Reconstitution of Acetylcholine Receptor Function Using Purified Receptor Protein†

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ABSTRACT: Membrane preparations containing only the four acetylcholine receptor polypeptide subunits (40, 50, 60, and 65 × 10^3 daltons) were purified from *Torpedo californica* electroplax. The receptor protein was extracted from these membranes with 2% aqueous sodium cholate, and complete dissolution into discrete molecular species was confirmed by sedimentation analysis. The solubilized preparation reassociated with exogenous phospholipids when the detergent was removed by dialysis and formed spherically sealed vesicles 400–600 Å in diameter. The reconstituted receptor preparations had nearly 90% of their α-bungarotoxin binding sites exposed on the exterior surface of the vesicles. In a reproducible manner, the reconstituted acetylcholine receptor responded to carbamoylcholine by exhibiting a rapid efflux of $^{22}Na^+$ from within the vesicles. Such preparations were successfully reconstituted only from acetylcholine receptor stabilized by asolectin above a minimal level upon dissolution of the original membrane preparation by detergent. In addition to the response to carbamoylcholine, the reconstituted preparations also exhibited pharmacological characteristics that resemble those observed for the original electroplax membranes. In terms of the carbamoylcholine-induced signal, a significantly large fraction of the total receptor was functionally reconstituted. The results also confirm the notion that only the four polypeptides considered to constitute the receptor are essential for acetylcholine-mediated cation translocation and rule out possible roles for other polypeptide species.

The acetylcholine receptor protein has been isolated and purified in amounts sufficient for extensive biochemical studies from the electroplax of the electric rays *Narcine* and *Torpedo* and also from *Electrophorus electricus* as well as more recently from denervated mammalian muscle [see Vandlen et al. (1979); Heidmann & Changeux (1978)]. It is now generally agreed that *Torpedo* AcChR[1] is composed of four polypeptide subunits of 40, 50, 60, and 65 × 10^3 daltons as first described by Raftery et al. (1974) and Weill et al. (1974), although there is not total accord in this regard (Sobel et al., 1977).

A wealth of information is available with respect to the interactions of a wide variety of cholinergic analogues with Torpedo receptor dissolved and purified in detergent solution and in receptor-enriched membranes [see Heidmann & Changeux (1978)]. In addition, affinity labels that act as antagonist (Weill et al., 1974) or agonist (Chang et al., 1977; Damle et al., 1978; Moore & Raftery, 1979) covalently label the 40 000-dalton polypeptide so that it is commonly believed that this subunit contains all or part of a cholinergic ligand binding site. Witzemann & Raftery (1977) used a different photosensitive antagonist as an affinity reagent and observed labeling of three of the four chains under different conditions, including the one of mol wt 40 000. This result indicates that other cholinergic ligand binding sites may exist within the AcChR subunit complex.

One major question is whether the purified receptor protein contains all the necessary structural and functional components to bind AcCh and also to effect cation translocation across a biomembrane in response to this binding. A second important question is whether this single protein complex manifests the known pharmacological properties of the AcChR delineated by electrophysiological studies in vivo, including agonist-induced receptor desensitization or block of agonist-activated cation translocation by cholinergic antagonists, α-neurotoxins, or histrionicotoxin. This latter toxin is now known to bind to what we have referred to above as the AcChR in membrane-bound form (Elliott & Raftery, 1979; Neubig et al., 1979; Elliott et al., 1979) rather than to a protein distinct from the receptor (Eldefrawi et al., 1977) or to a specific protein of 43 000 daltons associated with AcChR-enriched membranes (Sobel et al., 1978).

Although the notion that *Torpedo* AcChR is composed of four polypeptide subunits is now widely accepted, evidence that this complex constitutes the complete physiological receptor is incomplete. Reconstitution has generally been viewed as a viable approach to test this. Successful reconstitution studies have been reported (Hazelbauer & Changeux, 1974; Epstein & Racker, 1978) which use crude membrane preparations that contained protein species other than the AcChR. Utilizing purified AcChR, Michaelson & Raftery (1974) described reconstitution approaches that yielded both active and inactive preparations with regard to $^{22}Na^+$ flux in response to Carb while others (Karlin et al., 1975; McNamee et al., 1975; Howell et al., 1978) reported similar studies in which none of the preparations were active.

We have recently presented in preliminary form (Wu & Raftery, 1979) an account of the studies described here. In this report we present evidence that we have successfully reconstituted a large fraction of the purified AcChR protein. A quantitative analysis of the response to Carb by the reconstituted preparations has allowed us to rule out any possible functional roles for minor contaminants.

Materials and Methods

Materials

AcChR membranes were prepared from electric organs of *Torpedo californica* obtained live in local Pacific waters. Fresh

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[1] Abbreviations used: AcChR, acetylcholine receptor; α-BuTx, α-bungarotoxin; Carb, carbamoylcholine; DEAE, diethylaminoethyl; [^{3}H]PC, L-α-dipalmitoyl(2-[9,10-^{3}H]palmitoyl)phosphatidylcholine; HTX, histrionicotoxin; H_{12}-HTX, perhydrohistrionicotoxin; ONPG, *O*-nitrophenyl β-D-galactopyranoside; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EM, electron microscopy.

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organs were used immediately or stored at −90 °C following freezing in liquid nitrogen. Sodium cholate was obtained from Consolidated Midland Corp., Brewster, NY. DE-81 DEAE disks were purchased from Whatman, Ltd., and Sepharose 4B resin was from Pharmacia Co. Radioiodine (carrier-free $Na^{125}I$) was obtained from New England Nuclear Corp. and $^{22}NaCl$ (21.1 Ci/nmol) was from Amersham Corp. [^{3}H]PC [L-α-dipalmitoyl(2-[9,10-^{3}H]palmitoyl)phosphatidylcholine] (13 Ci/mmol) was purchased from Applied Science Laboratories, Inc., State College, PA. Asolectin was purchased from Associated Concentrates, Woodside, NY. β-Galactosidase in 50% ammonium sulfate was given to us by Audry Fowler and catalase was purchased from Boeringer Manheim Biochemicals, Indianapolis, IN. H_{12}-HTX was a generous gift of Dr. Y. Kishi. Gramacidin D was purchased from Sigma Chemical Co., St. Louis, MO.

