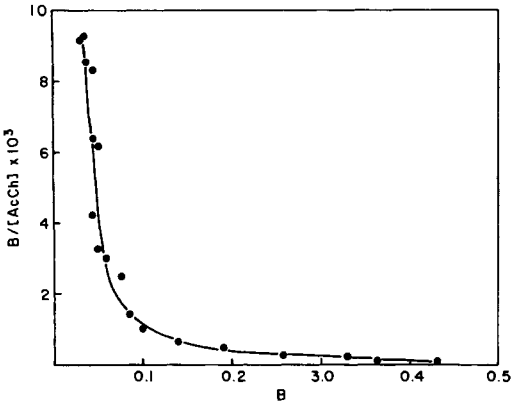

FIGURE 4: Scatchard plot for a typical delipidated receptor preparation. Delipidation and binding studies were carried out as described in Materials and Methods. *B*, nmol of ligand bound per nmol of α-[^{125}I]bungarotoxin binding sites. [AcCh], molar concentration of free [^{3}H]acetylcholine after equilibration.

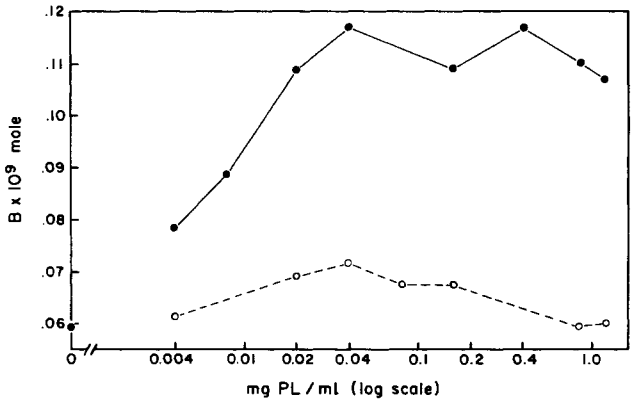

FIGURE 5: Acetylcholine equilibrium binding against 40 nM [^{3}H]acetylcholine of delipidated receptor (see Materials and Methods) with various amounts of phosphatidyl inositol (●—●) and phosphatidylcholine (O---O) added. *B*, [^{3}H]acetylcholine bound per nmol of α-toxin binding sites; PL, phospholipid.

acetylcholine equilibrium concentration. This increased affinity showed a strong dependence on the particular phospholipid used and on its concentration (Figure 5). Incubation of delipidated receptor with 0.05 mg/mL of phosphatidyl inositol, the most effective of the phospholipids tested, resulted in about a 90% increase in acetylcholine binding relative to the same receptor without added phospholipid. Nevertheless, this corresponds to only about 40% of the binding capacity of the receptor before delipidation at this acetylcholine concentration. The 40 nM acetylcholine concentration used in these experiments was chosen because at this concentration about 91% of the high affinity sites ($K_d \sim 4$ nM) are occupied compared with only 16% and 4% of the medium ($K_d \sim 0.2$ μM) and low ($K_d \sim 1$ μM) affinity sites, respectively. Thus an increase in binding upon addition of phospholipids would reflect recovery mainly of high affinity sites.

Discussion

The problems associated with the heterogeneity of acetylcholine affinities of acetylcholine receptor from *Torpedo* have been very complex and their physical chemical basis has essentially remained unknown. Recently we have shown that the two ranges of dissociation constants ($K_d \sim 10^{-9}$ and 10^{-8} M) reported even for the crude detergent extract from *fresh Torpedo* tissue (O'Brien & Gibson, 1975) are attributable, at least in part, to the presence of two molecular forms of the acetylcholine receptor, the 13S form (H) and 9S form (L), obtained in various proportions depending on tissue homogenization conditions (Chang & Bock, 1977). Furthermore, these high affinities of the receptor for acetylcholine have been shown to decrease ($K_d \sim 10^{-7}$ and 10^{-6} M) during affinity chromatography purification (Eldefrawi et al., 1975; Sugiyama & Changeux, 1975; O'Brien & Gibson, 1975; Raftery et al., 1975; Chang & Neumann, 1976). The extent of change was variable but usually involved more than 80% of the total sites and in some cases close to 100% (Moody et al., 1974). This change probably is not due to proteolysis or oxidation alone because a similar change does not occur in *crude* detergent extracts stored for the same period of time as that required for purification. This suggests that there are specific *stabilizing factors* for the high affinity state of the receptor in the crude extract. It is conceivable that detergent solutions used to dissociate the acetylcholine receptor protein from the membrane only slowly displace certain specific lipids closely associated with the receptor. Examples of such tight lipid–protein interactions have been well documented for integral membrane enzyme systems (Tanford & Reynolds, 1976; LeMaire et al., 1976). However, some of these tightly bound lipids may eventually be displaced by detergent molecules during the affinity chromatography purification step. The extent of phospholipid displacement by detergent during affinity chromatography should depend on the concentrations and the volumes of the detergent used for extraction and purification as well as on the effectiveness of the surfactants. This expectation has been confirmed with several receptor preparations described here.

