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Purified Acetylcholine Receptor: Its Reconstitution to a Chemically Excitable Membrane*

(acetylcholine binding/ion translocation)

DANIEL M. MICHAELSON AND MICHAEL A. RAFTERY

Church Laboratory of Chemical Biology, Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, Calif. 91109

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ABSTRACT **Association of purified acetylcholine receptor from *Torpedo californica* electroplax with lipids from the same organism results in a vesicular membrane system in which the receptor protein is oriented so that all neurotoxin binding sites appear to be on the outer surface. The reconstituted system is chemically excitable by acetylcholine and carbamylcholine, as measured by $^{22}Na^+$ efflux. This excitability is specifically blocked by the antagonist α-bungarotoxin. These results demonstrate that the purified receptor macromolecule contains not only the specific neurotransmitter binding site but also the molecular elements necessary for ion translocation in order to effect postsynaptic depolarization.**

Postsynaptic depolarization at the vertebrate neuromuscular junction or in the electric organs of *Torpedo* species and *Electrophorus electricus* is mediated by the neurotransmitter acetylcholine. Two elementary steps have been recognized in this process: (*a*) interaction of the neurotransmitter with its macromolecular receptor and (*b*) cation translocation across the postsynaptic membrane to effect depolarization. The macromolecular receptor for acetylcholine has been identified and purified recently in a number of laboratories (1–9, 12) and its binding of cholinergic ligands has been clearly demonstrated (10–13). To date it is not known whether this purified receptor contains the molecular apparatus necessary for ion translocation, be this an ionophore, an ion channel, or another structural element. This important question can be answered only by functional reconstitution of purified acetylcholine receptor complex (AcChR) in a membrane system, since ion translocation is a membrane-specific phenomenon. Recently, regeneration of membrane-specific functions have been reported for several systems other than that considered here (14–19). A reasonable approach for study of membrane-specific functionality of AcChR is provided by the assay system used by Kasai and Changeux (20) for study of $^{22}Na^+$ flux from membrane fragments of *Electrophorus electricus* electroplax. Their assay has been recently extended to membrane fragments from *Torpedo marmorata*, which also have been shown to form sealed vesicles, to accumulate $^{22}Na^+$, and to respond to addition of carbamylcholine with increase in the rate of release of trapped $^{22}Na^+$ (21). The work of Changeux and his colleagues showed that electroplax membrane fragments contain all of the molecular apparatus necessary for neurotransmitter recognition and ion translocation. Moreover, these authors (21) have demonstrated that, after dissolution of membrane fragments by sodium cholate, removal of the detergent allowed reformation of membrane structures with renaturation of a chemically excitable membrane. These results show that, in principle, reconstitution is possible.

Since AcChR-rich membrane fragments from *Torpedo* electroplax (21, 22, 33) contain protein components other than AcChR, the question of whether the AcChR macromolecule alone was responsible for neurotransmitter recognition *and* ion translocation is unanswered. In this communication we describe the successful reconstitution of isolated purified AcChR with *Torpedo* lipids to form sealed vesicles which retain $^{22}Na^+$ and which respond to acetylcholine and to carbamylcholine by an increase in $^{22}Na^+$ efflux. This effect can be completely abolished by the irreversible cholinergic antagonist α-bungarotoxin (α-Bgt).

EXPERIMENTAL

Materials

AcChR was prepared by published procedures employing affinity chromatography (1, 2). *Torpedo californica* was obtained from Pacific Biomarine Labs, Venice, Calif. *Torpedo* lipids were prepared as described below. [^{3}H]Triton X-100 was a gift from Rohm and Haas. [^{14}C]Cholic acid, sodium salt, and ^{22}NaCl were purchased from the Radiochemical Centre, Amersham, Great Britain. [^{3}H]Phosphatidylcholine was obtained from Dhom Products Limited.

Methods

Detergent Exchange. About 15 mg of purified AcChR in 10 mM sodium phosphate buffer, pH 7.4, containing 30 mM NaCl and 0.1% Triton X-100 were loaded on a DEAE-cellulose column (inside diameter 2.5 cm, height 10 cm). The column was first washed (three column volumes) with 10 mM Tris·HCl buffer, pH 7.4, containing 100 mM NaCl and 0.5% sodium cholate, after which the cholate concentration was raised to 2% and washing was continued for approximately 20 hr at a flow rate of 1.5 ml/min. The receptor was eluted with 0.5 M NaCl in 10 mM Tris, pH 7.4, containing 2% cholate. The eluted receptor, about 10 mg in 50 ml, was concentrated with an XM-50 Amicon membrane and then resuspended in 10 mM Tris buffer at pH 7.4. The receptor had a lower α-Bgt binding (23) in cholate than in Triton. This effect is reversed by excess Triton, consequently toxin binding activity of the receptor in cholate was monitored in the presence of about a fourfold excess of Triton X-100.

