Biochimica et Biophysica Acta, 691 (1982) 249–260
Elsevier Biomedical Press

BBA 71350

ACETYLCHOLINE RECEPTOR FROM *TORPEDO*

PREFERENTIAL SOLUBILIZATION AND EFFICIENT REINTEGRATION INTO LIPID VESICLES

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(Received January 26th, 1982)
(Revised manuscript received June 7th, 1982)

Key words: Acetylcholine receptor; Solubilization; Membrane reconstitution; Na^+ efflux; (Torpedo californica)

The acetylcholine receptor has been effectively solubilized from *Torpedo californica* electroplax under defined conditions with the nonionic detergent, β-D-octylglucopyranoside. Preferential solubilization of the receptor protein, with regard to yield and specific α-bungarotoxin binding activity, occurs in the absence of salt and diminishes when NaCl is present in the solubilization media (≥50 mM). Conversely, elevated salt concentrations increase the solubilization of bulk membrane proteins including the peripheral membrane enzyme, acetylcholine esterase. Additional selectivity for the solubilization of acetylcholine receptor can be obtained by adjusting the detergent to membrane phospholipid molar ratio within a narrow optimum range (4.1 to 6.7). Purified acetylcholine receptor and electroplax total lipid are utilized to reconstitute chemically excitable membrane vesicles. Reconstitution is achieved by dialysis of octylglucopyranoside from lipid/detergent/receptor protein mixed micelles and the resulting vesicles are analyzed by sucrose density gradient centrifugation. Extensive incorporation of the acetylcholine receptor within the lipid vesicles is obtained at lipid concentrations greater than 18 mg/ml with lipid/protein ratios ranging from 12/1 to 60/1 (w/w). Reconstituted receptor vesicles and native receptor-enriched membranes exhibit similar agonist-induced effluxes of $^{22}Na^+$ with 50% of the maximum response occurring at carbamylcholine concentrations of $1.8 \cdot 10^{-5}$ M and $3.4 \cdot 10^{-5}$ M, respectively. At saturating carbamylcholine concentrations (10^{-2} M) the agonist-induced efflux of $^{22}Na^+$ for both native and reconstituted acetylcholine receptor is $(6–7) \cdot 10^{13}$ cpm $^{22}Na^+$ per mol of receptor. The efflux responses exhibited by either preparation can be effectively blocked by preincubation with carbamylcholine ('desensitization'). The similar behavior of native and reconstituted acetylcholine receptor indicates that octylglucopyranoside-purified receptor retains all of the necessary determinants for proper ligand binding and ion translocation.

Introduction

The study of the properties of biological membranes has been greatly facilitated in recent years with the advent of techniques for the solubilization, purification and reconstitution of membrane proteins. A transmembrane protein which has generated considerable interest has been the nicotinic acetylcholine receptor for which many structural and functional aspects are now beginning to be understood (reviewed in Refs. 1–3). Numerous lines of evidence have shown that the receptor

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Abbreviations: octylglucoside, β-D-octylglucopyranoside; SDS, sodium dodecyl sulfate.

0005-2736/82/0000–0000/$02.75

plays an essential role during postsynaptic depolarization at the neuromuscular junction and in the electric organ of certain fish. The purified receptor, solubilized from *Torpedo californica* electroplax, has a molecular weight of 270000 [4] and is composed of four nonidentical polypeptide chains with apparent molecular weights of 40000, 50000, 60000 and 65000 [5–10]. Numerous studies have indicated that the 40000 subunit exhibits water exposed regions which are directly responsible for ligand binding [6,7,11–13], whereas knowledge concerning the topography of the other subunits in the postsynaptic membrane has been limited [14]. Despite being one of the most intensively studied receptor systems in neurobiology, controversial questions remain unanswered regarding the molecular arrangement of the functional acetylcholine receptor in the membrane and the dynamic processes by which the receptor translocates ions in the initiation of membrane depolarization. Because of the complexity of postsynaptic membranes, one approach in resolving these questions would be the reconstitution of lipid vesicles which contain exclusively the purified receptor protein. The prerequisite for this approach is the solubilization and purification of the receptor which is usually accomplished with the aid of detergents, and its subsequent reincorporation within a lipid bilayer which should resemble the native membrane environment. It is not surprising that problems in previous attempts at reconstituting receptor functionality can be partly ascribed to tightly bound detergent, such as Triton X-100 [4,15,16]. Receptor-bound detergent would not only hinder the precise characterization of the purified receptor, but presumably interfere with the reintegration of the protein into a simulated membrane environment by possibly modifying important protein-lipid boundaries.

The problem of detergent interference has been circumvented in part with the introduction of the nonionic detergent β-D-octylglucopyranoside (octylglucoside) into the realm of membrane research [10,17–23]. Octylglucoside appears to be technically advantageous over the more commonly utilized polyoxyethylene ether-type detergents (Triton, Brij, Lubrol, etc.) in the solubilization and characterization of certain membrane proteins [17,21,23]. Because octylglucoside can be effectively removed by dialysis, this detergent has proved to be quite suitable for the reconstitution of functional membrane vesicles from detergent/lipid/protein mixed micelles [10,19,21,24]. We recently reported the reconstitution of acetylcholine receptor vesicles which exhibited many of the structural and functional properties characteristic of receptor-enriched membranes [10]. One problem we encountered during these studies was the inability of the experimental system to completely incorporate the available receptor protein into the vesicles resulting in a large fraction of unincorporated receptor. In addition, the only available technique to estimate cation translocation was based upon vacuum filtration of the vesicles and was less than suitable in clearly resolving the agonist-induced efflux of $^{22}Na^+$.

