RECONSTITUTION OF THE PURIFIED ACETYLCHOLINE RECEPTOR FROM *TORPEDO CALIFORNICA*

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1. Introduction

The acetylcholine receptor from *Torpedo californica* and similar sources has been purified in several laboratories using affinity chromatography [1–3]. Previous attempts in our and other laboratories to reconstitute these purified preparations failed to give reproducible results; it was not clear whether the receptor was damaged or had lost a component essential for channel formation [4–6]. We have therefore used crude acetylcholine receptor-enriched membrane preparations [7] to develop a biological ion flux assay based on the reconstitution into liposomes [8].

We now report the purification of a solubilized acetylcholine receptor which is fully active in reconstitution. We also show that the carbamylcholine-dependent ion flux in these reconstituted vesicles is sensitive to inhibitors such as α-bungarotoxin and that specific antibodies inhibit the reconstitution of active vesicles. The reconstituted liposomes exhibited the characteristic phenomenon of desensitization.

2. Materials and methods

Materials were as described in [8].

2.1. *Preparation of membranes and liposomes*

Membranes from *Torpedo californica* were prepared according to [7] as modified [8]. The middle band on the discontinuous sucrose gradient was used for all experiments. Crude soybean phospholipids were suspended in 10 mM NaP_i (pH 8.0) at 100 mg/ml by sonication in the bath-type sonicator under nitrogen for 20 min.

2.2. *Assays*

Ion flux measurements were performed as in [8] except that 2–4 μCi ^{86}Rb was substituted for ^{22}Na. All fluxes were measured after 10 s in the absence and presence of 200 μM carbamylcholine. ^{125}I-Labeled α-bungarotoxin binding measurements were performed as in [9]. SDS–gel electrophoresis was performed according to [10]. Protein determinations were preformed as in [11].

2.3. *Preparation of choline carboxymethyl affinity resin*

The affinity resin was prepared according to [12] and contained 3 μmol choline carboxymethyl groups/ml packed resin.

2.4. *Solubilization and purification of receptor*

Membranes were diluted to 2.5 mg/ml protein in buffer I (60 mM KCl, 100 mM NaCl, 10 mM NaP_i (pH 8.0)) and the pH adjusted to 10.6 with 1 N NaOH [13]. After 20 min at 20°C they were centrifuged 20 min at 130 000 × *g* and the pellet resuspended with buffer I to the original volume. Na-cholate (20%) was then added to 1% final conc. After 20 min at 4°C, insoluble material was removed by centrifugation at 130 000 × *g* for 30 min. The cholate extract was added to 1/10 vol. packed choline carboxymethyl affinity resin and stirred gently for 2 h at 4°C. The resin was centrifuged at 750 × *g* for 2 min and washed 4 times with 20 vol. buffer I containing 1% Na-cholate and 1 mg/ml soybean phospholipids. The receptor complex was eluted 3 times with 2 bed vol. washing buffer containing 10 mM carbamylcholine and the eluants were pooled.

2.5. *Reconstitution methods*

2.5.1. Cholate dialysis

Membranes, solubilized extract, or the purified receptor (~ 1–2 nmol α-bungarotoxin binding sites) were reconstituted as in [8] except that they were adjusted to 1 ml in 2% Na-cholate, 25 mg/ml soybean phospholipids final conc. in buffer I just before dialysis. When purified receptor containing 10 mM carbamylcholine was used, dialysis for ~36 h with 4 buffer changes was required.

2.5.2. Cholate dilution

The solubilized extract (1–2 nmol α-bungarotoxin binding sites) was diluted with 1 vol. buffer I to bring cholate to 0.5%. To 0.4 ml of this extract 0.11 ml of 94 mg/ml liposomes in buffer I containing 1.3% Na-cholate was added and incubated 30 min at 20°C. The mixture was diluted with 20 vol. cold buffer I, incubated for 15 min at 4°C and centrifuged 130 000 × *g* for 1.5 h. The pellet was resuspended in 0.55 ml 100 mM NaCl, 10 mM NaP_i (pH 8.0) and incubated at 20°C for 30 min, chilled and assayed.

2.5.3. Freeze–thaw sonication

To 0.4 ml (1–2 nmol α-bungarotoxin binding sites) of the solubilized extract, soybean phospholipids (100 mg/ml) were added to 7.5 mg/ml final conc. and the protein was precipitated by the addition of saturated dibasic ammonium phosphate to 60% saturation. After centrifugation at 100 000 × *g* for 20 min, the pellet was resuspended in 1 ml 25 mg/ml liposomes in 100 mM NaCl, 10 mM NaP_i (pH 8.0). This suspension was immersed for 2 min in liquid nitrogen, thawed at room temperature then sonicated for 1 min in a bath-type sonicator.