Abbreviations: AcChR, acetylcholine receptor complex; α-Bgt, α-bungarotoxin.

* Contribution no. 4947.

TABLE 1. *Torpedo lipids—composition*

	% of total phospholipids*
Phosphatidylcholine	40.6
Phosphatidylethanolamine	29.1
Phosphatidylserine	10.7
Sphingomyelin	9.3
Phosphatidylinositol	1.7
Cardiolipin	0.9
Phosphatidic acid	0.9

* The lipid extract contained 75% phospholipids and 25% neutral lipids.

Extraction and Sonication of Lipids. Lipids were extracted from *Torpedo* electric organ. The procedure was similar to that employed in the purification of the AcChR except that instead of the Triton extraction step the lipids were chloroform–methanol extracted following the Bligh and Dyer method (24). The lipid extract was further purified by passage through a Sephadex G-50 column employing organic solvents (25). The eluted lipids were 75% phospholipids, as determined by phosphate analysis, and 25% neutral lipids. They were dissolved in chloroform and stored under argon at −20°. Sonicated *Torpedo* lipids in 10 mM Tris buffer, pH 7.4, containing 200 mM NaCl were prepared as follows. The lipids, approximately 50 mg in chloroform, were evaporated to dryness in a round bottom flask. About 5 ml of buffer and two glass beads were added to the flask which was then vortexed for 10 min. The milky suspension was transferred to a conical centrifuge tube and sonicated in a bath (Laboratory Supplies Co., Inc.) for 1 hr at 4° under argon.

Reconstitution of Lipids and Receptor. Reconstitution was achieved by mixing sonicated *Torpedo* lipids with AcChR in cholate (lipid:protein = 10:1 w/w) and dialyzing at room temperature against 200 mM NaCl in 10 mM Tris, pH 7.4, containing 0.02% Na azide and 2 mg/liter of butylated hydroxytoluene. The sample was dialyzed for 68 hr with eight buffer changes.

After dialysis the reconstituted material was more opaque than at the beginning. The product was concentrated about 2-fold by placing the dialysis bags in contact with Bio-gel P-300 beads. The preparation was spun for 15 min on a desk centrifuge to pellet some precipitated material. The supernatant, which contained most of the lipid and protein, was employed in the ^{22}Na efflux studies.

Measurement of ^{22}Na Efflux. ^{22}Na efflux was measured by adsorbing aliquots (0.5 ml) of the reconstituted vesicle preparation onto tightly packed DEAE-cellulose columns and by elution of the internal ^{22}Na with Triton. For $^{22}Na^+$ flux the preparation was preincubated overnight at room temperature with $^{22}NaCl$ at about 2×10^7 cpm/ml. Assays were conducted by dilution of the preparation 20-fold with the same medium (buffer and unlabeled NaCl) with or without a cholinergic effector. At desired time intervals 0.5-ml samples were rapidly adsorbed to columns (vacuum packed DEAE-cellulose, 1.5 ml), and washed within 1 min with 4 ml of buffer. ^{22}Na retained by the vesicles was eluted with 3 ml of 1% Triton X-100 in 10 mM Tris, pH 7.4, after which the eluate was dried by air-flow and ^{22}Na measured in a dioxane-based scintillation fluid. In the experiments where α-Bgt was employed, the sample was pre-incubated for 1 hr at room temperature with a 3-fold excess of toxin to toxin binding sites.