Recently, we observed that octylglucoside was similar in many respects to Triton X-100 in the solubilization of *Torpedo* electroplax membranes [23]. Concurrently, a number of reports appeared in which the chemical milieu was shown to be an important factor in the solubilization of membrane proteins by octylglucoside. Brotherus et al. [25] observed that inactivation of $(Na^+ + K^+)$-ATPase from *Electrophorus electricus* occurred at well-defined threshold concentrations for various detergents, including octylglucoside. These threshold concentrations also appeared to be dependent on the absolute amount of membrane lipid being solubilized. In addition, Helenius and Kartenbeck [26] have shown that the interaction between spike proteins and the nucleocapsid of the Semliki Forest virus in the presence of octylglucoside was extremely sensitive to the pH and the ionic strength.

In this communication we initially examine the effect of the ionic strength and the detergent to membrane phospholipid ratio on the effective solubilization of electroplax membranes by octylglucoside. Our results will be related in such a way that they can be accurately evaluated and reproduced since absolute concentrations of membrane phospholipid in addition to detergent are reported [27]. We also examine those conditions necessary for the extensive reintegration of octylglucoside-purified receptor into lipid vesicles. The ion translocating properties of native and reconstituted acetylcholine receptor will be compared

with the aid of a novel assay we developed for the measurement of sodium fluxes. The octylglucoside-purified receptor, when reintegrated into electroplax lipid vesicles, appears to provide an ideal system to study acetylcholine receptor functionality.

Materials and Methods

Materials. *Torpedo californica* electroplax was purchased from Pacific Biomarine Supply Co. and β-D-octylglucopyranoside was obtained from Calbiochem. α-Bungarotoxin was purified from *Bungarus multicinctus* venom (Sigma) following published procedures [28] and ^{14}C-labeled α-bungarotoxin was prepared as described [29]. ^{125}I-Labeled α-bungarotoxin, ^{22}NaCl, and cholesteryl [1-^{14}C]oleate were purchased from New England Nuclear. α-Cobratoxin was purified from *Naja naja siamensis* venom (Sigma) by the method of Ong and Brady [30].

Solubilization of electroplax membranes. Frozen electroplax tissue was sliced into approx. 1 cm cubes and placed in an equal volume of receptor buffer (10 mM sodium phosphate, pH 7.4/1 mM EDTA/0.1 mM phenylmethanesulfonyl fluoride/0.02% NaN_3) containing 0.1 M NaCl. The cubed tissue was homogenized in a Sorvall Omnimixer for 3×1 min at maximum speed and the homogenate was centrifuged for 30 min at $10000 \times g$ in a Sorvall GS-3 rotor. The supernatant was discarded and the more loosely packed portion of the pellet was resuspended in the same volume of receptor buffer and rehomogenized for 1 min at 75% speed. After another low speed centrifugation, the pellet (crude membranes) was carefully weighed and appropriate portions were used for the solubilization studies. The crude membranes were solubilized by vigorous stirring for 15 h with an appropriate amount of receptor buffer containing octylglucoside ranging from 3.4 to 136 mM and NaCl ranging from 0 to 500 mM. The solubilized tissue was centrifuged either for 3 h at $100000 \times g$ in a Beckman Ti 50.2 rotor or for 30 min at 30 $(\text{lb} \cdot \text{inch}^{-2}) \cdot g$ in a Beckman Airfuge (small-scale preparation). The supernatants (crude solubilized extracts) were carefully decanted and used for further studies.

Solubilized acetylcholine receptor was purified by α-cobratoxin affinity chromatography from the solubilized crude extracts as previously described [14], except that octylglucoside replaced Triton X-100 throughout the procedure. Acetylcholine receptor-enriched membranes were prepared by the method of Delegeane and McNamee [31] and exhibited a specific activity of 1.35 nmol of α-bungarotoxin bound per mg protein. Protein concentration was determined by the method of Lowry et al. [32] and radiolabeled α-bungarotoxin binding to acetylcholine receptor was determined using a DEAE-cellulose filter disk assay procedure [33]. Acetylcholine esterase activity was determined by the colorimetric assay of Ellman et al. [34]. Total lipids from electroplax crude membranes were extracted as described [35] and lipid phosphorus was determined by a modification of published procedures [36].

SDS-polyacrylamide gel electrophoresis. Exponential gradient SDS-polyacrylamide slab gel (5–20%) electrophoresis was performed with a 3% stacking gel and the discontinuous buffer system of Laemmli [37]. After staining with Coomassie blue, the molecular weights of the polypeptide bands were determined by comparison with the electrophoretic mobility of protein standards. Alternatively, samples were electrophoresed on 12% SDS-polyacrylamide tube gels for direct scanning of unstained gels at 280 nm with a Cary 210 spectrophotometer.

Reconstitution procedure. Octylglucoside and total electroplax lipid in a 4/1 (w/w) ratio were dissolved in benzene. Appropriate aliquots of the mixture were transferred to test tubes and lyophilized. Purified acetylcholine receptor in receptor buffer containing 1.5 mM octylglucoside was added to the detergent/lipid mixtures and was gently and continuously stirred until the samples were completely clarified. When reconstituted vesicles containing greater than 1 mg/ml receptor were desired, the clarified detergent/lipid/receptor mixed micelles were concentrated by ultrafiltration. All samples were dialyzed at room temperature against reconstitution buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl and 0.02% NaN_3) until greater than 99% of the octylglucoside was removed as estimated by using the radioactive tracer, β-D-octyl[U-^{14}C]glucoside. In some experiments an aliquot of cholesteryl [1-^{14}C]oleate was

added to the detergent/lipid mixtures in order to estimate the recovery of total lipid upon dialysis (greater than 90% in all cases). Sucrose density gradient centrifugation of the reconstituted vesicles was performed as previously described [10].

