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RECONSTITUTION OF A PURIFIED ACETYLCHOLINE RECEPTOR

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Dialysis of the purified acetylcholine receptor from Torpedo californica electroplax with lipids from the same organ results in a vesicular membrane system in which the receptor is embedded in the bilayer and oriented so that most of the neurotoxin-binding sites appear to be on the outer surface. The reconstituted vesicles are chemically excitable by acetylcholine and carbamylcholine, as measured by $^{22}Na^{+}$ efflux. The excitability is specifically blocked by the antagonist α-bungarotoxin. These results demonstrate that the purified reconstituted receptor system not only can specifically bind neurotransmitter but also can trigger ion translocation. It therefore has the properties necessary to effect postsynaptic depolarization in vivo.

INTRODUCTION

Postsynaptic depolarization at the vertebrate neuromuscular junction and 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 in Torpedo californica has been identified and purified recently in a number of laboratories (1–6) and its binding of cholinergic ligands has been clearly demonstrated (7). 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 other structural elements. Recently, Hazelbauer and Changeux (8) have shown that electroplax membrane fragments enriched in acetylcholine receptor (AcChR) may be dissolved with detergent and renatured to form sealed vesicles. The renatured vesicles respond specifically to the addition of carbamylcholine by an increase in $^{22}Na^{+}$ efflux. This work has shown that the membrane fragments contain the molecular components necessary for neurotransmitter recognition and ion translocation, and that the system may be dissolved and renatured to form chemically excitable vesicles. However, the question of whether the AcChR alone is responsible for neurotransmitter recognition and ion translocation is unanswered, because the fragments contain protein components other than the AcChR. Since ion translocation is a membrane-specific function, we have attempted to answer this question by means of reconstitution of the purified acetylcholine receptor into a functional membrane system.

In this communication we describe the reconstitution of the purified Torpedo acetylcholine receptor with Torpedo lipids to form sealed vesicles which retain $^{22}Na^+$ and which respond to acetylcholine and carbamylcholine by an increase in $^{22}Na^+$ efflux. This effect is abolished by the irreversible cholinergic antagonist α-bungarotoxin (α-Bgt).

EXPERIMENTAL PROCEDURES

AcChR was prepared by published procedures employing affinity chromatography 1, 7). Torpedo lipids were extracted and sonicated as previously described (9) except that after 0.5 hr of sonication, concentrated cholate solution was added and the suspension further sonicated to clarity. Two procedures were employed in the reconstitution experiments. The first procedure, described in reference 9, employed AcChR in a buffer in which the detergent Triton X-100 was exchanged for cholate by means of a DEAE-cellulose column. The second procedure was similar to the first, except that the detergent exchange was eliminated; instead, AcChR was concentrated by loading it, at 4°C, on to a DEAE-cellulose column and slowly eluting with 0.3 M NaCl, 0.05% Triton X-100 in 10 mM Tris, pH 7.4. This procedure resulted in a protein concentration of approximately 5 mg/ml. Since, after the detergent exchange employed in the first procedure, the ratio of protein to Triton X-100 was about 1:1 (wt/wt), it was decided to use the second procedure which was faster and yielded comparable Triton X-100-to-protein ratios. Reconstitution was achieved by codialysis of the sonicated Torpedo lipids and the AcChR (lipid-to-protein = 10:1 wt/wt) at room temperature against 10 mM Tris buffer, pH 7.4 containing 200 mM NaCl, 0.02% azide, and 2 mg/liter butylated hydroxytoluene (9). The last 24 hr of the dialysis were performed against 0.5 M sucrose, 0.02% azide, in 0.5 mM Tris, pH 7.4. $^{22}Na^+$ efflux was measured as described previously (9) except that overnight incubation with $^{22}Na^+$ was done in 10 mM Tris buffer, pH 7.4, containing 0.5 M sucrose and 10 mM NaCl. Dilution of the sample at the beginning of the efflux experiment was done with nonradioactive 1.5 mM sodium phosphate buffer, pH 7.4, containing 255 mM KCl, 4 mM $CaCl_2$, 2 mM $MnCl_2$, and 0.02% azide. This procedure resulted in a higher percentage of excitable preparations. Quantitation of ^{125}I-labeled α-Bgt binding was conducted by the DEAE-paper disk method of Schmidt and Raftery (10). Protein concentration was measured by the method of Lowry et al. (11) and phosphate analysis was done by the method of See and Fitt (12).