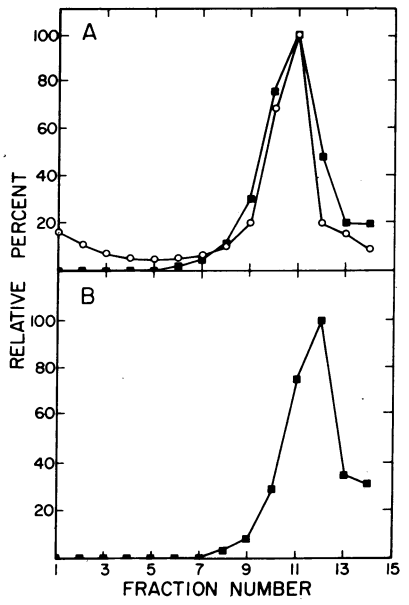

FIG. 1. Sucrose density profiles of reconstituted AcChR vesicles (A) and of sonicated lipid vesicles (B). Lipid concentration (■) was monitored by [^{3}H]phosphatidylcholine, a trace of which was added to the *Torpedo* lipids prior to sonication. α-Bgt sites (○) were assayed as previously described (23). The scale for the ordinate is such that 100% corresponds respectively to the highest concentration of [^{3}H]phosphatidylcholine and to the maximum α-Bgt binding. The gradient is from 8 to 27% sucrose in 10 mM Tris, pH 7.4, containing 200 mM NaCl; it was spun in a Spinco SW 41 rotor at 40,000 rpm for 18 hrs. Fraction 1 is bottom of gradient.

Analytical Methods. Quantitation of ^{125}I-labeled α-Bgt was conducted by the DEAE-paper disc method of Schmidt and Raftery (23). Phosphate analysis was done by standard procedures, using ammonium molybdate.

RESULTS

Purity of Materials. The AcChR isolated from *Torpedo californica* and used in these reconstitution experiments had previously been characterized as a single macromolecular species by gel electrophoresis under nondenaturing conditions (3, 29). It has also been shown, by sodium dodecyl sulfate–gel electrophoresis, to be composed of one major polypeptide subunit of 40,000 molecular weight and, in addition, to contain small amounts of polypeptides of 50,000 and 65,000 molecular weight. The other material used was lipid isolated from *Torpedo californica*. Table 1 summarizes its composition.

Demonstration of a Lipoprotein Complex. Following dialysis it could be shown that the AcChR and lipids were associated. The results obtained from sucrose density gradient centrifugation of the receptor–lipid preparation (Fig. 1) show that the AcChR co-sediments with the lipid, and at a slightly faster rate than the lipids alone do. Not all of the AcChR associates with the lipid, some receptor being found at the bottom of the centrifugation tube. Similar sedimentation studies of free AcChR demonstrate that in the absence of lipid, the receptor is concentrated at the bottom of the tube.

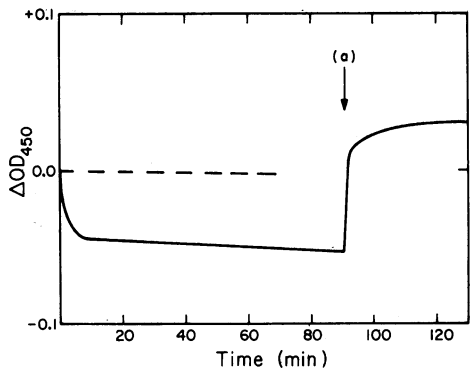

FIG. 2. Osmotic activity of the reconstituted vesicles. At zero time the vesicles were diluted twofold with isotonic buffer (broken line) or with H_2O (solid line). At (a) the buffer was made hypertonic by addition of concentrated salt solution.

In Table 2, quantitation of the results is presented. Since not all of the AcChR associates with lipids, the lipid-protein ratio in the centrifuged product (Fig. 1) is higher (14.8:1 w/w) than in the original protein mixture (10:1 w/w). The use of radioactive detergents allows determination of residual Triton X-100 or sodium cholate in reconstituted preparations. With [^{3}H]Triton or [^{14}C]cholate it was found that at the end of dialysis only 10.5 μg of [^{3}H]Triton remained per mg of protein. In parallel experiments it could be shown that 10 μg of [^{14}C]cholate per mg of lipid remained in the preparation.

Demonstration of Sealed Vesicles. The AcChR–lipid complex was shown to consist of sealed vesicles by two methods: (*a*) upon dilution with hypotonic and hypertonic buffers the sample showed osmotic activity (Fig. 2), as monitored by light scattering (26); (*b*) after overnight equilibration of the AcChR–lipid complex with ^{22}NaCl it could be demonstrated that the preparation retains $^{22}Na^+$. After dilution of $^{22}Na^+$ loaded vesicles with isotonic unlabeled buffer, a comparison of the amount of $^{22}Na^+$ retained by the vesicles with the total known concentration of ^{22}Na yielded the fractional volume engulfed by the vesicles. This was shown to be of the order of 20–50 μl/mg of lipid.