Methods

A preliminary account of the experimental procedures has been reported earlier (Wu & Raftery, 1979) and is described here in detail.

Preparation of α-[^{125}I]Bungarotoxin. α-BuTx was purified from lyophilized venom of *Bungarus multicinctus* (Sigma Chemical Co.) as described by Clark et al. (1972) and radiolabeled with ^{125}I according to the method of Blanchard et al. (1979). Monoiodolabeled α-[^{125}I]BuTx was used in all binding studies.

Preparation of Membrane Fragments. Purified membranes enriched in AcChR were prepared by using sucrose step gradients in a Beckman VTi 50 vertical rotor (Elliott et al., 1980). Membrane fractions recovered from the middle band (approximately 36% sucrose, w/w) of the gradients were pooled and diluted twofold into 10 mM Tris-Cl, pH 7.4, before centrifuging for 1 h at 30000 rpm in a Beckman Type 35 rotor. The membranes were resuspended with a Virtis-23 homogenizer in the same buffer and assayed for protein and α-[^{125}I]BuTx binding sites (Schmidt & Raftery, 1973). The specific activities of such membrane preparations were 1.0–1.5 nmol of α-BuTx sites per mg of protein.

Alkali Treatment. Purified AcChR-enriched membrane preparations containing 10–15 mg of protein per mL were diluted 10-fold into distilled water (room temperature) and the pH was carefully adjusted to 11.0 with 0.2 N NaOH at room temperature (Neubig et al., 1979; Elliott et al., 1979). It was found that more quantitative removal of the 90×10^3 dalton polypeptide(s) was obtained when this step was performed at room temperature rather than at 0 °C. The membranes were stirred at 4 °C for 1 h and then centrifuged at 18000 rpm in a Sorvall SS-34 rotor for 1 h. The supernatant and the light particulate fraction ("soft pellet") sedimenting on top of the membrane pellet were removed and saved for analyses. The membrane pellets were resuspended in distilled water and the pH treatment and centrifugation steps were repeated. The final membrane pellets were resuspended in 10 mM Tris-Cl, pH 7.4. The specific activity of alkali-treated membrane preparations was typically 5.5 nmol of α-[^{125}I]BuTx bound per mg of protein.

Assays. Protein concentrations were determined by the method of Lowry et al. (1951) with bovine serum albumin as standard. The concentration of α-[^{125}I]BuTx binding sites was determined by the DEAE-cellulose disk assay of Schmidt & Raftery (1973). Enzymatic assays of catalase and β-galactosidase activities were adapted from the procedures described in the Worthington "Enzyme" manual.

Solubilization and Reconstitution of AcChR. For membrane solubilization, a mixture containing the appropriate concentrations of sonicated asolectin, sodium cholate, NaCl, and Tris-Cl, pH 7.4, was stirred at room temperature for 10 min. Alkali-treated membrane suspensions containing 2.0 mg of protein were then added to this mixture to a final volume of 1.5 mL and final concentrations of 20 mg/mL (or other desired final concentrations) asolectin, 2% (w/v) cholate, 200 mM NaCl, and 10 mM Tris-Cl, pH 7.4. The mixture was sonicated twice for 15 s in a bath sonicator. After being stirred at 4 °C for 30 min, the extract was centrifuged in a Beckman Type 65 rotor at 40000 rpm (140000*g*) for 1 h. The supernatant was removed and dialyzed against 3 L of 200 mM NaCl in 10 mM Tris-Cl, pH 7.4, for 20 h at room temperature. For solubilization in sodium cholate solutions containing lower lipid concentrations, appropriate volumes of sonicated asolectin (100 mg/mL) were added to cholate mixtures prior to addition of membranes. Following centrifugation, sonicated asolectin was added to the supernatant such that the final volume and the overall amounts of lipid added remained the same, i.e., 20 mg/mL asolectin in 1.5-mL total volume. The mixtures were stirred at 4 °C for 15 min before being dialyzed.

$^{22}Na^+$ Flux Assay. For the measurement of agonist-induced cation efflux, the reconstituted preparations (~1 μM in α-BuTx sites) were incubated with 0.87 μM $^{22}NaCl$ (Amersham, 21.1 Ci/nmol) for 12 h at 4 °C to allow equilibration of $^{22}Na^+$ inside and outside the vesicles. The flux assay was started at time zero by diluting the radioactive vesicle suspension 20-fold into nonradioactive dilution buffer with or without Carb at room temperature. (The dilution buffer consisted of 200 mM NaCl in 10 mM Tris-Cl, pH 7.4.) The mixture was immediately vortexed and at time intervals of 10 s 200-μL aliquots were transfered with an Eppendorf pipet onto three layers of DEAE-cellulose disks (Whatman DE-81) which had been presoaked in the dilution buffer and mounted on a Millipore manifold apparatus. The filters were immediately washed twice with 7.5 mL of nonradioactive dilution buffer and counted on a Beckman gamma 4000 counter with the counting windows optimized for $^{22}Na^+$. The four time points obtained (at 10, 20, 30, and 40 s) from each assay were fit to a single exponential. In some cases scattering of data points resulted in lines with positive slopes when the points were fit to a single exponential formula. Since an exponential decay was not truly significant at the time points taken (very close to linear and Na efflux had essentially all terminated), the sign of the slopes was ignored in these cases.

Gel Electrophoresis. Polyacrylamide gels (8.75%) were run in 0.1% $NaDodSO_4$ (Laemmli, 1970). The gels were stained for protein with 0.05% (w/v) Coomassie Brilliant Blue in 10% (v/v) acetic acid and 25% (v/v) methanol and destained in the same solution without the dye. Gel strips cut from slabs were scanned at 550 nm by using a Gilford 240 spectrophotometer equipped with a linear-transport accessory.