$^{22}Na^+$ flux assay. The absorption filtration assay (Paraschos et al., unpublished data) described below is in part a modification of (i) the 'hygroscopic desorption' technique of Conrad and Singer [38] for measuring the binding of amphipathic compounds to cell membranes and phospholipid vesicles and (ii) the serial washing procedure by Thomas et al. [39] for the isolation of DNA/protein complexes. Native membranes and reconstituted vesicles were equilibrated overnight with 75 μCi/ml $^{22}Na^+$. Four Whatman 934-AH glass fiber filter disks (2.4 cm diameter) were placed in a row on parafilm and a double layer of Whatman GF/C glass fiber filter disks 7 mm in diameter were centered onto the first 934-AH disk. The GF/C disks were presoaked with 10 μl of the appropriate buffer, and 10 μl of the $^{22}Na^+$ equilibrated samples was then added to the center of the GF/C disks. The deposited samples were carefully washed dropwise with 100 μl buffer in a manner such that each drop covered the surface of the GF/C disks before being absorbed into the 934-AH disk. The GF/C disks were then transferred onto the next 934-AH disk, washed with 100 μl buffer, transferred and washed again. At this point essentially all of the extravesicular $^{22}Na^+$ was washed into the three 934-AH disks, whereas the intravesicular $^{22}Na^+$ remained with the deposited samples on the GF/C disks. The pair of GF/C disks were then transferred onto the remaining 934-AH disk and washed with 50 μl buffer with or without carbamylcholine (efflux step). In a typical experiment with reconstituted vesicles, greater than 95% of the cholesteryl [1-^{14}C]oleate lipid tracer and 7.5% of the $^{22}Na^+$ remained with the GF/C disks at the termination of the assay. Conversely, in the absence of vesicles, free $^{22}Na^+$ was extensively washed into the 934-AH disks with less than 0.08% (90 cpm) remaining with the GF/C disks.

For desensitization experiments, the $^{22}Na^+$ equilibrated samples were preincubated with $1 \cdot 10^{-6}$ M carbamylcholine for 30 minutes and assayed as described above except that the washing buffer also contained $1 \cdot 10^{-6}$ M carbamylcholine. When the washing buffer did not contain carbamylcholine, desensitization was completely reversed.

Results

Preferential solubilization and purification of acetylcholine receptor

The effect of varying the NaCl concentration on the solubilization of *Torpedo* crude membranes by 34 mM octylglucoside was examined (Fig. 1). Maximal solubilization of acetylcholine receptor, expressed in terms of nmol α-bungarotoxin sites recovered per μmol of crude membrane lipid phosphorus, occurs in the absence of salt. Conversely, there is a 40% decrease in recovery of receptor in

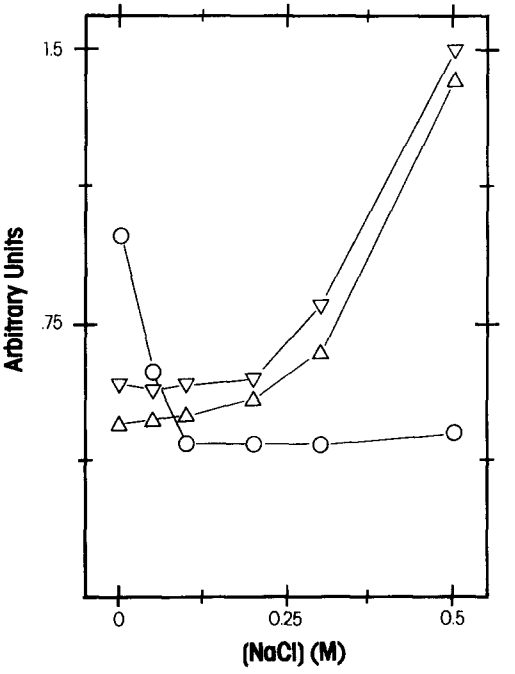

Fig. 1. Effect of salt on the solubilization profile of *Torpedo californica* electroplax. Crude electroplax membranes (1.9 mM lipid phosphorus) were solubilized with 34 mM octylglucoside at various NaCl concentrations and centrifuged at 100000 × *g* for 3 h. The supernatants (solubilized crude extracts) were assayed for protein (△), ^{14}C-labeled α-bungarotoxin binding (○) and acetylcholine esterase activity (▽). Our arbitrary unit equals 1 mg protein/μmol lipid phosphorus, 0.1 nmol α-bungarotoxin sites/μmol lipid phosphorus and 5 mol acetylthiocholine hydrolyzed/liter per min.

the presence of 0.05 M NaCl with a maximum decrease of 60% at concentrations of 0.1 M NaCl or greater. The specific activity of the solubilized extracts in terms of nmol α-bungarotoxin bound per mg protein follows a similar pattern, since the amount of total protein solubilized per μmol lipid phosphorus gradually increases with increasing NaCl concentrations. It appears that at lower NaCl concentrations the receptor protein is being preferentially solubilized since maximal yields and specific activities are obtained.

