Reconstitution of functional membrane-bound acetylcholine receptor from isolated *Torpedo californica* receptor protein and electroplax lipids

(acetylcholine/membrane reconstitution/octyl glucoside/desensitization/sodium fluxes)

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ABSTRACT Purified acetylcholine receptor and total lipids, both extracted from *Torpedo californica* electroplax, were utilized to reconstitute chemically excitable membrane vesicles. Reconstitution was achieved by dialysis of the extraction detergent, octyl β-D-glucoside, from protein/lipid incubation mixtures. The reconstituted preparations could be fractionated by sucrose density gradient centrifugation and consisted of vesicular structures visible in electron micrographs. In addition, the reconstituted vesicles exhibited the following properties characteristic of native receptor-enriched membranes: (i) an external distribution of α-bungarotoxin-binding sites, (ii) a time-dependent binding of α-bungarotoxin that is depressed by preincubation with the cholinergic agonist carbamoylcholine ("desensitization"), (iii) an ability to retain $^{22}\text{Na}^+$ that is lost in the presence of detergents or gramicidin A, and (iv) a carbamoylcholine-induced acceleration of $^{22}\text{Na}^+$ efflux that can be blocked by α-bungarotoxin. The purified acetylcholine receptor that was utilized in the reconstitution experiments apparently does not require other protein components for ligand recognition or ion translocation.

Postynaptic depolarization at the neuromuscular junction and in the electric organs of certain fish is a membrane phenomenon. Upon the recognition of the neurotransmitter acetylcholine, ions are translocated through the postynaptic membrane, effecting depolarization. The acetylcholine receptor (AcChR) has been isolated in solubilized and membrane-bound forms (1) and appears to be directly implicated in ligand binding. On the other hand, the identity of the membrane component(s) involved in ion translocation has not been definitively established.

Reconstitution of a membrane from specific constituents can aid in gaining an understanding of fundamental membrane processes at the molecular level. In the case of the AcChR, it should be possible to combine separate pools of purified AcChR and total lipids and reconstitute membrane vesicles that exhibit the following well-known properties of native AcChR-enriched membrane vesicles: (i) a "right-side-out" orientation of α-bungarotoxin (α-Bgt) binding sites, (ii) the ability to bind cholinergic ligands, (iii) the capacity to undergo affinity state transitions in response to cholinergic agonists, and (iv) an agonist-induced stimulation of $^{22}\text{Na}^+$ translocation. Previous attempts to reconstitute AcChR membranes have never met all the above criteria, whether purified (2–4) or partially purified (5–9) AcChR was used. With either type of receptor preparation, problems in reconstitution can be partly ascribed to the presence of tightly bound detergent (10). Furthermore, purified AcChR could be irreversibly altered by conventional purification methods (10), and thus could prove unsuitable for functional reconstitution. Alternatively, attempts at reconstituting with total solubilized protein from essentially intact membrane fragments generally yielded incompletely characterized membranes of low specific activity (i.e., α-neurotoxin binding) (5–9).

In this paper, we discuss the reconstitution of excitable membrane vesicles that are composed of purified AcChR and total lipids isolated from *Torpedo californica* electroplax. The reconstituted vesicles exhibit all the above-mentioned properties inherent to native AcChR-enriched membrane vesicles. The results suggest that AcChR purified by α-cobratoxin affinity chromatography contains all of the molecular components necessary for neurotransmitter recognition and ion translocation.

MATERIALS AND METHODS

Materials. *T. californica* electroplax was purchased from Pacific Bio-Marine Supply (Venice, CA). α-Bgt was purified from *Bungarus multicinctus* venom (Sigma). $^{125}$I-Labeled α-Bgt, $^{22}\text{NaCl}$, cholesteryl [1-14C]oleate, and octyl β-D-[U-14C]glucoside were purchased from New England Nuclear. d-Tubocurarine di[14C]methyl ether iodide was obtained from Amersham.

α-Cobratoxin was purified from *Naja naja siamensis* venom (Sigma) and was used to prepare the α-cobratoxin-Sepharose 4B affinity gel as described (11). Nonradioactive octyl β-D-glucoside (OcGlc) was purchased from Calbiochem.