Samples were prepared for electron microscope examination by sucrose density gradient centrifugation, as described in the legend to Fig. 1, and fractions containing the vesicles were dialyzed extensively against 5 mM sodium phosphate, pH 7.4. Aliquots of the dialyzed suspensions were diluted with 4 vol of phosphate-buffered saline (PBS) (13) and centrifuged at 78,000 g_{max} for 30 min at 3°C. The resulting pellets were resuspended with 1 ml 2% glutaraldehyde in PBS and incubated for 30 min at 0°C. The fixed suspensions were pelleted as described above and washed in PBS by resuspension and centrifugation. The final pellet was resuspended with 10% glycerol in PBS and centrifuged for 1 hr at 78,000 g_{max} at 3°C. A portion of the glycerol-impregnated specimen was freeze cleaved in a Balzers' apparatus equipped with a double-replica cleavage device. The fracture face thus obtained was platinum-palladium shadowed with the Balzers' electron beam gun. The replicas were observed in a Phillips EM300 electron microscope.

RESULTS

The acetylcholine receptor purified from Torpedo californica and employed in the

reconstitution experiments has been characterized as a single macromolecular species by gel electrophoresis under nondenaturing conditions (4). It has further been shown by sodium dodecyl sulfate-gel electrophoresis that this molecule is composed of four subunits (3, 4). The Torpedo lipids employed contain 75% phospholipids and 25% neutral lipids. Table I summarizes their composition.

TABLE I. Composition of Torpedo Lipids

Phospholipid	Percent 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% phospholipid and 25% neutral lipids.

Following dialysis of the AcChR with sonicated Torpedo lipids, it could be shown, by sucrose density gradient centrifugation, that the lipids and the protein were associated in a complex. Results of such an experiment are shown in Fig. 1. Most of the protein co-sediments with the lipids and at a slightly faster rate than do the lipids alone. A small fraction of the protein, however, sedimented to the bottom of the gradient, as did the purified receptor in the absence of lipids. Quantitation of these results is given in Table II. The lipid-to-protein ratio (wt/wt) in the centrifuged product was 14:8 compared to the starting ratio of 10:1. After dialysis, the protein retained about 75% of its initial α-Bgt binding capability and the reconstituted receptor-lipid complex contained only minimal amounts of detergent. Experiments with [^{3}H] Triton and [^{14}C]cholate revealed that after dialysis only about 10.5 μg [^{3}H] Triton per mg protein or 10 μg [^{14}C] cholate per 10 mg of phospholipids are present. Thus, the phospholipids successfully replaced the detergent requirement for maintaining α-Bgt binding ability.

Several experimental approaches were employed to show that the receptor-lipid complex consists of sealed vesicles. Upon dilution with hypotonic and hypertonic solutions, the vesicles manifested osmotic activity, as monitored by light scattering (14). The preparation was also shown to retain $^{22}Na^+$; a comparison of the radioactivity retained by the preparation with the total radioactivity yielded the volume engulfed by the vesicles. The volume obtained, about 10 μl/mg phospholipids, is slightly smaller than that obtained when the sonicated lipids were not completely dissolved with cholate prior to reconstitution by dialysis (9).