At elevated NaCl concentrations (> 0.2 M), the sharp increase in the total protein being solubilized is closely paralleled by the appearance in the solubilized extract of the peripheral membrane enzyme, acetylcholine esterase (Fig. 1). Note that over the wide range of salt concentration, the specific activity of the esterase remains essentially constant.

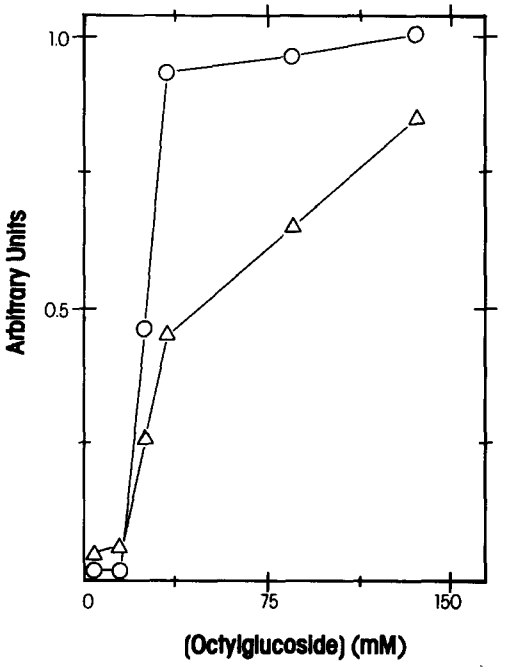

Fig. 2. Effect of detergent concentration on the solubilization profile of *Torpedo californica* electroplax. Crude electroplax membranes (1.7 mM lipid phosphorus) were solubilized in the absence of NaCl with increasing concentrations of octylglucoside and centrifuged at 100000 × *g* for 3 h. The supernatants were assayed for protein (△) and ^{14}C-labeled α-bungarotoxin binding (○). One arbitrary unit equals 1 mg protein/μmol lipid phosphorus and 0.1 nmol α-bungarotoxin sites/μmol lipid phosphorus.

The optimal concentration of octylglucoside necessary for maximal solubilization of electroplax membranes was determined. As shown in Fig. 2, a constant amount of crude membranes containing 29 μmol of lipid phosphorus was maintained while the octylglucoside concentration in the absence of salt was varied (3.4 to 136 mM). A dramatic increase in both the number of nmol α-bungarotoxin sites and mg protein solubilized per μmol crude membrane lipid phosphorus is observed at a detergent concentration of 34 mM (optimal concentration). Above this concentration, there is a plateau in the recovery of α-toxin binding sites and thus a decrease in the amount of α-toxin bound/mg protein in the solubilized extracts. In addition, the optimal concentration is identical whether the membranes are solubilized in the absence or presence [23] of salt. The yield and specific activity of crude extracts solubilized by 34 mM octylglucoside are quite similar to those values obtained with extracts solubilized by 1% Triton X-100 under comparable conditions.

The effect of detergent/phospholipid ratios on the solubilization of receptor was examined by maintaining the octylglucoside concentration at 34 mM in the absence of salt and varying the amount of crude membranes to be solubilized (data not shown). The yield (0.11 nmol α-bungarotoxin sites/μmol lipid phosphorus) and specific activity (0.2 nmol α-bungarotoxin bound/mg protein) remains essentially constant at octylglucoside/phospholipid molar ratios ranging from 7 to 133. A moderate increase in yield and a 2-fold increase in specific activity, however, is observed at octylglucoside/phospholipid molar ratios from 4.1 to 6.7. At lower detergent/phospholipid ratios the recovery of α-toxin binding sites sharply decreases to a minimum. When optimal solubilizing conditions are employed with receptor-enriched membranes instead of crude membranes, the specific activity is observed to increase from 1.35 for intact membranes to 2.34 nmol α-bungarotoxin bound/mg protein for the solubilized extracts.

Solubilization of crude membranes in the absence of salt at optimal octylglucoside/phospholipid molar ratios provides yields of purified receptor 3-fold higher than that previously reported [23]. Acetylcholine receptor purified by α-cobratoxin affinity chromatography always ex-

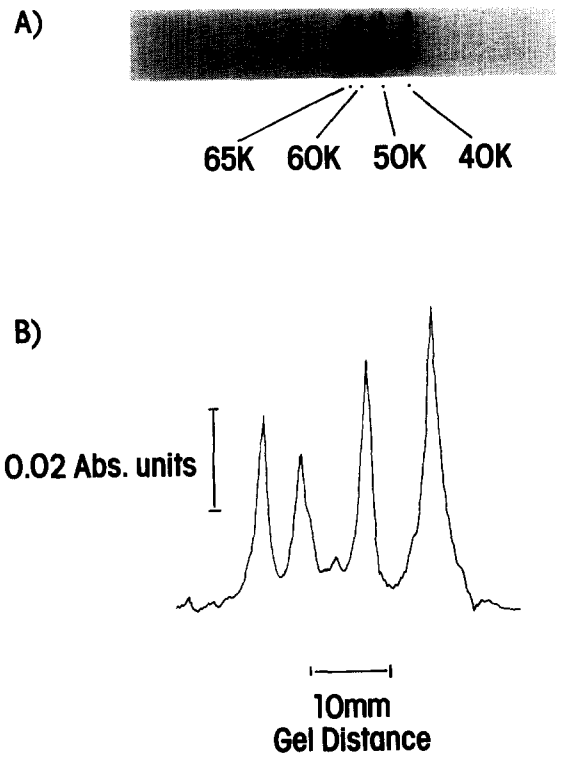

Fig. 3. Polypeptide patterns on SDS-polyacrylamide gels of octylglucoside purified acetylcholine receptor. A, Coomassie blue stained gel; B, absorption scan of an unstained tube gel at 280 nm.