Preparation of Solubilized AcChR. Purified AcChR was obtained by α-cobratoxin affinity chromatography subsequent to solubilization of *Torpedo* electroplax with OcGlc instead of Triton X-100. Protein concentration of the purified AcChR was calculated on the basis of its absorption spectrum by assuming a molar absorptivity of 533,000 M⁻¹ cm⁻¹ and a molecular weight of 270,000 (12). The specific activities of the purified preparations were determined by the binding of $^{125}$I-labeled α-Bgt, using the DEAE filter disc assay procedure (13). Total lipids from electroplax membrane fragments were extracted as described (14).

Reconstitution Procedure. Reconstitution of purified AcChR into electroplax total lipid vesicles was obtained after extensively dialyzing OcGlc from a soluble mixture containing AcChR and total lipid. Routinely, 18–20 mg of electroplax total lipid, dissolved in benzene, was evaporated to dryness in a test tube under a stream of argon. About 8 mg of AcChR in receptor buffer (10 mM sodium phosphate, pH 7.4/1 mM EDTA/0.1 mM phenylmethylsulfonyl fluoride/0.02% NaN₃) containing 1.4 mM OcGlc was added to the lipid mixture and was gently and continuously stirred. One volume of a concentrated OcGlc solution was slowly added to give a final

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Abbreviations: α-Bgt, α-bungarotoxin; AcChR, acetylcholine receptor; OcGlc, octyl β-D-glucoside; CbmCho, carbamoylcholine.
RESULTS

Solubilization of electroplax membranes with OcGlc and subsequent purification by affinity chromatography renders a preparation of AcChoR with macromolecular properties identical to those of preparations in which Triton X-100 is the solubilizing detergent (unpublished). Purified receptor is an oligomeric protein with a molecular weight of 270,000 (12), which is composed of four different polypeptide chains (40,000, 50,000, 60,000, and 65,000) in an unknown stoichiometry. The total lipid extract from Torpedo electroplax membranes is known to be composed of about 70% various phospholipid classes and about 25% neutral lipids, mainly cholesterol (6, 7).

The effect of dialysis against three 1-liter changes of buffer on the receptor–lipid incubation mixtures is shown in Table 1. Total protein and specific activity (α-Bgt-binding sites per mg of protein) as well as total lipids, estimated by the radioactive lipid tracer, were almost entirely recovered. Furthermore, the subunit pattern of the reconstituted material on NaDodsO₄ gels (data not shown) was identical to the starting purified AcChoR, indicating no loss of individual polypeptide chains during the experiment. Conversely, OcGlc was exhaustively removed (99.9%) upon dialysis, as determined by the recovery of the radioactive tracer, octyl β-D-[U-14C]glucoside. The amount of detergent remaining in the dialyzed sample was estimated to be less than 35 μg OcGlc per mg of protein.

**Formation of sealed vesicle-like structures**

 Upon coidialysis, the AcChoR protein associated with the electroplax lipids. Results obtained from sucrose density gradient centrifugation in a high ionic strength medium demonstrated that in the reconstituted sample AcChoR cosedimented with the lipids at a faster rate than the lipids alone (Fig. 1). Nevertheless, the association between AcChoR and the lipid vesicles was not completely quantitative. Fractions containing either free AcChoR or plain lipid vesicles were detected near the bottom or at the top of the gradient, respectively. This association phenomenon did not, however, obey a simple association equilibrium, because the fractions containing the superimposed peaks (Fig. 1B) behaved as a single component in a subsequent sucrose density gradient centrifugation.