3. Results and discussion

3.1. *Extraction and reconstitution of acetylcholine receptor*

Various cholate concentrations were explored for the extraction of the receptor from acetylcholine-enriched membranes. Without phospholipids, which were added in [8] to stabilize the receptor in the presence of 2% cholate, optimal extraction was obtained at 1% cholate. Lower concentrations (0.5%) did not effectively solubilize the receptor, while 2% cholate led to inactivation of reconstitutive activity without impairing α-bungarotoxin binding. Triton X-100 (1%) was used as an alternative procedure of solubilization followed by removal of the detergent [14] prior to reconstitution by cholate dialysis. Both cholate and Triton X-100 extracts (without additional phospholipids) lost ~ 25% activity after 18 h at 4°C, while at 0.5% cholate only ~10% was lost for each day of storage.

Three different reconstitution procedures have been used. As shown in table 1, the cholate dialysis procedure gave the highest ion flux values, but cholate dilution and freeze–thaw sonication [15] are more rapid procedures and often useful for the exploration of the properties of the receptor.

3.2. *Properties of solubilized acetylcholine receptor*

It was shown that the carbamylcholine-dependent ion flux of reconstituted vesicles was sensitive to α-bungarotoxin as well as to several other inhibitors [8]. It can be seen in table 2 that an antiserum produced either in a rat or in a rabbit against purified receptor preparations, effectively inhibited the reconstitution of the ion flux activity of the channel. Addition of antiserum to reconstituted proteoliposomes, however,

Table 1
Reconstitution methods

Procedures	Ion flux (nmol . 10 s^{-1} . mg^{-1}) −Carbamylcholine	Ion flux (nmol . 10 s^{-1} . mg^{-1}) +Carbamylcholine	Ion flux (Δnmol . 10 s^{-1} . mg^{-1})
Cholate dialysis	95	384	289
Cholate dilution	65	215	150
Freeze–thaw sonication	67	207	140

Table 2
Inhibition of reconstitution by anti-receptor serum

Sample	Ion flux (nmol . 10 s^{-1} . mg^{-1}) −Carbamylcholine	Ion flux (nmol . 10 s^{-1} . mg^{-1}) +Carbamylcholine	Ion flux (Δnmol . 10 s^{-1} . mg^{-1})	Inhibition
Expt. 1[a]				
Control	76	256	180	0%
Rabbit normal serum	60	236	176	2%
Rabbit anti-receptor serum	84	163	79	66%
Expt. 2				
Control	68	340	273	–
Rat normal serum	59	285	226	17%
Rat anti-receptor serum	60	109	49	82%

[a] Membranes containing 2 nmol α-bungarotoxin binding sites were incubated with 0.5 ml serum (or buffer I) for 1 h at 20°C and were washed twice with 20 vol. buffer I. The membranes were then solubilized with cholate and reconstituted by cholate dialysis as in section 2

Table 3
Purification of acetylcholine receptor active in reconstitution

Fraction	Ion flux		α-Bungarotoxin binding	
	Specific activity, nmol . 10 s^{-1} . mg^{-1}	Total units, nmol . 10 s^{-1}	Specific activity nmol . mg^{-1}	Total units nmol
Membranes	183	915 (100%)	2.0	10 (100%)
pH treated	226	904 (99%)	2.5	10 (100%)
Extract	280	672 (73%)	3.4	7.7 (77%)
Purified	903	595 (65%)	9.3[a]	6.2 (62%)

[a] According to analyses performed by Drs V. N. Damle and A. Karlin, 7.6 nmol [^{3}H]methyltoxin and 3.9 nmol 4-(*N*-maleimido)benzyltri-[^{3}H]methylammonium iodide was bound per milligram of this preparation

had little or no effect on ion flux activity. Studies with antibodies have been most useful in the elucidation of the role of coupling factors in oxidative phosphorylation [16,17] and could play a similar role in the analysis of the function of various receptor protein components.