Assay of AcChR Orientation. For determination of the proportion of receptors reconstituted with the correct orientation for interaction with cholinergic ligands, the fraction of the total α-[^{125}I]BuTx sites exposed on the exterior surface of vesicles was assayed. Reconstituted AcChR vesicles were incubated 30 min at room temperature in 0.5 mL of 200 mM NaCl and 10 mM Tris, pH 7.4, containing a 2-fold excess (~1 μg/mL) of α-[^{125}I]BuTx. Aliquots of 25 μL were transferred to each of five 0.25-mL wash buffer solutions (10 mM NaP_i, pH 7.4, 50 mM NaCl, and 0.1% Triton X-100) (total sites) or to each of five 0.25-mL wash buffer solutions containing 3.3 μg/mL of unlabeled α-BuTx (exterior sites). The samples were incubated at room temperature for 30 min, and 0.1-mL aliquots of each were transferred onto DEAE-cellulose disks.

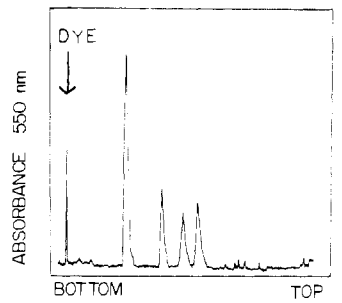

FIGURE 1: Densitometer scans of Coomassie Brilliant Blue stained $NaDodSO_4$ gel. The scan reveals the gel pattern of membranes from selected peak α-BuTx binding fractions following further purification of alkali-treated membranes through a reorienting sucrose gradient.

The 10 disks were immersed in 250-mL wash buffer solution and stirred for 30 min with two changes of buffer following each successive 10-min period. The disks were then counted in a Beckman gamma 4000 counter. As in all α-[^{125}I]BuTx site assays, the values obtained were corrected for background binding of α-[^{125}I]BuTx to DEAE disks.

Electron Microscopy. In preparation for EM studies membrane suspensions were negatively stained in the following manner. Ten microliters of alkali-treated membranes (0.15 mg/mL protein) was placed on a carbon-coated copper grid for 1 min and then dried on filter paper. The grid was stained with 10 mL of 2% phosphotungstic acid, pH 7.4, for 2 min and excess solution was removed by placing the grid on filter paper. For staining of reconstituted AcChR membranes, reconstituted preparations were diluted 10-fold into 2% phosphotungstic acid, pH 7.4, and incubated for 5 min. Ten-microliter aliquots were transferred to a carbon-coated copper grid. After 1 min, excess solution was removed with filter paper. Following negative staining, all samples were examined in a Phillips 201 electron microscope.

Results

Purification of Membranes. Membranes purified in reorienting sucrose gradients (Elliott et al., 1980) are highly enriched in acetylcholine receptor and typically contain 1.0–1.5 nmol of α-BuTx sites per mg of protein. However, at least two major nonreceptor polypeptides, of mol wt 40×10^3 and 90×10^3, in addition to several minor ones, copurify with the membranes. When such preparations were subjected to alkaline pH followed by centrifugation, essentially all of the nonreceptor polypeptides were separated from intact AcChR membranes. Following centrifugation, the receptor protein and the contaminating polypeptides were differentially recovered in three distinct fractions; the 43×10^3 dalton polypeptide was recovered in the supernatant while over 95% of the 90×10^3 dalton species occurred in a light particulate fraction ("soft pellet") that sedimented on top of the dense membrane pellet. The "soft pellet" was also enriched in the two minor polypeptides of mol wt less than 40×10^3 daltons. A scan of an $NaDodSO_4$ gel revealed the high purity of membranes extracted twice with alkali, which were essentially devoid of any nonreceptor polypeptides (Wu & Raftery, 1979), with the possible exception of trace amounts of the 90×10^3 dalton species. Such alkali-treated membranes typically represent a 3–4-fold increase in specific α-BuTx binding activity over the corresponding untreated preparation. Preparations treated twice with alkali were used for all reconstitution studies reported here. It was also shown that residual small amounts of the contaminating 90×10^3 dalton species were further reduced (Figure 1) when the treated membranes were rerun through the same type of reorienting sucrose gradient

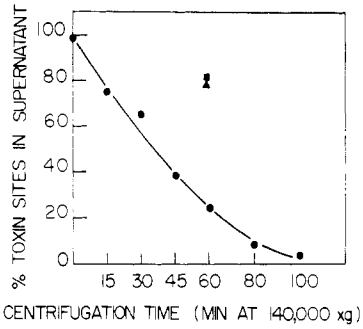

FIGURE 2: Sedimentation at 140000g of AcChR in cholate extract. Membranes (1.3 mg/mL protein) were solubilized in 2% sodium cholate, 200 mM NaCl, and 10 mM Tris-Cl, pH 7.4, containing either 20 (●), 2.67 (▲), or 0 mg/mL (■) of asolectin as described under Methods and then centrifuged in a Beckman type 65 rotor at 40000 rpm for the indicated time periods.

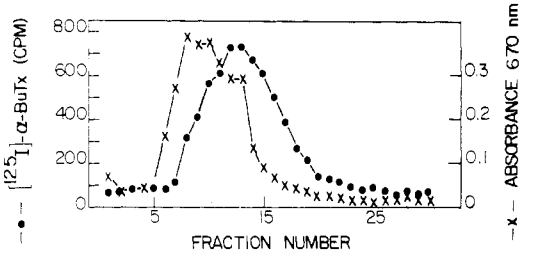

FIGURE 3: Sepharose 4B column chromatograph of AcChR cholate extract. Alkali-treated membranes were solubilized as described under Methods, in 2% sodium cholate solution containing 20 mg/mL asolectin and centrifuged for 1 h. To a 200-μL aliquot of the supernatant was added 23 ng of α-[^{125}I]BuTx and the mixture was incubated for 30 min at room temperature. Small quantities of crystalline Blue Dextran were dissolved before the mixture was layered on a Sepharose 4B column (2 × 12 cm) that had been preequilibrated with 10 mM Tris, pH 7.4, 200 mM NaCl, 2% sodium cholate, and 20 mg/mL sonicated asolectin. The material was eluted with the same solution and 30–12-drop fractions (0.32 mL) were collected. The fractions were counted for ^{125}I on a Beckman gamma counter and were assayed for Blue Dextran content by determining absorbance at 670 nm.

used in purification of the receptor-enriched membranes.