The lipoprotein complexes behaved as sealed vesicle-like structures as indicated by three different criteria: osmotic activity, loading and retention of ⁴²Na, and electron microscopic examination. The osmotic activity was monitored by light scattering upon dilution of the vesicles with hypotonic and hypertonic buffers. Unlike free AcChoR solutions, light scattering of the reconstituted preparation dramatically increased upon dilution in 1 M NaCl. In addition, the vesicles could be loaded with ²²Na. The permeability coefficient for ²²Na in the vesicles appeared to be low compared to native (freshly isolated) AcChoR-enriched vesicles, because longer periods of time were required for equilibration. Actually, quasilinear increases in

Table 1. Effect of dialysis on OcGlc-solubilized AcChoR/electroplax lipids mixtures

<table>
<thead>
<tr>
<th>Property</th>
<th>%*</th>
</tr>
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<tbody>
<tr>
<td>Total protein content</td>
<td>95.4 ± 2.0</td>
</tr>
<tr>
<td>α-Bgt binding</td>
<td>96.8 ± 2.3</td>
</tr>
<tr>
<td>Lipid content</td>
<td>98.4 ± 1.5</td>
</tr>
<tr>
<td>OcGlc removal*</td>
<td>99.9 ± 0.1</td>
</tr>
</tbody>
</table>

* Data expressed as percentages of the initial values in the reconstituted samples, mean ± SD.
* Estimated by using the radioactive tracers cholesteryl [1-14C]oleate and octyl β-D-[U-14C]glucoside, respectively.
22Na uptake were detected in the first 72 hr for the reconstituted vesicles (data not shown). In agreement with these results, the retention of 22Na in efflux assays was higher in the reconstituted vesicles than in the native vesicles. In other words, the reconstituted preparation was less "leaky" to Na+ than native AcChoR-enriched vesicles. Upon addition of OcGlc at concentrations below the critical micelle concentration, or upon preincubation with gramacidin A, the amount of retained 22Na was dramatically reduced (Fig. 4). Finally, the vesicular nature of the reconstituted samples was explored by electron microscopy after negative staining. The micrographs reveal a heterogeneous population of vesicle-like structures with an average diameter of approximately 2500 Å (Fig. 2) similar to those obtained with native Torpedo membrane vesicles.

In order to determine the orientation of the AcChoR molecules within the reconstituted vesicles, α-Bgt-binding assays were conducted on untreated vesicles (to label available external toxin sites) and on vesicles after their solubilization with 1% Triton X-100. The results indicated that 90–100% of the total α-Bgt-binding sites are located on the external surface of the reconstituted vesicles, resembling the "right-side-out" orientation detected for this receptor in native AcChoR-enriched vesicles from Torpedo electroplax (18).

**Functional properties of reconstituted AcChoR vesicles**

Ligand binding to both the reconstituted membrane-bound and the detergent-solubilized AcChoR was measured by using the di-[14C]methyl derivative of the cholinergic antagonist d-tubocurarine. After equilibrium dialysis in the presence of the radioactive antagonist, the ligand binding parameters were analyzed through Scatchard plots. With either OcGlc or Triton X-100-solubilized AcChoR, the results indicate that there is one d-tubocurarine-binding site per two α-Bgt-binding sites, and the apparent dissociation constant for d-tubocurarine is about 0.2 μM. Similar results were obtained with the reconstituted vesicles.

We conclude that the reconstituted vesicles are functionally similar to AcChoR-enriched membranes, because the vesicles exhibited the following well-recognized characteristics of the membrane-bound AcChoR: (i) an ability to undergo reversible affinity state transitions as a consequence of prolonged exposure to agonists, and (ii) an increase in ion permeability in response to the presence of agonists. Unlike solubilized receptor, the reconstituted vesicles exhibited the ability to undergo agonist-induced transitions from low to high ligand affinity states. This phenomenon resembles pharmacologic desensitization of postsynaptic membranes induced by cholinergic agonists (1). The existence of this affinity state transition can be examined by measuring the binding of 125I-α-Bgt (16) to both lipid-bound and solubilized receptor. Samples were incubated with or without (control) 10 μM CbmCho at time zero or were preincubated 30 min in the presence of 10 μM CbmCho prior to the addition of 125I-α-Bgt. =, Overnight (=15-hr) incubation periods.

