THE EFFECT OF CHOLESTEROL ON AGONIST-INDUCED FLUX IN RECONSTITUTED ACETYLCHOLINE RECEPTOR VESICLES

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Received 19 September 1980

1. Introduction

The AChR has been successfully incorporated into functional vesicles and planar bilayer membranes by several procedures [1–9], using soybean lipids [1,3–5,7], total lipids isolated from electroplax membranes [6], or mixtures of lipids resembling the composition of electroplax membranes [2]. In [9] a neutral lipid fraction from asolectin was required to obtain a maximum agonist-induced ion flux, and α-tocopherol, phylloquinone or coenzyme Q_{10} were able to replace the neutral lipid component.

Here, we describe the reconstitution of the AChR from *Torpedo californica* electroplax tissue in defined lipid mixtures. In agreement with [9], phospholipids alone do not result in effective reconstitution of the permeability control properties of the receptor. However, if cholesterol is included with the phospholipids at concentrations comparable to those found in native membranes, ion permeability control can be recovered.

2. Materials and methods

2.1. *Preparation of* Torpedo *membranes*

Torpedo californica electroplax tissue (stored in liquid nitrogen) was thawed (200 g) and immediately homogenized in 240 ml homogenizing buffer (3 mM EGTA, 0.1 mM PMSF, 10 mM Tris–HCl, 0.02% NaN_3 (pH 7.4) 4°C) using a Brinkmann Polytron. The homogenate was centrifuged at 5000 × *g* for 10 min and the supernatant filtered through 4 layers of cheesecloth. The filtrate was centrifuged for 10 min at 5000 × *g* and the final supernatant layered on 25 ml 35% (w/v) sucrose in buffer I (100 mM NaCl, 60 mM KCl, 10 mM NaH_2PO_4, 0.02% NaN_3 (pH 8)) and centrifuged at 95 000 × *g* for 2 h. The soft pellet was suspended in distilled water (0°C) and centrifuged at 95 000 × *g* for 20 min. The membrane pellet was resuspended in distilled water at room temperature and adjusted to pH 11 to remove peripheral proteins [5,10]. After 30 min the suspension was centrifuged at 40 000 × *g* for 45 min, and the pellet was resuspended in buffer I and stored in liquid nitrogen.

2.2. *Solubilization and reconstitution of receptor*

The reconstitution procedure is modified from [4]. AChR-enriched membranes were diluted to 1.9 mg protein/ml in buffer I containing 1% sodium cholate. After 20 min at 0°C the insoluble material was removed by centrifugation at 130 000 × *g* for 30 min.

Lipids were prepared by drying mixtures of purified lipids under nitrogen. Each preparation contained ~9 nCi [^{14}C]DPPC/μmol lipid. The mixtures were redissolved in chloroform and again dried under nitrogen. Residual chloroform was removed in vacuo for 2 h. Buffer I was added to give a 120 mM suspension of lipids. The lipids were dispersed by a combination of rapid vortexing and bath sonication (30°C) in a nitrogen atmosphere for ~1 h. At this point some of the mixtures remained turbid but no additional change in absorbance was observed with further sonication. Sodium cholate (20%) was added to the lipid vesicles to give a final concentration of 4% cholate. After 30 min at room temperature the suspensions were sonicated for 15 min.

The solubilized AChR extract was added to the lipid suspensions (0°C) to give a lipid: protein ratio of ~37:1 (w/w) and a final lipid concentration of 30 mM

Abbreviations: AChR, acetylcholine receptor; carb., carbamylcholine; ^{125}I-α-Bgt, iodinated α-bungarotoxin; PE, phosphatidylethanolamine; PS, phosphatidylserine; PMSF, phenylmethanesulfonyl fluoride; [^{14}C]DPPC, 1,2-[1-^{14}C]dipalmitoyl-*sn*-glycero-3-phosphocholine

in 2% sodium cholate. This mixture was dialyzed against 800 vol. dialysis buffer (100 mM NaCl, 4 mM NaH_2PO_4, 0.1 mM EDTA (pH 7.4)) for 48 h at 4°C with one change of buffer. The reconstituted vesicles were frozen in liquid nitrogen before measuring the $^{86}Rb^+$ influx.

2.3. *$^{86}Rb^+$ influx assay*

Agonist-induced cation influx was measured by adding 100 μl reconstituted membranes to 15 μl dialysis buffer (0°C) containing 5×10^6 cpm of $^{86}Rb^+$ and no carb. or 7.7 mM carb. (to give 1 mM carb. final conc.). After 30 s incubation at 0°C the sample was diluted with 2 ml dialysis buffer, immediately filtered through a cold Millipore filter (HAWP, 0.45 μm) and washed 3 times with 2 ml ice-cold dialysis buffer. Filters were counted in 10 ml PCS (Amersham-Searle): xylene (2:1) in a liquid scintillation counter. The results for each preparation were normalized to the average amount of [^{14}C]DPPC trapped by the filter which was typically 26–33% of the applied lipid. A student's *t*-test for the difference between 2 means was used to test the significance of the response to carb. [11].