hibits four polypeptide bands on SDS-polyacrylamide gels with apparent molecular weights of 40000, 50000, 60000 and 65000 (Fig. 3A). Similar values have been reported for the α, β, γ and δ subunits, respectively, of receptor preparations purified in the presence of other detergents [6,8,9]. The stoichiometry of the four subunits (α, β, γ and δ), determined by scanning an unstained gel at 280 nm, is approximately 2:1:1:1 (Fig. 3B) in agreement with molar ratios estimated previously by other methods [40,41]. The specific activity of the octylglucoside-purified receptor is equal to or greater than 9 nmol of α-bungarotoxin bound per mg protein which is comparable to values obtained with our best preparations of Triton X-100 purified receptor.

Reconstitution of acetylcholine receptor vesicles

We previously reintegrated the octylglucoside-purified receptor into *Torpedo* electroplax lipid vesicles by the detergent-dialysis method [10]. Upon codialysis, the receptor protein associated with the electroplax lipid in the formation of vesicles which were analyzed by centrifugation through linear sucrose density gradients. In a typical experiment, where the initial lipid concentration is 3 mg/ml and the lipid/protein ratio is 5/1 (w/w), a proportion of the receptor protein comigrates with the lipid through the sucrose gradi-

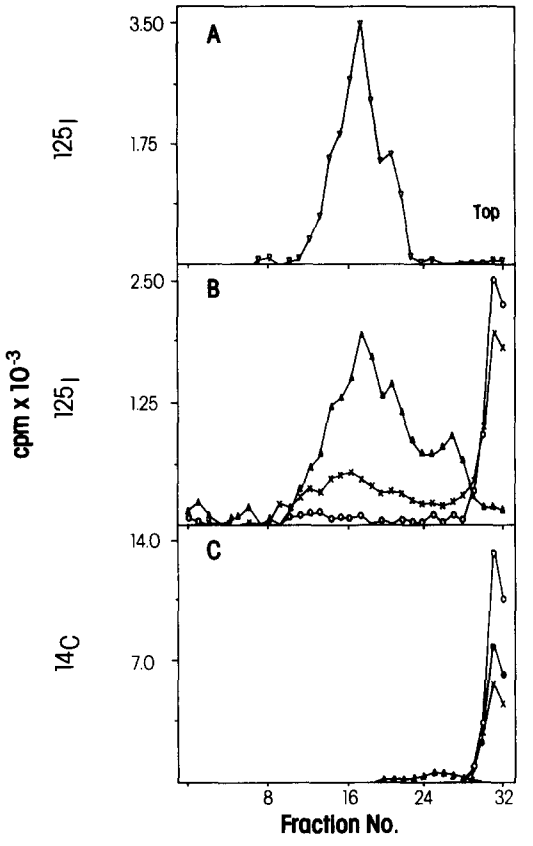

Fig. 4. Sucrose density gradient profiles of purified receptor protein (A), and reconstituted receptor protein (B) with corresponding electroplax lipid (C). Lipids were monitored with the radioactive tracer, cholesteryl [1-^{14}C]oleate. ^{125}I-labeled α-bungarotoxin binding was measured as described in Materials and Methods. The initial lipid concentration (mg/ml) and the lipid/protein ratio (w/w), respectively, for the different reconstituted samples were as follows: (▲) 3, 5/1; (×) 15, 30/1; (○) 28, 60/1. The initial lipid concentration for the plain lipid vesicles (●) was 15 mg/ml and the sedimentation profile is depicted in panel C.

ent at a faster rate than plain lipid vesicles, but at a slower rate than unassociated or 'free' receptor (Fig. 4B). Under these conditions, a large fraction of the receptor is not incorporated into the lipid vesicles (Fig 4B) and migrates characteristically in the sucrose gradient as free receptor (Fig. 4A). As the initial lipid concentration is increased, however, progressively more receptor is reintegrated into the lipid vesicles with extensive reconstitution occurring at an initial lipid concentration of 28 mg/ml with a lipid/protein ratio of 60/1 (Fig. 4B). In all cases, the migration of the reconstituted receptor coincides with the migration of the lipid tracer (Fig. 4C). In addition, as the initial lipid concentration is increased, the respective reconstituted vesicles apparently become more buoyant and migrate to a lesser extent in the sucrose gradient (Fig. 4B, C). Apparently, increasing the initial lipid/protein ratio (5 to 60/1) in the incubation mixtures results in an increased lipid/protein ratio (19 to 87/1) for the reconstituted vesicles which is reflected by greater buoyancy. Lipid vesicles prepared under comparable conditions (15 mg lipid/ml), with or without protein, migrate to a similar position in the sucrose gradients.

A number of reconstitution experiments have demonstrated that an initial lipid concentration of 18 mg/ml or greater is necessary for extensive reconstitution to occur (data not shown). At this lipid concentration the receptor protein can be efficiently reconstituted at lipid/protein ratios as low as 12/1. These reconstituted vesicles, which exhibit similar receptor densities as vesicles previously reconstituted by the cholate-dialysis procedure [42,43], are especially suited for the measurement of agonist-induced cation transport.