3.3. *Purification of reconstitutively active acetylcholine receptor*

The reconstitution into artificial liposomes of carbamylcholine-dependent ion flux with extracts from acetylcholine receptor-enriched membranes [8] was used as an assay during the purification of the channel. The importance of using such a biological assay is emphasized by our experience mentioned earlier that the α-bungarotoxin binding properties remain unimpaired at high cholate concentrations while reconstitutive activity is lost. Greater losses in reconstitutive activity than in α-bungarotoxin binding capacity were encountered with a purification procedure depending on an α-neurotoxin affinity column. In contrast, the purification procedure developed [12] which uses an affinity column prepared with bromoacetylcholine, gave excellent results as shown in table 3. The final preparation had a ^{125}I-labeled α-bungarotoxin binding activity of 9.3 nmol/mg protein and ion flux activity of 900 nmol . $mg^{-1} \times 10\ s^{-1}$ which represents a 4–5-fold purification above the receptor-enriched membrane and is still several-fold greater than the activity of preparations that have not been passed through an affinity column.

As shown in table 4, the carbamylcholine-dependent ion flux of the reconstituted vesicles containing purified receptor were also inhibited by α-bungarotoxin and procaine and showed the phenomenon of desensitization.

Table 4
Inhibition of ion flux of purified receptor

Additions	nmol . 10 s^{-1} [a]	nmol . 10 s^{-1} . mg^{-1}	Inhibition
None	19.8	1160	0%
+ 2.5 M α-Bungarotoxin[b]	0.6	35	97%
+ 1 mM Procaine	10.1	591	49%
+ 4 mM Procaine	2.0	117	90%
+ 200 μM Carbamylcholine added 20 s before ^{86}Rb	0.4	23	98%

[a] Expressed as carbamylcholine-stimulated flux
[b] All inhibitors were incubated with reconstituted vesicles for 20 min at room temperature before assay

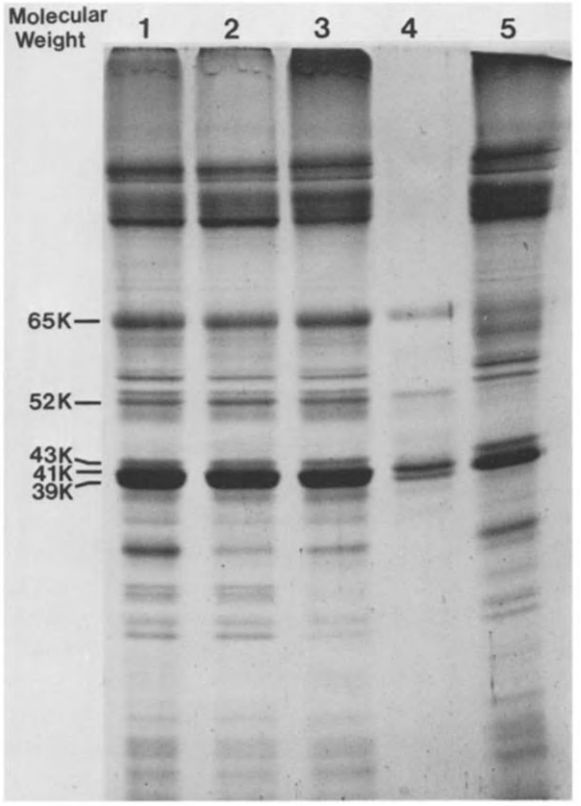

Fig.1. SDS–polyacrylamide gel electrophoresis of preparation: (1) receptor-enriched membranes (25 μg); (2) pH-treated membranes (25 μg); (3) cholate extract (15 μg); (4) purified receptor (6 μg); (5) extract after incubation with affinity resin (50 μg).

High recoveries of reconstitutive activity from the affinity column required the presence of phospholipids in the washing and elution buffers. Without phospholipids ~60% of the activity was lost. This is not surprising since it has been reported [18] that delipidation of the receptor causes losses of the high affinity binding sites for acetylcholine.

The purified receptor preparation showed four major polypeptide bands in SDS–acrylamide gels (fig.1) of 39 000, 41 000, 52 000, and 65 000 mol.wt. This differs from the patterns reported for the receptor purified with an α-neurotoxin affinity column [19]. The major difference is the almost total absence of a 60 000 mol.wt band and the appearance of a doublet in the 40 000 mol.wt region. Variations in the 60 000 mol.wt component have been reported [20] and our results suggest that it may not be required for either ion flux activity or desensitization. However, further experiments are required to rule out the possibility that more of this component is present during reconstitution than is revealed by SDS–gel electrophoresis.

During the preparation of this manuscript, reconstitution of the carbamylcholine-dependent ion flux from alkaline-treated membranes from *Torpedo californica* [21] and *Torpedo marmorata* [22] was reported by a method similar to the one described in [8].

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