2.4. *Receptor binding assay*

The equilibrium binding of toxin was measured by the DE-81 filter disc method in [12] except that ^{125}I-α-Bgt was used instead of *Naja naja siamensis* [^{3}H]-toxin. The iodinated toxin was prepared and the rate of toxin binding was measured as in [13]. Protein determinations used the Lowry method [14].

2.5. *Chemicals*

Soybean PE and bovine brain PS (>99% pure) were obtained from Avanti Biochemicals. Cholesterol (A grade) was obtained from Calbiochem, asolectin from Applied Concentrates and cholate from Sigma Chemical Co. [^{14}C]DPPC (100 mCi/mmol) and $^{86}Rb^+$ (0.5–10 Ci/g) were obtained from New England Nuclear.

3. Results

The cholate extract used in 8 different sets of reconstitution experiments had a mean ^{125}I-α-Bgt binding activity of 3.8 nmol/mg protein which represented a 3.8-fold purification of toxin sites compared to the original receptor-enriched membranes (table 1). By SDS electrophoresis, the 4 major polypeptide bands of the AChR [15] were the predominant bands in the cholate extract (not shown).

Table 1
Purification and solubilization of AChR-enriched membranes from *Torpedo californica*

	Yield %		^{125}I-α-Bgt-binding act. ± SD (pmol/mg protein)	Purification ×
	Protein	^{125}I-α-Bgt sites		
Original membranes	100	100	1356 ± 241	–
Base-extracted membranes	47	87	2549 ± 443	1.9
Cholate extract	19	52	3820 ± 978	2.8

For most of the studies the cholate extract was reconstituted with various amounts of cholesterol in the presence of a 3:1 molar ratio of PE:PS (fig.1). The total lipid concentration was maintained at a constant value of 30 mM and the lipid: protein ratio was typically 37:1 (w/w). The functional activity of the reconstituted membranes was measured by the agonist-stimulated uptake of $^{86}Rb^+$ into AChR vesicles. In the presence of 25–50 mol% cholesterol, a significant ($p < 0.01$) agonist-stimulated $^{86}Rb^+$ influx was observed. The $^{86}Rb^+$ influx response was comparable to results obtained using asolectin in the reconstitution procedure (not shown). With 0–10 mol% cholesterol the agonist-stimulated response was not significantly different from zero at the 95% confidence level but there was a trend in the data from two replicates to suggest a small response.

The $^{86}Rb^+$ counts trapped by the Millipore filters increased at higher cholesterol content (fig.1B). Since the filters always trap between 26 and 33% of the [^{14}C]DPPC, the trapping efficiency does not appear to be responsible for the increase in trapped $^{86}Rb^+$ counts. Freezing the reconstituted vesicles in liquid nitrogen appears to result in aggregates of vesicles, which are more effectively trapped by the filters.

The rate of ^{125}I-α-Bgt binding to the AChR was similar in the presence or absence of 25 mol% cholesterol (fig.2). Moreover, in both lipid environments coincubation with 5 μM carb. decreased the rate of toxin binding. Preincubation with carb. further decreased the rate of binding indicating the AChR

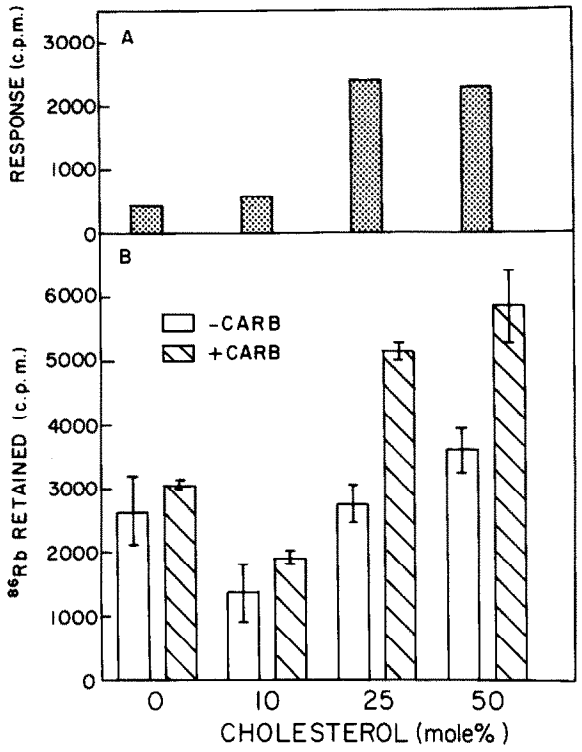

Fig.1. Effect of supplementing AChR-reconstituted vesicles with cholesterol on agonist-induced $^{86}Rb^+$ influx. (A) $^{86}Rb^+$ influx response to carb. (cpm(+ carb.)−cpm(− carb.)). (B) Total counts retained by Millipore filters. Counts obtained by filtering an equivalent amount of $^{86}Rb^+$ in the absence of membranes has been subtracted. The error bars indicate ± 1 SD (N = 4). The results are representative of 2 replicate reconstitution experiments.