TABLE I

PROPERTIES OF NATIVE AND RECONSTITUTED ACETYLCHOLINE RECEPTOR

No carb, carbamylcholine absent.

		cpm $^{22}Na^+$			$\bar{F}$	cpm $^{22}Na^+$	
		Intravesicular [a]	Effluxed [b]	Retained [c]		Intravesicular per mg lipid	Effluxed per mol receptor ($\times 10^{-13}$)
Native membranes	No carb	3833	639	3194	0.167	42588	4.03
		3965	710	3254	0.179	44055	4.48
	Carb	3606	1479	2127	0.410	40066	9.33
		3978	1797	2125	0.452	44200	11.34
	Δ		963	1069			6.08
Reconstituted receptor vesicles	No carb	96625	6723	89901	0.070	539202	12.25
		97862	6937	90924	0.071	546104	12.64
	Carb	98117	10762	87353	0.110	547527	19.60
		96369	10467	85900	0.109	537773	19.07
	Δ		3785	3786			6.89
Plain lipid vesicles	No carb	178264	10888	167375	0.061	1037625	–
		178071	12740	165330	0.072	1036501	–
	Carb	177637	10790	166847	0.061	1033975	–
		179085	12975	166109	0.073	1042403	–
	Δ		69	126			

[a] $^{22}Na^+$ remaining on GF/C filter after three successive 100 μl wash and transfer cycles. Each preparation was equilibrated overnight with 75 μCi $^{22}Na^+$ per ml which corresponded to $1 \cdot 10^6$ cpm $^{22}Na^+$ per 10 μl sample.

[b] $^{22}Na^+$ effluxed into 934-AH filter with or without $1 \cdot 10^{-2}$ M carbamylcholine (50 μl) after washing cycles.

[c] $^{22}Na^+$ retained on GF/C filter after termination of assay.

[d] $\bar{F}$ = cpm $^{22}Na^+$ effluxed/cpm $^{22}Na^+$ intravesicular.

Properties of reconstituted acetylcholine receptor vesicles

We have previously shown [10] that the reconstituted acetylcholine receptor vesicles exhibit the following well-known properties characteristic of native receptor-enriched membranes: (i) a totally external distribution of α-bungarotoxin binding sites, (ii) a time-dependent binding of α-bungarotoxin that is depressed by preincubation with carbamylcholine ('desensitization'), and (iii) a carbamylcholine-induced acceleration of $^{22}Na^+$ translocation that can be blocked by α-bungarotoxin. We have extended these results by comparing the cation permeability control properties of native membranes and lipid vesicles assembled either with or without receptor as assessed by the absorption filtration assay (Table I). After extensive removal of extravesicular $^{22}Na^+$, the apparent internal volume (tested with gramicidin A) of the plain lipid vesicles appears to be twice that of the reconstituted receptor vesicles in terms of cpm intravesicular $^{22}Na^+$ per mg lipid. Lipid vesicles assembled either with or without receptor protein exhibit apparent internal volumes which are an order of magnitude greater than the native receptor-enriched membranes. In addition, the passive efflux (apparent 'leakiness') of $^{22}Na^+$ in the absence of carbamylcholine (no carb, $\bar{F}$) is 2-fold higher in the native membranes than in either vesicle preparation. The agonist-induced efflux of $^{22}Na^+$ is similar for native membranes and reconstituted receptor vesicles with 6.08 and $6.89 \cdot 10^{13}$ cpm $^{22}Na^+$ effluxed per mole of receptor, respectively. The amplitude of the flux response for the reconstituted vesicles is in close agreement with values obtained with the cholate-dialyzed reconstituted receptor vesicles for agonist-induced influx [44] and efflux [43] of $^{22}Na^+$ as measured by ion-exchange chromatography and vacuum filtration, respectively. As shown in Table I, the plain lipid vesicles which are assembled without receptor protein exhibit no carbamylcholine-induced efflux of $^{22}Na^+$.

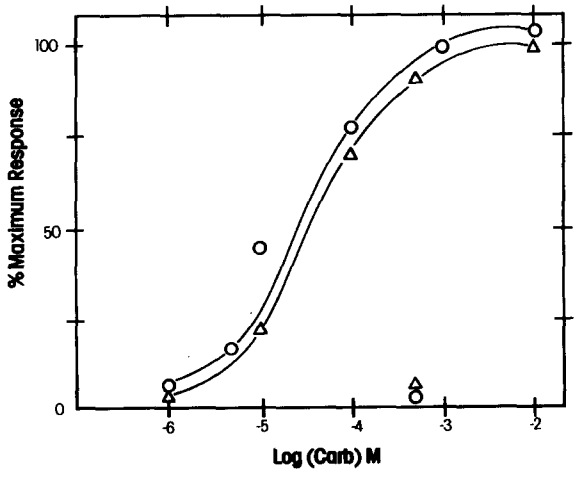

Fig. 5. Efflux of $^{22}Na^+$ from receptor-enriched membranes (△——△) and reconstituted receptor vesicles (○——○) at varying carbamylcholine concentrations. For agonist-induced desensitization, the samples were preincubated with $1 \cdot 10^{-6}$ M carbamylcholine for 30 min and effluxed at $0.5 \cdot 10^{-3}$ M carbamylcholine (△, ○; single points near abscissa).