Neurotoxin Binding Sites Available in AcChR Lipid Vesicles. Studies were conducted on the binding of ^{125}I-labeled α-Bgt to determine retention of specific neurotoxin binding and to estimate the fraction of neurotoxin sites on the external surface of the closed vesicles. The vesicles were first reacted with ^{125}I-labeled α-Bgt in isotonic buffer to label toxin sites on the outside. One-half of the sample was then dissolved with 1% Triton X-100 to expose neurotoxin binding sites which were previously on the inside. At the same time the other half of the sample was treated with excess unlabeled α-Bgt to terminate the reaction. The amount of ^{125}I-labeled α-Bgt–AcChR complexes formed in each sample was then determined (23). The results showed that within the experimental error ($\pm$5%) all ^{125}I-labeled α-Bgt binding sites were on the outside of the vesicles.

Chemical Excitability of the Reconstituted Vesicles. The most interesting property of the reconstituted AcChR–lipid vesicles is that the efflux of ^{22}Na is accelerated in the presence of carbamylcholine and that this effect is completely blocked by preincubation with saturating concentrations of α-Bgt (Fig. 3). The baseline ^{22}Na efflux from the reconstituted vesicles is similar to that obtained from vesicles composed only of *Torpedo* lipids (Fig. 4). Additionally, neither carbamylcholine nor α-Bgt have an effect on $^{22}Na^+$ efflux from such lipid vesicles. The $^{22}Na^+$ efflux has two components: first, a component that accounts for approximately 80% of the internal $^{22}Na^+$, which has a fast half-equilibration time of 8–20 min in the absence of carbamylcholine, and a second, slower, component that accounts for approximately 20% of the internal $^{22}Na^+$. In the presence of carbamylcholine the fast component of $^{22}Na^+$ efflux has a half-equilibration time of 1 min.

TABLE 2. *Reconstitution of acetylcholine receptor with sonicated Torpedo lipids*

	mg lipid/ mg protein	α-Bgt sites, % of initial
Prior to dialysis*	10	100
After dialysis	10	76
Density gradient peak	14.8	49

* A typical preparation has a volume of 3 ml and contains 0.5 mg of receptor per ml and 5 mg of lipid per ml after mixing of protein and lipid solutions.

The excitability, defined as $\tau_0/(\tau - 1)$ (τ_0 and τ are the half-times for $^{22}Na^+$ equilibration in the absence and presence of 100 μM carbamylcholine, respectively), varies for different preparations; the best value obtained was 10.

Additional Observations. As expected, acetylcholine dramatically increased the $^{22}Na^+$ efflux from reconstituted vesicles in a manner similar to that described for carbamylcholine. However, not all receptor preparations yielded chemically excitable reconstituted vesicles. A given electric organ yielded an AcChR preparation which either did or did not yield excitability. In addition there was no simple correlation between the ability of a given AcChR preparation to reconstitute an excitable system and its capacity to bind ^{125}I-labeled α-Bgt.

It was found that 48 hr of dialysis were sufficient to yield excitable vesicles. There was a slow decline in excitability and after 3 days at room temperature following the end of the dialysis period excitability was virtually lost.

Some effects of temperature appear important for control of excitability in reconstituted AcChR-lipid vesicles. Dialysis at 4° instead of room temperature consistently yielded preparations with no excitability. Additionally, when dialysis was conducted at room temperature but activity was assayed at 4°, negative results were also obtained.

DISCUSSION

The results presented in this communication clearly show that the single macromolecular species, which we isolate as AcChR on the basis of its ability to complex quaternary ammonium ligands, can be reconstituted with native lipids from the same organism. The reconstituted system contains both lipids and AcChR in a membrane-like physical state. These model membranes occur in the form of closed vesicular structures, as evidenced by their osmotic activity and their ability to retain $^{22}Na^+$ after equilibration with ^{22}NaCl. The AcChR incorporated into vesicles seems to have all of its neurotoxin binding sites on the outside of the vesicles. There

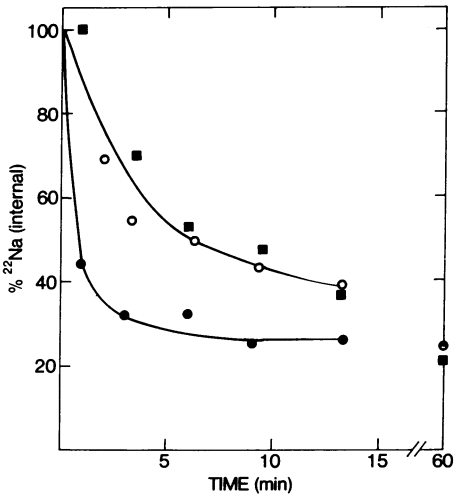

FIG. 3. ^{22}Na efflux from reconstituted AcChR–lipid vesicles; O represents efflux in 200 mM NaCl, 10 mM Tris, pH 7.4; ●, identical conditions plus 100 μM carbamylcholine; ■, preincubated with excess α-Bgt (3-fold excess over α-Bgt binding sites) followed by 100 μM carbamylcholine in the same buffer.