Cholate Solubilization of AcChR. Initially, extraction of the AcChR was achieved by solubilizing alkali treated membranes in 2% (w/v) sodium cholate in the presence of a 15-fold (w/v) excess of asolectin over protein. Prior to dialysis, the extract was centrifuged at 140000g for 1 h to remove any undissolved material. The AcChR in such detergent extracts apparently occurred as relatively large aggregates, since sedimentation of the α-BuTx binding component from the detergent solution was shown to occur rapidly at 140000g (Figure 2). However, the bulk of such "solubilized" structures is apparently smaller than the 2×10^6 dalton Blue Dextran when chromatographed through a Sepharose 4B column (Figure 3). Despite this we still could not rule out the possibility that the AcChR was composed of small intact membrane fragments in the detergent "extract". In an attempt to rule out such a possibility, alkali-treated membranes were solubilized in solutions of higher cholate concentrations while the same lipid concentration was maintained. Table I shows that following centrifugation the α-BuTx sites recovered in the supernatant solutions of cholate up to 6% did not differ significantly, indicating that 2% cholate was at or above the maximal required level for solubilization.

In order to achieve meaningful reconstitution, it must be demonstrated unequivocally that all of the receptor protein extracted in detergent was solubilized, i.e., that the receptor was present in the detergent solution as discrete structures with sizes comparable to those previously characterized in Triton

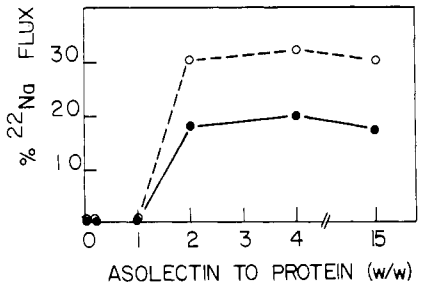

FIGURE 4: Lipid requirement in solubilization of AcChR. The dependence of detergent-solubilized AcChR functionality on the presence of asolectin was investigated by assaying the Carb-dependent excitability of membranes reconstituted from cholate extracts containing varying concentrations of asolectin. Alkali-treated membranes (1.3 mg of protein/mL) were solubilized in cholate solutions containing asolectin concentrations from 0 to 20 mg/mL, as described under Methods. The resulting reconstituted membrane preparations were assayed for Carb- and gramicidin-induced $^{22}Na^+$ efflux and the signals observed (average of four time points) were plotted against the corresponding concentrations of asolectin. The Carb-activated $^{22}Na^+$ flux signals are expressed here as percent of total entrapped counts (●) and as percent of gramicidin-releasable counts (○), while lipid concentrations are given in terms of asolectin:protein ratios (w/w).

Table I: Effect of Cholate Concentration and Omission of Asolectin on Recovery of α-[^{125}I]BuTx Sites in Cholate Supernatant

Na cholate concn (%)	asolectin:protein ratio	α-[^{125}I]BuTx recovery in cholate supernatant (%)
2	0:1	81.4
2	0:1-15:1[a]	69.8
2	15:1	22.8
3	15:1	27.8
4	15:1	24.9
6	15:1	19.8

[a] Membranes were solubilized in an asolectin:protein ratio of 0:1 (no added lipid) but following centrifugation at 140000*g* for 1 h to the supernatant was added asolectin to yield a final ratio of 15:1. In this case, toxin sites were assayed for the supernatant after asolectin addition.

X-100 solutions. This could be demonstrated by determining the sedimentation coefficient values for the α-BuTx binding components in the 2% cholate extract. Unfortunately, attempts to perform such determinations using sucrose gradients containing 2% cholate and equivalent lipid concentrations (20 mg/mL) did not yield meaningful results, because of the high lipid content. A minimal lipid concentration required to stabilize the receptor during cholate solubilization was determined to circumvent this problem. The plot in Figure 4 shows the relative excitability (in terms of Carb-induced $^{22}Na^+$ efflux) of membranes reconstituted from AcChR solubilized in the presence of various concentrations of asolectin. The results demonstrate that functional reconstituted membranes were obtained from receptor solubilized in cholate solutions that contained a lipid:protein ratio of 2 (w/w) (2.67 mg/mL in asolectin) or higher. In these experiments, lipids were again added to a final concentration of 15:1 (asolectin:protein) after centrifugation. This was necessary in order to obtain vesicles with appreciable interior volume. By use of the minimal required lipid to protein ratio demonstrated here, excitable AcChR membranes were reconstituted reproducibly, as shown here (Figure 7B,C) and previously (Wu & Raftery, 1979) for a higher lipid concentration (asolectin:protein 15:1).

AcChR solubilized in minimal lipid–cholate solution was subsequently used in experiments to determine sedimentation coefficient values. Figure 5 shows the results of such a determination, which utilized a 4–20% sucrose gradient containing 2% cholate and the same lipid concentration (2.67 mg/mL) present in the cholate extract. Two α-BuTx binding peaks of 14.7 S and 10.5 S representing the AcChR dimer and monomer, respectively, were observed. The *s* values for these species are slightly higher than the 13.7 and 9.0 S reported previously (Raftery et al., 1972) for AcChR solubilized in Triton X-100 free of exogenous lipids. The higher values observed here are possibly due to formation of larger AcChR lipid–detergent micelles.

Demonstration of AcChR Association with Lipids. When the detergent was removed by dialysis, it could be seen that a marked increase in light scattering of the reconstituted sample had occurred, usually mainfested as a 5-fold increase in absorbance at 450 nm. Direct demonstration of the formation of an AcChR–lipid complex was achieved by equilibrium centrifugation of reconstituted receptor–lipid preparations through a 5–27% sucrose density gradient. As shown in Figure 6C, the α-[^{125}I]BuTx binding and the [^{3}H]PC profiles dem-