The distribution of α-Bgt binding sites between the inner and the outer surfaces of the vesicles was determined by reacting the reconstituted preparation with α-Bgt in the presence and in the absence of Triton X-100 (9). The results varied depending upon the experimental procedure. In experiments where prior to dialysis the lipids were sonicated to clarity with cholate 70% ± 5% to 95% ± 5% of the α-Bgt binding sites were found on the outer surface of the vesicles; whereas, in experiments where the lipids were not sonicated to clarity prior to dialysis, all α-Bgt binding sites were found to reside on the outer surface. It was conceivable, however, that the protein was merely attached electrostatically to the surfaces of the vesicles and not incorporated into the lipid bilayer. Since

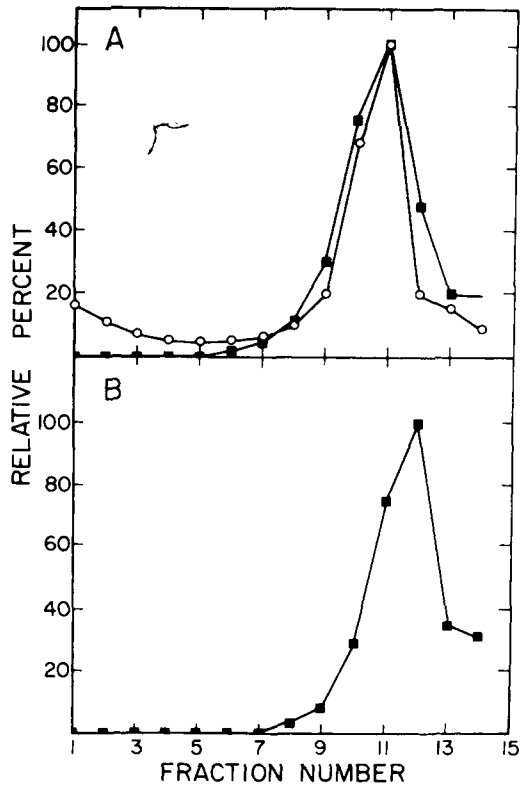

Fig. 1. Sucrose density gradient profiles of reconstituted AcChR vesicles (A) and of sonicated lipid vesicles (B). Lipid concentration was monitored by [^{3}H]PC, a trace of which was added to the Torpedo lipids prior to sonication. α-Bgt sites were assayed as previously described (9). The scale on the ordinate is such that 100% corresponds both to the highest concentration of [^{3}H]PC and to the maximum α-Bgt binding. The gradient is from 8% to 27% sucrose in 10 mM Tris, pH 7.4, containing 20 mM NaCl; it was spun in an SW 41 rotor at 4 × 10^4 rpm for 18 hr. Fraction 1 represents the bottom of the gradient.

TABLE II. Reconsitution of AcCh Receptor with Sonicated Torpedo Lipids

Preparation	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 receptor/ml and 5 mg/ml lipid, following mixing of protein and lipid solutions.