A definite relationship appears to exist between the amount of endogenous phospholipid remaining in the purified receptor and its retention of high affinity sites for acetylcholine (see Table I). A significant amount of endogenous phospholipid (20 ± 2 phospholipid molecules per α-toxin site) was still tightly associated with the purified receptor when the buffers used during purification contained low concentrations (0.3 and 0.01%) of the nonionic detergent Lubrol WX. These receptor preparations with limited delipidation showed almost complete retention of the high acetylcholine affinity ($K_d = 4 \pm 1 \times 10^{-9}$ M) sites seen in the corresponding crude extract (Figure 2). In contrast, the use of higher concentrations and/or more effective detergents during purification results in much lower phospholipid to α-toxin site ratios and greatly increased conversion of high affinity sites to a lower affinity state. For example, the use of 0.5–0.1% of the detergent Renex 30 yields a purified receptor preparation containing only 8 phospholipid molecules per α-toxin site and in which 65% of the high affinity sites seen in the corresponding crude detergent extract have been converted to lower affinity. The 10–15% low affinity sites shown in Figure 2 are generally observed in crude extracts prepared from frozen, rather than fresh, tissue (O'Brien & Gibson, 1975). Since this study has focused on the high affinity state of the receptor, the acetylcholine concentration range at which low affinity sites become apparent has not been carefully investigated here. The upward curvature in the Scatchard presentation in Figure 2 is consistent with earlier reports that positive cooperativity is involved in the binding of acetylcholine to the receptor (Eldefrawi & Eldefrawi, 1973; O'Brien & Gibson, 1975; Gibson, 1976). However, there are significant differences in the degree of positive cooperativity between the two binding curves for the crude and the purified receptor in Figure 2 and Table I suggesting that some structural modification may have occurred during purification. Our cumulative results of equilibrium binding studies with [^{3}H]acetylcholine using carefully standardized [^{3}H]acetyl-

choline concentrations consistently indicated 1.0 α-bungarotoxin binding site to a maximum of 0.5 acetylcholine binding site. This is in agreement with reports of Raftery et al (1975); however, for the receptor from *Torpedo marmorata* a 1:1 relationship has been reported (Sugiyama & Changeux, 1976; Eldefrawi et al., 1975).

The retention of high affinity sites in the purified receptor (preparation 1) appears to be due, at least in part, to the large amount of phospholipids retained by the receptor. This conclusion was indicated by the experiment involving phospholipase A_2 digestion. Very mild treatment of the purified receptor (preparation 1) with phospholipase A_2 resulted in a 68% loss of high affinity sites relative to the control. $NaDodSO_4$ gel electrophoresis confirmed that this loss did not arise from proteolysis. The sensitivity of acetylcholine receptor to phospholipase A_2 with respect to acetylcholine binding has been reported previously (O'Brien & Gibson, 1974). Complete delipidation of the receptor (see Materials and Methods) results in an even larger conversion (90%) of high affinity sites to a lower affinity state (Table I and Figure 4).

Figure 5 indicates that the addition of certain phospholipids to delipidated receptor preparations leads to *partial* recovery of the high affinity sites that are otherwise lost by delipidation. The greater efficacy of phosphatidylinositol relative to other phospholipids investigated suggests that the effect may be due to a specific protein–phospholipid interaction. Whether this specificity resides in the polar head group or is due to the length of the particular hydrophobic chain has not been determined. However, a specificity for phosphatidylinositol is not substantiated by TLC phospholipid analyses of our preparation 1, where no obvious enrichment in endogenous phosphatidylinositol over three other common phospholipids is evident. Repeated experiments with delipidated receptor indicate that the effect of relipidation is critically dependent on the *time* elapsed after the delipidation. This suggests that the structural integrity of the receptor protein requires a highly specific hydrophobic environment, perhaps defined by endogenous phospholipids.

Reversible transitions between high affinity and low affinity states of the receptor have been reported to take place by varying the concentration of anionic detergent (sodium cholate) after solubilizing receptor-rich membrane fragment from *Torpedo marmorata* (Sugiyama & Changeux, 1975). These phenomena can be interpreted in the following way: crude receptor extracts in nonionic detergents (1% Triton X-100 or Lubrol WX) exhibit the high affinity state because specific interactions between phospholipids and the receptor still exist; however, in 3% sodium cholate extracts such interaction does not occur, and thus sites are converted to lower affinity states. It has been shown that sodium cholate is an extremely effective delipidating agent (Schiebler & Hucho, 1978). Upon lowering the sodium cholate concentration in extracts either by dilution or by dialysis, the receptor appears to reassociate with phospholipids, recovering the original high affinity state as suggested by our delipidation–relipidation experiments. The inability of the receptor to revert to the high affinity state after prolonged storage (>72 h) in sodium cholate (Sugiyama & Changeux, 1975) as well as our relipidation experiments suggest that some irreversible structural change, such as intramolecular sulfhydryl oxidation, is facilitated by the absence of protective phospholipids.