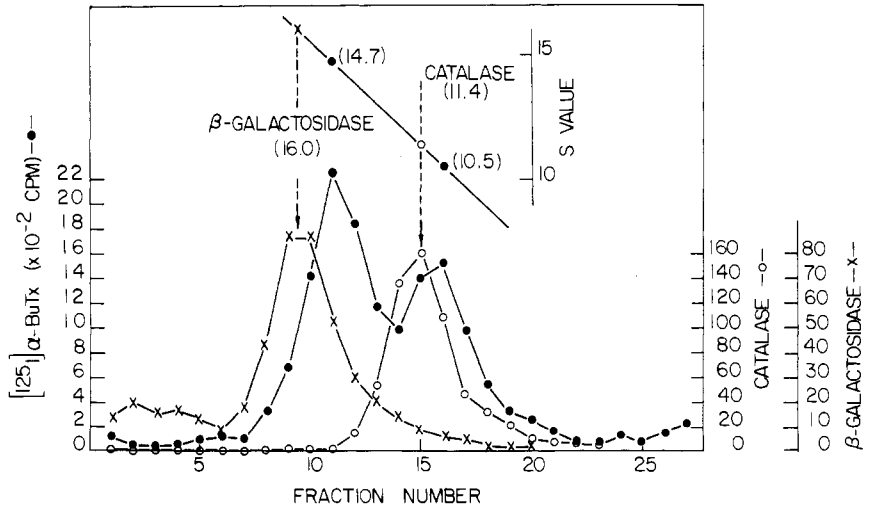

FIGURE 5: *s* value determination of solubilized AcChR. To an aliqout (50 μL) of a 2% cholate extract of AcChR (asolectin:protein ratio of 2) were added 0.5 mg of β-galactosidase and 0.8 mg of catalase. The sample was layered on a 12-mL 4–20% (w/v) linear sucrose gradient containing 10 mM Tris, pH 7.4, 200 mM NaCl, 2% sodium cholate, and 2.67 mg/mL sonicated asolectin. The gradient was centrifuged in a Beckman SW 41 rotor at 40000 rpm (195700*g*) for 14.5 h. Following centrifugation, 18-drop fractions (0.5 mL) were collected. The fractions were assayed for α-[^{125}I]BuTx sites (●) and for the enzymatic activities of catalase (○) and β-galactosidase (×), which are expressed here as $\Delta A_{240\ nm}$/15 s and $\Delta A_{405\ nm}$/15 s, respectively.

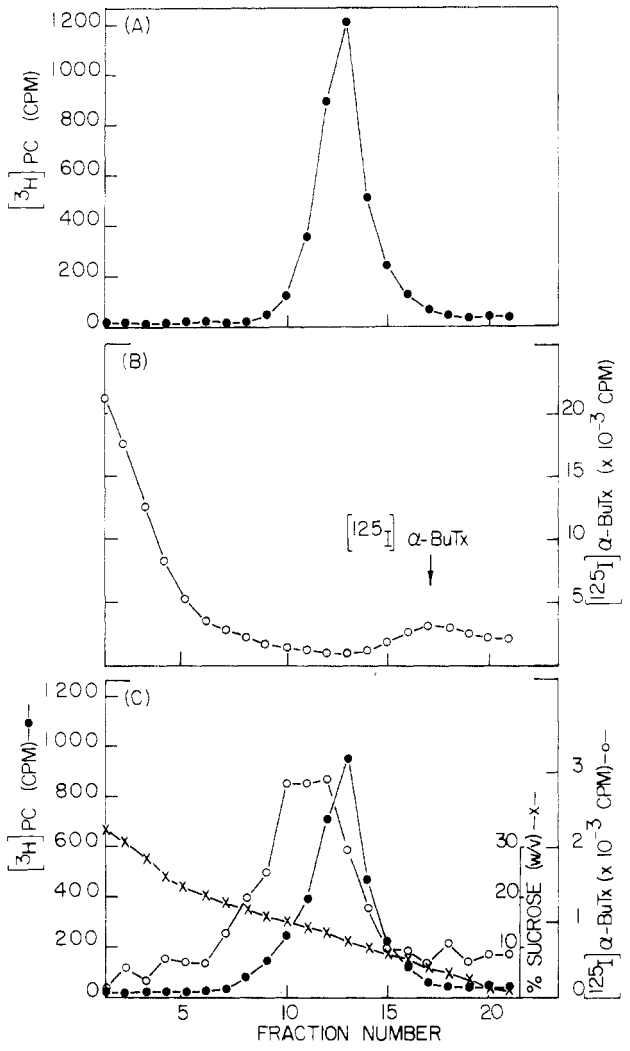

FIGURE 6: Linear sucrose gradient (5–27%, w/v) profiles of reconstituted membranes. (A) Sucrose gradient profile of AcChR-free liposomes prepared in a manner similar to the procedure for the reconstitution of AcChR membranes. (B and C) Profiles of AcChR reconstituted with 0 and 20 mg/mL asolectin, respectively. [^{3}H]PC was added in trace amounts to aqueous asolectin suspensions prior to sonication. The sucrose gradients were in 200 mM NaCl and 10 mM Tris, pH 7.4, and were centrifuged in a Beckman SW 41 rotor at 40000 rpm for 16 h. Fractions of 15 drops (0.57 mL) were collected and were counted for ^{3}H in aquasol and for ^{125}I on a Beckman gamma counter.

onstrate cosedimentation of the toxin binding peak with the denser fraction of the lipids. Similar centrifugations were also carried out for protein-free liposomes (Figure 6A) and for AcChR "reconstituted" in the absence of exogenous lipids, which sedimented to the bottom of the gradient (Figure 6B).

Orientation of AcChR in Reconstituted Membrane Vesicles. Formation of receptor–lipid vesicles could occur such that the AcChR-associated ligand binding site(s) faced either in or out. For determination of the proportion of receptors reconstituted with the desired orientation for interaction with cholinergic ligands (ligand binding sites exposed on the outside), experiments were carried out to assay the α-[^{125}I]BuTx sites accessible from the exterior face relative to the total sites. The assay (described under Materials and Methods), originally used for studies of both reconstituted (Michaelson & Raftery, 1974) or intact native membrane vesicles (Hartig & Raftery, 1979), involved saturation of externally exposed sites on reconstituted vesicles with α-[^{125}I]BuTx followed by membrane disruption with detergents to expose sites on the vesicle interior for binding of either ^{125}I-labeled or excess unlabeled α-BuTx