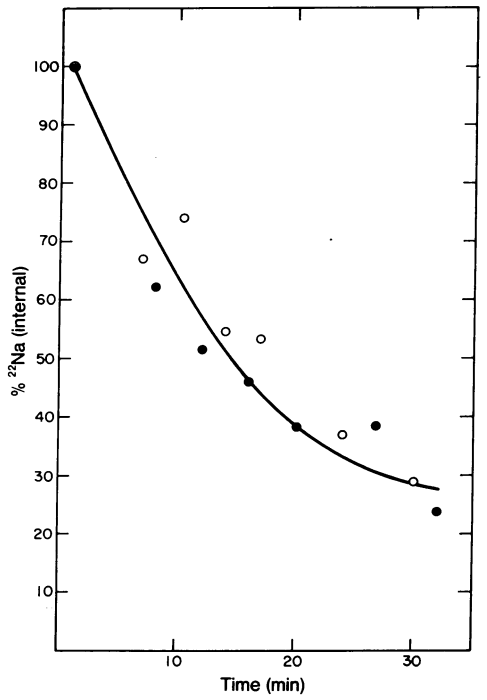

FIG. 4. ^{22}Na efflux from reconstituted AcChR vesicles (O) and from sonicated *Torpedo* lipid vesicles (●). Both preparations are in 10 mM Tris, pH 7.4, containing 200 mM NaCl.

are two possible explanations for this effect: either the receptor gets intercalated into preexisting lipid bilayers in a manner such that all neurotoxin binding sites are on the outside, or alternatively the lipid-incorporated AcChR can reorientate within the bilayer during short time periods. At the present time it is not possible to differentiate clearly between these alternatives, although it seems unlikely that the AcChR, which is a glycoprotein, would rotate through a lipid bilayer. It is possible that the sonicated lipid vesicles may be composed of asymmetric bilayers (27) which may in turn induce an asymmetric distribution of the receptor between the two surfaces. Whatever the mechanism, it gives rise to asymmetry in the distribution of neurotoxin binding sites in the reconstituted system. This may be significant, since in native membranes the receptor exhibits polarity in response to neurotransmitter (28).

The most important observation presented here is that isolated purified AcChR, a singular macromolecular complex with a multi-subunit composition, can be incorporated into lipid vesicles so that it retains the property of binding neurotoxin (α-Bgt) and furthermore, upon the addition of cholinergic agonists, it enhances cation flux from within the closed vesicular structures. The importance of this finding is that the molecular species isolated by affinity chromatography methods not only recognizes the neurotransmitter acetylcholine, but in addition it contains whatever molecular features are necessary for ion translocation. This represents the first clear demonstration that an acetylcholine binding protein contains all of the molecular machinery necessary for postsynaptic depolarization.

^{22}Na efflux from the reconstituted vesicles is quite fast even without cholinergic agonists. However, it appears that this is probably due to the lipid composition of the vesicles, since lipid vesicles without incorporated AcChR have an essentially identical leakage rate. Preliminary experiments of $^{22}Na^+$ leakage from lipid vesicles comprised of *Torpedo* lipids supplemented with synthetic phospholipids have given encouraging results with regard to slowing down the intrinsic leakage rate. Such studies in the future will hopefully make the study of agonist-induced cation efflux easier and more quantitative.

Several reports have recently appeared on the incorporation of various preparations of AcChR or AcChE into black lipid membranes (30–32). Conductance changes were observed in all cases as a function of added cholinergic agonist. In no case, however, was the AcChR or the acetylcholinesterase preparation used chemically characterized as a macromolecular species. We have also incorporated the AcChR–lipid vesicles described here into black lipid membranes formed from the same *Torpedo* lipids described in the experimental section. Large conductance changes have been observed upon the addition of carbamylcholine, with its effect being completely blocked by prior addition of α-Bgt (D. Miller, H. Lester, D. Michaelson, and M. Raftery, unpublished observations, 1974).

The significance of the experiments described in this communication is that the macromolecule isolated on the basis of cholinergic ligand binding and shown (2, 3) to contain the specific neurotransmitter binding site is now known to contain the molecular constituents necessary for ion translocation upon binding of cholinergic agonist to its specific binding site. This finding renders possible future studies directed towards identification of the molecular features of the isolated macromolecule necessary for the translocation of cations to effect postsynaptic depolarization.

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