**FIG. 1.** Sucrose density gradient profiles of AcChoR protein (A), reconstituted AcChoR/electroplax lipid preparations (B), and electroplax lipid vesicles (C). Lipids (△) were monitored with the radioactive tracer cholesteryl [1-14C]oleate. 125I-α-Bgt binding (O) was measured as described in Materials and Methods. Refractometry measurements (x), exemplified in C, are representative of all the sucrose density gradients.

**FIG. 2.** Negative stain electron micrograph of a reconstituted AcChoR/electroplax lipid preparation after dialysis. The inserted bar represents 1 μm.

**FIG. 3.** Time-dependent 125I-α-Bgt binding to purified AcChoR protein (A) and reconstituted AcChoR/electroplax lipid preparations (B). All receptor concentrations were 0.1 μM in α-Bgt-binding sites and the 125I-α-Bgt concentration was 1 μM. O, 125I-α-Bgt binding in the absence of CbmCho; △, CbmCho (final concentration of 10 μM) and 125I-α-Bgt added simultaneously at zero time; X, samples preincubated 30 min in the presence of 10 μM CbmCho prior to the addition of 125I-α-Bgt.
cubated with 10 μM CmChGo for 30 min (Fig. 3). The results indicate that the “sensitized” state (low affinity) is absent in the solubilized AcGoR, but reappears when the receptor is reintegrated into a lipid environment, resembling the behavior of “sensitized” native AcGoR-enriched membranes (16).

Finally, the excitability of the reconstituted vesicles was examined by measuring the efflux of trapped $^{22}\text{Na}^+$. As expected for sealed vesicles, a small $^{22}\text{Na}^+$ efflux was observed upon a 1:25 dilution of the vesicles in the absence of agonists (Fig. 4A). A similar baseline efflux of $^{22}\text{Na}^+$ was obtained from plain Torpedo lipid vesicles that contained no protein (Fig. 4B). The retention of $^{22}\text{Na}^+$ was greatly reduced in both types of vesicles when they were either preincubated with the ion-pore-forming polypeptide gramicidin A or solubilized with OcGlc at concentrations below the critical micelle concentration. Conversely, only the vesicles containing AcGoR were stimulated to release Na$^+$ in response to 0.5 mM CmChGo present in the dilution buffer. Furthermore, this response was selectively blocked by preincubation of the vesicles with an excess of either α-Bgt or d-tubocurarine (data not shown), suggesting that the AcGoR is responsible for agonist-induced increases in ion permeability.

**DISCUSSION**

Postsynaptic depolarization at the neuromuscular junction and in the electric organs of certain fish is mediated by two fundamental processes: (i) the specific interaction of the neurotransmitter (acetylcholine) with the membrane-bound receptor, followed by (ii) an increase in ion permeability of the postsynaptic membrane. Investigating these events, various groups have purified the AcGoR in solubilized and membrane-bound forms (1). Studies have demonstrated that the purified receptor is exclusively responsible for ligand binding; however, the role of ion translocation has not been unequivocally attributed to the AcGoR or to any other membrane component.

Several attempts at recovering membrane ionophoretic responses from the incorporation of undefined extracts enriched with AcGoR or acetylcholinesterase into black lipid membranes (for review see refs. 1 and 3) led to seldom reproducible results. The transient conductance events induced by ligands in these artificial membranes were quantitatively and qualitatively different from the physiological membrane system. Other groups have attempted the reconstitution of either crude solubilized extracts from AcGoR-enriched membrane fragments (5–9) or AcGoR purified by affinity chromatography (2–4) into various types of lipid vesicles with mixed, and even contradictory, results. Changeux and coworkers (5, 6), Epstein and Racker (8), and Schiebler and Hucho (7) did not utilize purified AcGoR, because they believe that there is an apparent partial modification of the receptor during the purification by affinity chromatography (19). However, under those conditions, the assignment of specific functional properties to AcGoR in the reconstituted preparation becomes ambiguous due to the presence of protein contaminants. In addition, the use of non-native lipid mixtures (7–9) might give rise to a host of physical phenomena that could alter bulk membrane properties by mechanisms of little relevance to processes occurring in the native system.