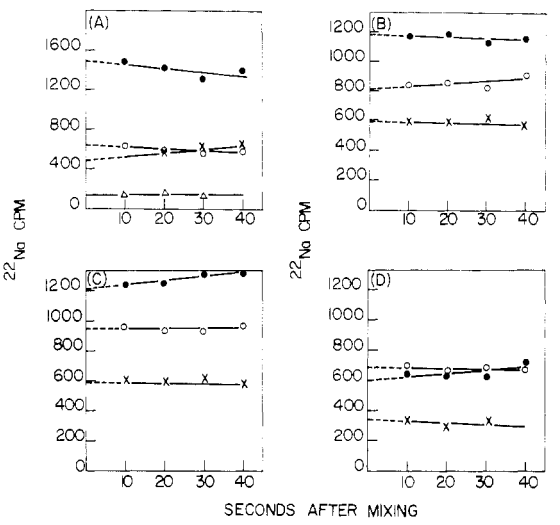

FIGURE 7: Carbamoylcholine- and gramicidin-induced $^{22}Na^+$ efflux from alkali-treated and reconstituted membrane vesicles. (A) Alkali-treated membranes or (B and C) reconstituted AcChR-containing membrane vesicles preequilibrated with $^{22}Na^+$ were isotonically diluted 20-fold at time zero into buffer (10 mM Tris-Cl, pH 7.4) [for (A)] or dilution buffer (200 mM NaCl in 10 mM Tris-HCl, pH 7.4) [for (B) and (C)], respectively, containing 100 (○) or 0 μM (●) Carb or 10 μg/mL gramicidin D (×). Time points were taken as described under Methods for the $^{22}Na^+$ flux assay of reconstituted preparations. (D) $^{22}Na^+$ efflux from AcChR-free liposomes. Liposomes were prepared in the same way as reconstituted AcChR vesicles except without addition of AcChR. The phospholipid vesicles were assayed for $^{22}Na^+$ efflux by isotonically diluting the preparation preequilibrated with the isotope into dilution buffer containing 100 (○) or 0 μM (●) Carb or 10 μg/mL gramicidin D (×) and assayed for $^{22}Na^+$ retained as a function of time as described under Methods. Counts obtained for each time point were corrected for dilution errors by directly counting duplicate aliquots removed from each diluted radioactive suspension. All curves were fit to a single exponential.

to prevent binding of α-[^{125}I]toxin. Thus, the sample exposed to the large excess of unlabeled α-BuTx after membrane disruption contains only exterior sites radiolabeled, while the sample exposed to α-[^{125}I]BuTx throughout yields the total sites. When a reconstituted receptor sample was assayed by this method, it was found that 87% of the α-BuTx binding sites were on the exterior surface. A similar value of 90% was obtained for a preparation reconstituted from a cholate extract containing the minimal required lipids. This extent of binding sites exposed on the exterior of the vesicle precludes the possibility of significant amounts of the reconstituted AcChR being oriented with binding sites on the interior membrane surface or being associated with multilamellar structures.

Functionality and Pharmacology of Reconstituted AcChR. Reconstituted AcChR–lipid preparations were shown to contain significant interior volume, as indicated by the gramicidin-mediated release of 50% of the total entrapped $^{22}Na^+$. Figure 7B,C shows that in duplicate experiments these reconstituted AcChR vesicles responded to carbamoylcholine by releasing 27% and 26% of the total entrapped $^{22}Na^+$, respectively. The Carb-activated $^{22}Na^+$ efflux from reconstituted membranes was rapid in onset and was complete prior to the first time point taken, as demonstrated for native membranes (Figure 7A, Miller et al. (1978) and Moore et al. (1979a)]. The size of such Carb-induced signals was reproducible for a given reconstituted preparation but varied somewhat among different preparations.

The Carb-induced effect typically represented ~40% of the total gramicidin-releasable pool of $^{22}Na^+$; most of the remaining 60% was likely stored in vesicles lacking functional AcChR. Liposomes without added AcChR prepared in a manner similar to the reconstitution procedure, by using the

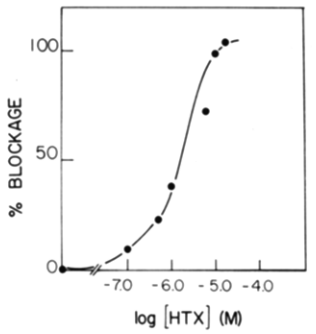

FIGURE 8: Dose–response blockage by H_{12}-HTX of Carb-induced $^{22}Na^+$ efflux from reconstituted AcChR vesicles. Vesicle preparations were incubated with various concentrations of H_{12}-HTX at 0 °C for 30 min prior to isotonic dilution into dilution buffer containing 100 μM Carb and the same H_{12}-HTX concentrations as the corresponding incubation medium. HTX blockage in each case was determined from the difference between the $^{22}Na^+$ released in the presence of the given HTX concentration and that released in the absence of HTX. The percent of maximum blockage is plotted here against the log of the added HTX concentration. The response was determined from the average of four time points from each flux assay.

same concentration of added lipids, apparently contained smaller interior volume when monitored by gramicidin (Figure 7D). Although the gramicidin-released counts in liposomes represented a similar fraction of the total entrapped $^{22}Na^+$ when compared with that of the AcChR-containing preparations, the absolute size of the signal, which is a more accurate estimate of total vesicular interior volume, was smaller. EM studies of negatively stained samples which revealed significantly larger vesicles formed in those preparations reconstituted with AcChR were consistent with this observation. Addition of Carb to liposomes did not affect the permeability of the entrapped $^{22}Na^+$, demonstrating that the ligand was not acting nonspecifically on lipids to produce the Carb effect observed in Figure 7B,C.

In addition to the Carb-dependent excitability, the reconstituted AcChR vesicles also exhibited the expected pharmacology for a nicotinic AcCh receptor. The Carb-induced efflux of $^{22}Na^+$ from reconstituted membranes was completely blocked by α-BuTx as well as by HTX. Carb-induced desensitization of the reconstituted system also abolished the Carb-activated efflux, demonstrating that the mechanism responsible for the desensitization phenomenon was apparently restored. As previously demonstrated (Wu & Raftery, 1979) the reconstituted AcChR responded to Carb in a concentration-dependent manner. The preparation also exhibited a dose-dependent blockage by HTX of Carb-induced sodium efflux (Figure 8).