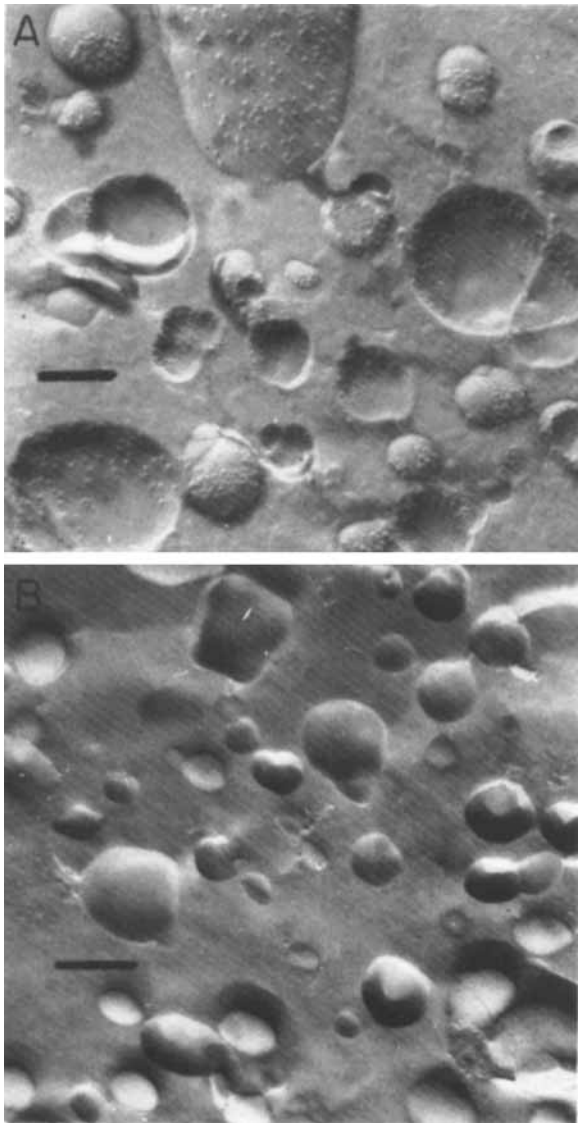

Fig. 2. Freeze-fracture micrographs of the reconstituted AcChR-lipid vesicles (a), and of Torpedo lipid vesicles (b). Bar = 0.2 μm.

freeze-fracture electron microscopy exposes to view interior surfaces of the lipid bilayers (15, 16), we employed this technique to study this problem. Figure 2 shows freeze-fracture electron micrographs of the reconstituted AcChR-lipid vesicles and of a control experiment of Torpedo lipid vesicles without added protein. Comparison of the micrographs reveals the presence of particles in the reconstituted vesicles and their absence in the vesicles composed of lipids alone. The particles, about 100–150 Å in diameter, are present in the concave and convex membrane surfaces, and not in the ice matrix. The particle density (about one particle per 2×10^5 Å^2) is close to that expected from the known lipid-to-protein ratio.

Studies of $^{22}Na^+$ efflux from the reconstituted AcChR vesicles showed that they lose internal sodium quite rapidly. This rapid efflux is due to the Torpedo lipids used