Close scrutiny of the data presented in Table I illustrates some of the problems intrinsic to the more conventional vacuum filtration technique. Note the small percentage difference between the cpm $^{22}Na^+$ retained (< 5%) in the presence and absence of agonist with the reconstituted receptor vesicles. This problem is exacerbated when these data are obtained by vacuum filtration which generates less reproducible results. Conversely, absorption filtration permits an accurate assessment of the cpm $^{22}Na^+$ effluxed whereby the flux signal in the presence and absence of carbamylcholine can be easily differentiated (> 35%). A resolvable efflux signal has enabled an examination of the concentration effect of carbamylcholine in the agonist-induced tracer efflux with the reconstituted receptor vesicles (Fig. 5). The dose-response curves are similar for both native and reconstituted receptor with 50% of the maximum response occurring at $3.4 \cdot 10^{-5}$ M and $1.8 \cdot 10^{-5}$ M carbamylcholine, respectively. Both preparations appear to be completely desensitized when preincubated with $1 \cdot 10^{-6}$ M carbamylcholine since $^{22}Na^+$ efflux is inhibited at an agonist concentration normally sufficient to elicit a 90% maximum response (Fig. 5). Desensitization can be reversed upon removal of the conditioning carbamylcholine which returns the agonist-induced flux activity to that observed with the 'resting' sensitized acetylcholine receptor.

Discussion

After initial studies with the membrane-bound form, a number of investigators were able to solubilize and purify the acetylcholine receptor with the aid of detergents (reviewed in Refs. 1, 2). Thorough understanding of receptor functionality, however, has been severely hindered by the presence of some of these detergents which bind tightly to the protein thus interfering with the characterization of the soluble receptor and its integration into lipid vesicles. With these considerations, the acetylcholine receptor was solubilized, purified, and reconstituted with the use of β-D-octylglucopyranoside which appears to produce minimum structural and functional modification [10,23]. We presently extend our results by introducing methods whereby (i) the receptor can be preferentially solubilized by octylglucoside from *Torpedo* membranes, and (ii) the purified receptor protein can be efficiently reincorporated into electroplax lipid vesicles providing a suitable defined model system to study membrane-bound receptor functionality.

The receptor protein appears to be preferentially solubilized from crude electroplax membranes by 34 mM octylglucoside only at low NaCl concentrations (< 0.05 M). Both maximal yields and specific activities of the receptor are observed in the absence of salt, whereas the bulk protein is increasingly solubilized with elevated NaCl concentrations. The solubilization of the peripheral membrane enzyme, acetylcholine esterase, is closely paralled by the appearance of total protein with increasing salt thus the esterase specific activity remains essentially constant ('nonpreferential' solubilization). The effect of high salt on the solubilization of the acetylcholine esterase suggests that the association of this peripheral type protein to the lipid matrix, in contrast to an integral membrane protein (receptor), probably depends on charged groups. This notion is in agreement with the results of Helenius and Kartenbeck [26] where the interaction between the spike glycoprotein and the nucleocapsid of Semliki Forest virus was observed to be quite sensitive to elevated salt concentrations in the presence of octylglucoside.

We found that 34 mM octylglucoside, either in the presence [23] or absence of salt, is the optimum concentration for the solubilization of the receptor protein. We also examined the effect of the octylglucoside/membrane phospholipid ratio on the solubilization process. This parameter was shown to be an important factor in the stability of ($Na^+ + K^+$)-ATPase [25] which, like the receptor, is a transmembrane protein highly enriched in specialized organs such as electroplax. Although the yield and receptor specific activity remain constant over a wide range of octylglucoside/membrane lipid phosphorus molar ratios (7 to 133), there is a narrow range (4.7 to 6.1) where the specific activity in terms of nmol α-toxin bound/mg protein is 2-fold higher. Similar detergent/phospholipid ratios have been observed to be critical for the optimal extraction of functional receptor during solubilization of *Torpedo* membranes with cholate-soybean lipid mixtures [43,44].

Regardless of the conditions for solubilization, however, the 'purified' receptor protein exhibits the four conventional polypeptide bands on SDS-polyacrylamide gels and similar specific activities (9 nmol α-bungarotoxin bound/mg protein). The protocol described above for the solubilization of the receptor provides for the purification of receptor in high yield (40 to 50 mg of purified protein from 500 g of electroplax tissue). In addition, receptor extracts with high specific activity are obtained by a one step solubilization of very crude membranes under carefully controlled conditions. With receptor-enriched membranes, detergent extracts can be obtained which exhibit a 2-fold increase in specific α-toxin binding activity. This approach to the solubilization of receptor may be quite useful in studies with mammalian tissue where the inability to purify receptor in workable quantity necessitates the direct characterization of detergent extracts with high specific activity.

Efficient incorporation of purified acetylcholine receptor into lipid vesicles is obtained when the initial lipid concentration is greater than 18 mg/ml with lipid/protein (w/w) ratios ranging from 12/1 to 60/1. Similarly, lipid/protein (w/w) ratios of 16/1 or greater have been reported to be optimal for the reconstitution of receptor vesicles by the cholate-dialysis procedure [42]. The extensive reincorporation of receptor not only obviates the tedious fractionation of reconstituted receptor from free protein, but also allows for direct control in reconstituting any number of receptor molecules