Electron Microscopy Studies. Alkali-treated membranes (Figure 9A) exhibited the arrays of densely packed rosette structures (65–85 Å) that are characteristic of individual membrane-bound acetylcholine receptor molecules. Examination of reconstituted AcChR preparations by EM (Figure 9B) revealed the expected spherical structures that were significantly smaller than the alkali-treated membranes. The reconstituted vesicles appear to be fairly homogeneous in size: 80–90% of the spherical structures were within the range of 400–600 Å in diameter, roughly one-tenth that of purified native membrane vesicles. A few reconstituted vesicles larger than 1000 Å were also observed.

Discussion

The electroplax membrane preparation from *Torpedo californica* which we describe here and use for all the reconstitution procedures has been shown to contain only the ace-

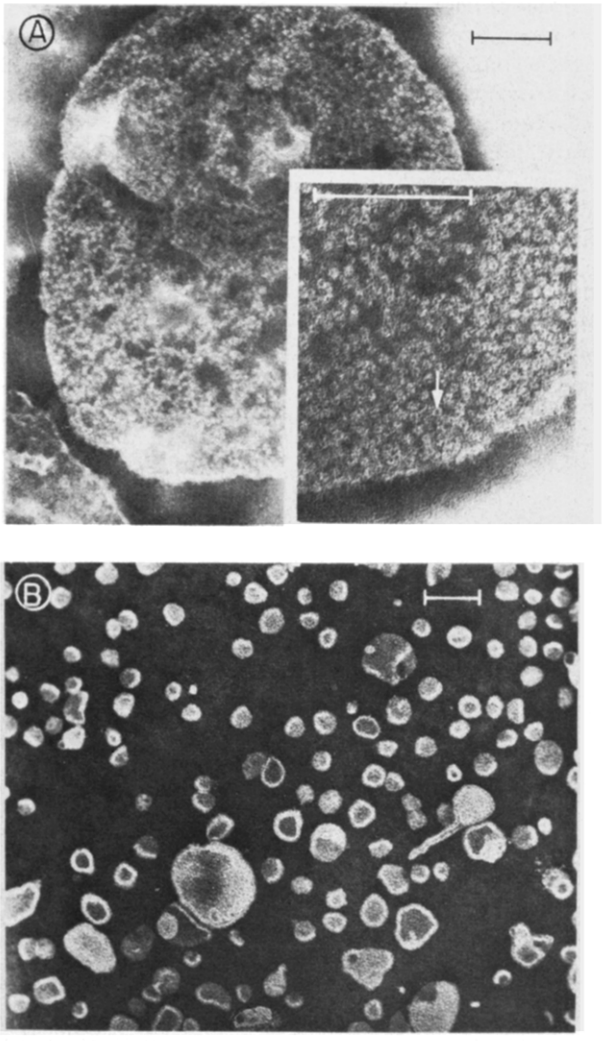

FIGURE 9: Electron micrographs of negatively stained preparations of alkali-treated membranes (A) and reconstituted AcChR membrane vesicles (B). Arrow points to one of the array of rosettes characteristic of individual AcChR molecules which are clearly visible on the surface of native membranes. Each bar represents 10^3 Å.

tylcholine receptor protein with no significant contamination by other polypeptide species (Figure 1). The initial sucrose density gradient centrifugation procedures (Duguid & Raftery, 1973) have been improved by minimizing the amount of time necessary for the purification by use of a reorienting gradient rotor (Elliott et al., 1980). The receptor-enriched preparation obtained by this method contains 15–20% of its total protein as AcChR. This can be further purified by extraction of nonreceptor peptides by exposure of the preparation to pH 11 at low ionic strength for short periods of time (Neubig et al., 1979; Elliott et al., 1979) as shown in Figure 1. The resultant membrane preparation contains the four polypeptides characteristic of the AcChR and no other protein components in significant amounts. The specific activities of these membranes are similar to the highest of those previously reported for membrane-bound preparations (Neubig et al., 1979; Huganir et al., 1979; Heidmann et al., 1980). Although analysis on $NaDodSO_4$–polyacrylamide gels reveals a comparable pattern of purity, the purified membranes are of lower specific α-BuTx binding activity than those of the AcChR purified by affinity chromatography from solubilized preparations. It is known (Moore et al., 1979b) that no significant loss of α-BuTx sites occurs in alkali treatment; total conservation of the sites was achieved in the various steps of the treatment. In any case, the $NaDodSO_4$–polyacrylamide gel analysis reveals the oc-

currence of only the four bands characteristic of the AcChR in these membranes. In addition, N-terminal amino acid sequence analysis of these four bands has shown each band to correspond to a single polypeptide (Raftery et al., 1980), and sequence analysis of whole alkali-treated membrane preparations has shown that no other protein species are present in the membranes. These alkali-extracted membranes have been shown to exhibit unaltered properties when compared with the starting material: (i) the rate for α-[^{125}I]BuTx receptor complex formation is unaltered (Elliott et al., 1979); (ii) a local anesthetic analogue (Neubig et al., 1979) and [^{3}H]H_{12}-HTX (Elliott et al., 1979) bindings are unaffected. In addition, Carb-induced translocation of $^{22}Na^+$ (Neubig et al., 1979; Moore et al., 1979a) was unaffected and had similar pharmacological properties (Moore et al., 1979a). These membranes represent a preparation most suitable for reconstitution studies since time-consuming chromatographic and analytical procedures are unnecessary. In addition, if specific phospholipids are required, such species are not lost due to chromatographic procedures.

A necessary prerequisite for meaningful reconstitution is that the native membrane be dissociated and that the protein to be reconstituted exists in solution as a monodisperse species. Toward this end we have defined the conditions necessary for obtaining receptor in sodium cholate solution in forms characteristic of the purified receptor protein, i.e., 13.7 and 9 S species, to indicate that the membrane was indeed disrupted and that the AcChR, while not necessarily free of lipid, was not present as a small membrane fragment or as a form aggregated to an extent greater than the dimer. The results shown in Figure 5 confirm this by demonstrating the absence of toxin binding components larger than that of the receptor dimer.