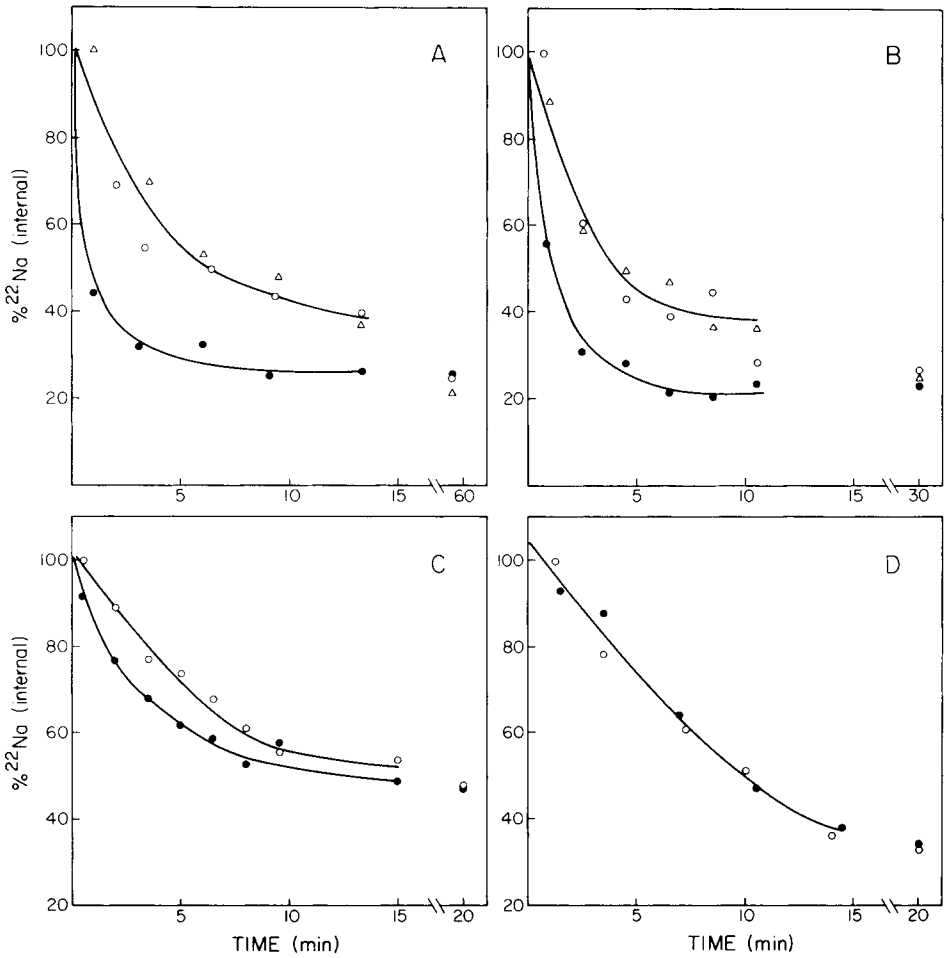

Fig. 3. $^{22}Na^{+}$ efflux from reconstituted AcChR-lipid vesicles; ○–○ represents efflux in 200 mM NaCl, 10 mM Tris pH 7.4; ●–● identical conditions plus 100 μM carbamylcholine; △–△ preincubated with excess of α-Bgt (threefold excess over α-Bgt binding sites) followed by 100 μM carbamylcholine in the same buffer. A, B, and D represent different reconstituted preparations. C represents ^{22}Na efflux from Torpedo californica membrane vesicles.

since in the absence of added receptor the flux rate is identical. Typical results obtained with a variety of reconstituted preparations are shown in Fig. 3. Some preparations show an accelerated $^{22}Na^{+}$ efflux upon addition of carbamylcholine which can be reversed by prior addition of α-Bgt (Fig. 3 A, B) while many preparations show no carbamylcholine induced excitability (Fig. 3 D). A comparison (Fig. 3 C) is also given of the excitability observed with Torpedo californica receptor-enriched membrane fragments isolated by centrifugation methods (17, 18). For the reconstituted preparations which show excitability (defined as $\frac{\tau_0}{\tau - 1}$ where τ_0 and τ and the half times $^{22}Na^{+}$ equilibration in the absence and in the presence of 100 μM carbamylcholine) values range from 2–10.

DISCUSSION

The results presented here show that the purified AcChR isolated from Torpedo californica electroplax and lipids isolated from the same organ can be reconstituted to form vesicles containing both components. The vesicles are closed, as evidenced by their osmotic activity and by their ability to retain $^{22}Na^{+}$. The receptor retains α-Bgt binding activity, it is embedded in the bilayer, and is asymmetrically oriented with most of the toxin-binding sites residing on the outer surface. This asymmetry is probably due to receptor molecules interacting with pre-existing lipid vesicles. This assertion is supported by the observation that, when the vesicles are not sonicated to clarity prior to dialysis, all the toxin-binding sites face the outside, whereas complete dissolution of the vesicles prior to dialysis results in a substantial fraction of the toxin-binding sites pointing to the inside, or at least being unavailable to α-Bgt.

The variability observed from one purified receptor preparation to the next in terms of successful reconstitution to an excitable membrane is not readily explained but probably arises from subtle differences in properties. It is quite possible that even in the best preparations only a small fraction of reconstituted receptor molecules show a response, and this fraction may be variable. Recent experiments conducted in our laboratory have demonstrated that membrane preparations from Torpedo californica electroplax can be isolated with either low or high affinity ligand binding properties. These preparations are interconvertible starting with the lower affinity form in a manner highly reminiscent of in vivo desensitization. In these preparations the high affinity agonist binding form would correspond to the desensitized state. The conditions used for preparation of membranes prior to affinity chromatography to obtain the receptor used in the reconstitution studies reported here result in desensitized preparations which may be largely irreversibly converted to that form. If this is the case, the use of receptor preparations with lower ligand affinity (i.e., not desensitized) could yield reconstituted preparations with superior properties.

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