per vesicle which may facilitate future examination of ion translocation events. To this end we have developed an ion transport assay which in our hands is more reproducible than other available methods such as vacuum filtration. The absorption filtration assay exhibits comprehensive data retrieval enabling the detection of extravesicular from intravesicular ions. Moreover, the amount of $^{22}Na^+$ effluxed can be distinguished from the remaining intravesicular pool after exposure of the reconstituted vesicles or native membranes to carbamylcholine. Desensitization processes can be dramatically reduced since the time of exposure to agonist can be limited to less than one second, that is the time necessary for a 10 μl drop of carbamylcholine to be absorbed through the upper GF/C disk to the underlying 934-AH disk. Due to the vast array of glass fiber filters and ion-exchange disks, the assay can be accommodated to the transport of ions and the binding of soluble molecules to a variety of intact cells, membranes and reconstituted vesicles of varying sizes and lipid composition (Paraschos et al., unpublished data). On the other hand, initial rates of agonist-induced flux, which can be measured by rapid-mix quenched-flow [45,46] or stopped-flow fluorescence quenching techniques [47,48], are not within the scope of the absorption filtration assay. Instead, absorption filtration allows for the measurement of time-averaged responses which qualitatively reflect flux amplitudes of native and reconstituted receptor after relatively brief exposure (1 to 2 s) to carbamylcholine.

Within the limitations of our assay, the lipid vesicles assembled with or without receptor protein appear to retain an order of magnitude more intravesicular $^{22}Na^+$ per mg lipid than the native membranes. In addition, the native membranes appear to be twice as 'leaky' as the lipid vesicles. These results can be partly ascribed to the fact that native receptor-enriched membranes are the result of subcellular fractionation whereby membrane fragments as well as sealed vesicular structures are produced. On the other hand, the lipid vesicles are assembled with high lipid to protein ratios with large internal volumes which may contribute to the significant retention of sodium ions and stable ion permeability characteristics.

We have previously shown that octylglucoside does not alter receptor functionality since reconstituted electroplax lipid vesicles containing octylglucoside-purified receptor exhibited many of the characteristics inherent to native receptor-enriched membranes [10]. We extended these results further by comparing the responses of native and reconstituted acetylcholine receptor to different concentrations of carbamylcholine. The ion flux dose-response curves for both preparations are quite similar. We additionally found that the native and reconstituted receptor preparations are equally susceptible to desensitization by preincubation with carbamylcholine whereby the agonist-induced efflux of $^{22}Na^+$ is completely blocked. With the advent of the absorption filtration assay, reversal of desensitization is achieved by removal of the carbamylcholine from the deposited samples by the normal wash and transfer cycles without dilution of the membranes or vesicles.

It is apparent that efficient reintegration of functional acetylcholine receptor into electroplax lipid vesicles can be achieved by the octylglucoside-dialysis method under appropriate lipid/detergent/protein ratios. Others [49] have reported failure in their attempts to reconstitute cholate-solubilized receptor with asolectin lipids by the octylglucoside-dialysis procedure and thus have ascribed our initial reports to the presence of residual amounts of 'active' receptor. Indeed, asolectin lipids have also been ineffective in our own hands for reconstituting octylglucoside-solubilized receptor (data not presented). We suspect that both the type of phospholipid as well as the presence of cholesterol may be important for the successful reconstitution of cation channel activity. A high cholesterol content, which is characteristic of native electroplax lipid [50], appears to be critical for optimal reconstitution whether asolectin or electroplax phospholipids provide the bulk lipid source (Gonzalez-Ros et al., unpublished data). Finally, the effects of mixing octylglucoside with cholate-solubilized receptor [49] have not been systematically investigated and are at best unclear, especially when followed by detergent-free dialysis of such a complex composite of mixed micelles.

Octylglucoside as well as sodium cholate have become increasingly utilized for the reconstitution

of a wide variety of membrane-bound proteins. Although physicochemically different, both detergents appear equally effective as solubilizing agents and as vehicles for the formation of lipid vesicles by the detergent-dialysis method. Recent publications [44,51] have appeared which emphasize the importance of maintaining the presence of lipids throughout the purification of the acetylcholine receptor when solubilized by sodium cholate. Apparently, high concentrations of sodium cholate or the absence of exogenous lipid irreversibly inactivates the receptor thus resulting in nonfunctional reconstitution. Nevertheless, we conclude that the presence of exogenous lipid may be unnecessary for the purification of octylglucoside-solublized receptor for the purpose of reconstituting receptor functionality. These apparent discrepancies may be partially explained by the charge difference between the anionic sodium cholate and the nonionic octylglucoside. Perhaps more significantly, however, the cholate-dialysis procedure for reconstitution usually results in the formation of small vesicles with average diameters ranging from 25 to 60 nm [24,43,52] which can be increased to 100 nm after freezing and thawing (Montal, M., personal communication). Conversely, reconstitution by the octylglucoside-dialysis method produces vesicles with an average diameter greater than 200 nm [10,24,53] with an internal volume 1000-fold larger than vesicles obtained by cholate-dialysis [53]. The larger size of the octylglucoside-dialyzed vesicles are advantageous for ion-transport studies since the intravesicular solute concentrations would be less restricted by small internal volumes [53]. In addition, the study of structure-function relationships in membranes would be more restricted by surface curvature restraints in the smaller sodium cholate-dialyzed vesicles than in the larger octylglucoside-dialyzed vesicles.

Acknowledgements

We wish to gratefully acknowledge the expect technical assistance of Miguel A. Gonzalez, and to thank Tina Fehskens and Judy Watts for typing the manuscript. This work was supported by Grant BNS 77-24715 of the National Science Foundation (to M.M.C.) and by Grant NS 17029 of the National Institutes of Health (to J.M.G.R.).

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