As observed previously with native membranes, the $^{22}Na^+$ flux induced by carbamoylcholine was complete by the first time point at 10 s, indicating that a rapid efflux of $^{22}Na^+$ had occurred (Figure 7). Several characteristics of the flux induced by carbamoylcholine support the notion that this was a specific effect: it was not observed in liposomes which did not contain AcChR, it was blocked by α-BuTx and by histrionicotoxin, and the effect was also abolished by preincubation with carbamoylcholine. This latter result indicates that agonist-induced AcChR desensitization had occurred. A dose–response curve was obtained for H_{12}-HTX blockage of the carbamoylcholine-induced efflux, using high concentrations of carbamoylcholine. The midpoint of this curve at 1.8 μM is close to the value obtained for the dissociation constant of HTX from such alkaline-treated membranes as measured by direct binding of [^{3}H]H_{12}-HTX (Elliott et al., 1979).

Successful reconstitution of AcChR from membranes containing proteins other than those characteristic of AcChR has been previously reported (Hazelbauer & Changeux, 1974; Epstein & Racker, 1978), as have partially successful reconstitution studies of purified AcChR (Michaelson & Raftery, 1974). We have recently presented a preliminary account (Wu & Raftery, 1979) of wholly reproducible studies using purified membrane-bound receptor. Changeux et al. (1979) and Huganir et al. (1979) have also described preliminary studies of Carb-induced $^{22}Na^+$ flux in a reconstituted system derived from alkali-treated membranes. The proportion of the total AcChR that was reconstituted functionally in mediating cation translocation in response to an agonist is an important question not previously addressed. The $^{22}Na^+$ efflux studies reported here provide an adequate means to assess the functional fraction of AcChR since (i) the agonist-induced signal ($^{22}Na^+$ efflux) was obtained as a percentage of the total entrapped $^{22}Na^+$ and (ii) use of an artificially added ion channel, such as gramicidin, allowed determination of the fraction of the total entrapped $^{22}Na^+$ that was mobile.

The average diameter of the reconstituted vesicles has been determined by electron microscopy. When the known concentrations of AcChR molecules and of phospholipids in the reconstituted preparation are used, the number of AcChR per vesicle was calculated (see Appendix). From this the fraction of vesicles that contain AcChR was obtained; this, when compared with the experimentally observed Na^+ transport signal, revealed that most of the AcChR in the preparation was functional. The functional fraction calculated represents an estimate since some parameters, such as the surface area of the phospholipids used in the calculations, cannot be determined precisely. Nevertheless, the calculations indicate that a large proportion of the AcChR was functional in mediating agonist-induced Na^+ translocation.

The correlation of polypeptide composition with specific function is the central question which can possibly be answered by reconstitution experiments such as those described here. Polypeptides other than those known to be constituent subunits of the AcChR were present in the purified membrane preparations used for reconstitution in only trace amounts (i.e., 1–3% level). It is nevertheless important to determine whether any function is attributable to such minor components. As noted by Moore et al. (1979b), such components, if essential, could occur in native membranes to form active AcChR complexes at a sufficient level to release the total vesicular content of $^{22}Na^+$ when monitored by filtration methods on the time scale of seconds, since native membranes have a very high density of AcChR ($\sim 5 \times 50^3$ receptors/vesicle). The reconstituted AcChR described here, with one or a few molecules per vesicle, provides a suitable preparation to assess the possible role of such minor contaminating polypeptides since the removal of an essential molecular component is expected to lead to failure to flux cations. From the calculations (see Appendix) it is estimated that a large proportion of the total AcChR was functional in our reconstituted preparation. Thus, we find it unlikely for a contaminating polypeptide removed to residual amounts of 1–3% to play an essential role in mediating the cation flux we observed.

These results, therefore, confirm other indications (Moore et al., 1979a; Moore & Raftery, 1980) that the 43×10^3 and the 90×10^3 daltons species, as well as other polypeptides that were substantially removed during purification of the AcChR membranes, were not essential for the AcChR-mediated cation translocation.

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Appendix

Estimation of the Fraction of Reconstituted AcChR That Is Functional in Mediating Carb-Induced $^{22}Na^+$ Flux. The fraction of reconstituted receptors that is functional can be estimated by dividing the fraction of vesicles that exhibited Carb-induced $^{22}Na^+$ flux (determined experimentally) by the fraction (calculated) of the vesicles that contain receptor.

The proportion of AcChR-containing vesicles is calculated as follows: For a vesicle 500 Å in diameter and 50 Å in bilayer

thickness, the surface areas exposed to the outer and inner media are 7.85×10^{-15} m^2 and 5.03×10^{-15} m^2, respectively. If the polar group of each phospholipid of asolectin occupies 55 Å^2 (S. Chan, personal communication; Chrzeszczyk et al., 1977), there are $[(7.85 + 5) \times 10^{-15}\ m^2 \div 5.5 \times 10^{-19}\ m^2] = 2.33 \times 10^4$ phospholipid molecules per vesicle. From the experimentally determined concentrations (for preparations solubilized in minimal asolectin) of phospholipid (1.67×10^{-2} M) and α-BuTx sites (1.2×10^{-6} M) (all determined following reconstitution), 1 α-BuTx site or 0.5 receptor monomer per 1.4×10^4 phospholipid molecules was obtained (assuming that 2 α-BuTx sites are associated with each monomer in the reconstituted preparation). This means that on the average 0.83 AcChR monomer is associated with one vesicle. From the actual distribution of AcChR occurring as monomer and dimer (shown in Figure 5) and taking into account that 90% of the α-BuTx sites face the exterior of vesicle surface, we obtain 0.52 as the effective number of AcChR oligomer per vesicle. If the distribution of receptors among vesicles follows a Poisson-type behavior (N. Davidson, personal communication), the fraction of total vesicles containing at least 1 AcChR oligomer is 0.41.

Experimentally, it was determined that Carb activated the release of 40% of the $^{22}Na^+$ trapped in the vesicle interior. The total entrapped $^{22}Na^+$ is defined as the radioactivity released by gramicidin. Thus, 40% of the reconstituted vesicles translocated $^{22}Na^+$ in response to Carb under the experimental conditions.

Comparison of the percentage of vesicles that exhibited $^{22}Na^+$ efflux with the fraction of vesicles that contained AcChR reveals that most (97% calculated here) of the AcChR in the reconstituted preparation was active in Na^+ translocation.

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