In order to obtain H-form (13S) receptor, which we believe to be the native form of *Torpedo* receptor, 5 mM NEM is used in the buffers for homogenization of tissue and the following salt extraction step (Figure 1). Unreacted NEM is removed in the subsequent steps prior to the detergent solubilization of the receptor. Despite this exposure to NEM, the sulfhydryl group concentration in the crude extract is 0.1 mM, equivalent to a ratio of 24 exposed sulfhydryl groups per receptor α-toxin site. This concentration presumably represents sulfhydryl groups from all proteins that had been protected in the membrane from NEM alkylation and exposed only by the detergent solubilization. Other investigators have also observed that detergent solubilization of the membrane from *Torpedo californica* exposes additional sulfhydryl groups when compared to those found in the intact membrane (Suárez-Isla & Hucho, 1977).

Purified acetylcholine receptor protein is known to contain sulfhydryl groups, but the actual number present when the receptor is in the membrane bound state is not known. The high reactivity of many of these sulfhydryls apparently causes oxidative disulfide bridge formation during the purification process (Eldefrawi et al., 1975; Chang & Bock, 1977). It appears that, everything else being equal, receptor preparations with higher phospholipid contents also retain more sulfhydryl groups, suggesting that phospholipids are protecting receptor sulfhydryl groups from air oxidation. If purified receptor in which over 80% of the acetylcholine binding sites are in the high affinity state is reacted with either DTNB or [^{14}C]NEM in the presence of $NaDodSO_4$, 4–5 sulfhydryl groups per α-toxin binding component (i.e., $\sim$100 000 daltons) are observed. This value for total sulfhydryl groups is about twice the maximum number reported previously for purified receptor prepared in carefully deaerated buffers (Eldefrawi et al., 1975; Chang & Bock, 1977). These sulfhydryl groups belong to at least two classes: 2 or 3 sulfhydryl groups readily titratable under nondenaturing conditions and 2 sulfhydryl groups accessible only after denaturation. Sulfhydryl group determination immediately after delipidation indicates that a total of only 2 sulfhydryls, one from each class, have been retained. Furthermore, $NaDodSO_4$ gel electrophoresis of the delipidated receptor in the absence of disulfide reduction (unpublished work) suggests that this apparent sulfhydryl oxidation accompanying delipidation results in *inter*subunit disulfide bond formation. It is noteworthy that one of the sulfhydryl groups that formerly required $NaDodSO_4$ in order to react with DTNB or NEM and, therefore, presumably is protected in the interior of the molecule, is oxidized before all the exposed sulfhydryl are oxidized.

Electrophysiological data have suggested that only the low affinity state ($K_d > 10^{-6}$ M) can be related to the receptor activation process, because the lifetime (or the reciprocal of the dissociation rate constant) of the activated receptor–acetylcholine complex must be equal to or less than the end-plate current decay time constant of 2–3 ms (see Rang, 1975). In addition, the bimolecular rate constant for the perhaps diffusion-controlled binding step cannot exceed about 10^9 M^{-1} s^{-1}. Since the equilibrium dissociation constant is the ratio between the dissociation and association rate constants, the predicted lifetime for the high acetylcholine affinity state (K_d, $\sim 10^{-9}$ M) is about 1 s; this is about 3 orders of magnitude larger than the lifetime of activated conducting receptor state. The higher affinity form found in both fresh membrane fragments and detergent solubilized extracts is therefore often suspected of being a "desensitized" receptor (Sugiyama et al., 1976; Weiland et al., 1976; Quast et al., 1978).

The current evidence based on the present study of the isolated receptor suggests that the lower affinity states observed in equilibrium acetylcholine binding studies with the receptor arise primarily as a consequence of the loss of endogenous

phospholipids and of sulfhydryl oxidation. Conventional methods for determining dissociation constants by equilibrium dialysis expose the receptor to acetylcholine for long periods (≥16 h), a condition known from electrophysiological evidence to promote the receptor inactivation–"desensitization" phenomenon (Katz & Thesleff, 1957). Unless a method can be devised to detect binding of acetylcholine to the receptor under fast mixing conditions, the question of the "true" dissociation constant involved in receptor activation may not be answered. If the high affinity equilibrium constant determined is indeed associated with the desensitized form of receptor, the fact that the receptor is able to change to a form which resembles the desensitized state during prolonged exposure to acetylcholine may be a useful indication that it is still functionally active.

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