Raftery and his colleagues (2, 4) overcame some of these drawbacks by using native lipids and purified AcGoR in their experiments. Nevertheless, they isolated AcGoR from Triton X-100-solubilized extracts of electroplax tissue. This detergent is thought to alter AcGoR irreversibly (10), bind tightly to the receptor protein (12), and be difficult to remove from solubilized preparations. Therefore, the method of choice could be the utilization of native lipids from electroplax membranes and purified AcGoR that was solubilized in an easily removable nonionic detergent, which produces minimum, or at least reversible, structural or functional modification. Reconstituted functional AcGoR membrane vesicles can be prepared from electroplax lipids and AcGoR extracted and purified in the presence of the dialyzable nonionic detergent OcGlc. Upon dialysis of the incubation mixture, virtually all of the total lipid, AcGoR protein, and specific activity is recovered, whereas the detergent is essentially removed. In addition, the lipid–protein association detected in the sucrose density gradients in the presence of 0.2 M NaCl is probably not due to nonspecific electrostatic interactions. Furthermore, the association between lipids and receptor is decreased if the lipids are dissolved first in OcGlc at concentrations above the critical micelle concentration. The reconstituted system exhibits an important property of intact AcGoR membranes, the ability to undergo reversible transitions from low to high affinity states for cholinergic ligands in response to prolonged exposure to agonists. This property has always been absent in detergent-solubilized AcGoR preparations. Indeed, OcGlc-solubilized and purified AcGoR behaves in the same manner when the procedure is carried out in the presence of other detergents. This “desensitization” process is recovered upon the reintegration of the receptor into its lipid environment (Fig. 3). Hence, the hypothesis of irreversible modification of AcGoR upon purification by affinity chromatography (19) does not appear to be applicable in our case, in which affinity chromatography is the purification procedure. The vesicular nature of the reconstituted samples is apparent by criteria such as electron microscopy, osmotic activity detected by means of light-scattering measurements, and the loading and retention of $^{22}\text{Na}$. The longer period of time required for $^{22}\text{Na}$ loading and the better retention of $^{22}\text{Na}$ in the reconstituted vesicles, compared to the native membranes, may be partially explained by either the lower protein-to-lipid ratio or the presence of additional types of protein in the native vesicles.

An interesting feature of the reconstituted vesicles is the asymmetrical distribution (i.e., right-side-out) of the receptor protein within the simulated membrane. This condition also
greatly resembles the "in situ" features of native AcChoR membranes (18) and may have been present in some reconstituted systems (2, 4, 20, 21), whereas other authors (6) report a random distribution of toxin sites. Nevertheless, at present, it is difficult to ascertain whether the right-side-out distribution is due to the intrinsic asymmetry of the receptor molecule or if it is induced by the lipid phase.

Finally, the reconstituted AcChoR vesicles were stimulated by CbmCho to increase ^22Na efflux in a similar manner to native Torpedo membrane vesicles (22). This response could be blocked by the preincubation of the loaded vesicles with α-Bgt. This contrasts with the behavior of plain lipid vesicles, containing no AcChoR protein, which do not display agonist-induced excitability, and is strongly supportive of our contention that AcChoR, as isolated after affinity chromatography, is directly responsible for ion translocation in membrane. Because the magnitude of the increase in ^22Na efflux induced by CbmCho is similar to that detected in native membranes, it is unlikely that a protein contaminant is responsible for ion translocation. Such a contaminant would have to copurify with the AcChoR in a high yield and be present in the reconstituted system in the same amount as in native membranes. This protein component is not detectable in the NaDodSO4/polyacrylamide gels, which show only the four polypeptides characteristic of Torpedo AcChoR. Furthermore, because our AcChoR preparations are devoid of the 43,000 molecular weight subunit, it is very unlikely that this membrane component could be ascribed a primary role in either ligand recognition or ion translocation (1).

In conclusion, we have described the preparation and characterization of reconstituted AcChoR vesicles that exhibit most of the inherent qualities of native AcChoR-enriched membranes. Our results strongly suggest that the AcChoR protein, isolated by conventional purification methods, contains all of the molecular determinants necessary for specific ligand binding and for ion translocation.

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