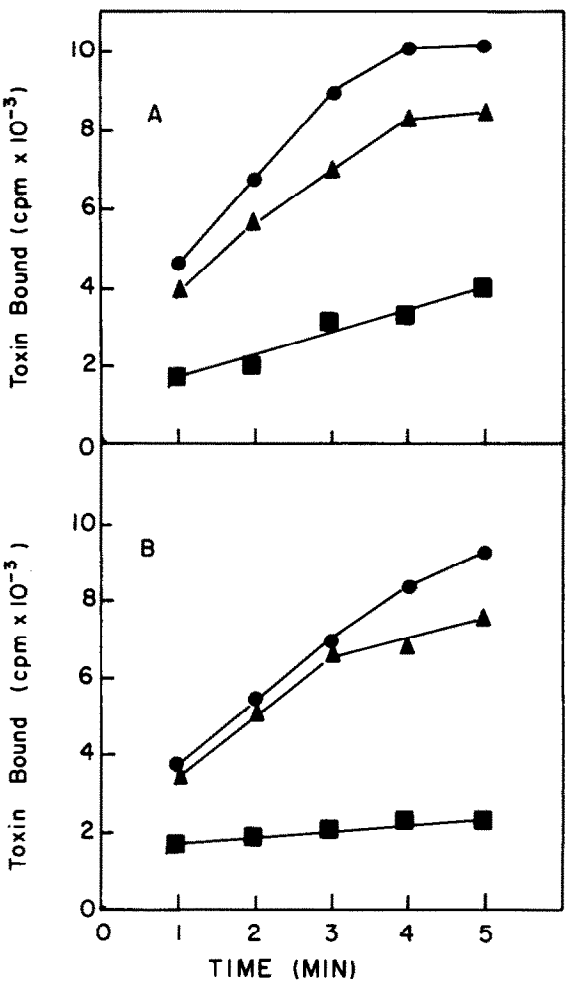

Fig.2. Rate of ^{125}I-α-Bgt binding to AChR reconstituted with: (A) 0 mol% or (B) 25 mol% cholesterol. (•) Absence of carb.; (▲) coincubation with carb.; (■) preincubation with carb.

underwent an agonist induced shift from a 'low' affinity to a 'high' affinity binding state for the agonist. This transition occurred independently of the ability of the AChR to allow an agonist-induced ion flux in these lipid environments. Although the shift to high affinity binding results in functional desensitization we had observed that ion flux can be blocked even though the AChR is in the low affinity state [13].

The carb. dose-response curve (fig.3) showed that the maximum agonist-induced ion flux response was

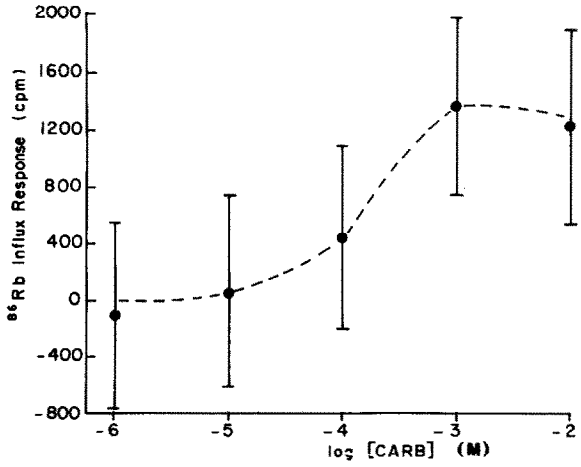

Fig.3. Effect of [carb.] on $^{86}Rb^+$ influx into reconstituted AChR vesicles containing PE:PS (3:1 molar ratio) with 25 mol% cholesterol. Error bars indicate the 95% confidence limits for the difference between the means of samples with and without carb. [11].

obtained at 10^{-3}–10^{-2} M carb. The 95% confidence limits are large but at lower [carb.] there was a trend in the flux response which suggests that half of the maximum response was obtained at 10^{-4}–10^{-3} M carb.

In preliminary experiments we have observed that several other phospholipid mixtures supplemented with cholesterol result in functionally active AChR. However, soybean phosphatidylcholine alone in the presence or absence of cholesterol gave poor reconstitution results.

4. Discussion

The AChR has been reconstituted in a defined system containing PE and PS (3:1 molar ratio). A significant agonist-induced ion flux was obtained only when reconstituted vesicles contained 25–50 mol% cholesterol. Native AChR enriched membrane lipids from *Torpedo californica* electric tissue contain 45 mol% cholesterol [2] and thus the cholesterol content required to produce the maximum agonist-induced ion flux (25–50 mol%) approaches the physiological range.

When reconstituted in the presence of 25 mol% cholesterol, the AChR had similar binding properties to native membranes. The agonist carb. competed for the α-Bgt-binding site and the low affinity carb.-binding site could be converted to a high affinity site by preincubation with carb. However, when the reconstituted AChR vesicles contained <1 mol% residual cholesterol (from the base-extracted membranes) the transition from low to high affinity states still took place although the agonist-induced ion flux was blocked. This suggests that cholesterol may be involved in modulating the ion channel itself without affecting the agonist-binding properties of the receptor. When α-tocopherol, phylloquinone or coenzyme Q_{10} were used to supplement mixtures of phospholipids similar to those used here, functional AChR vesicles were produced [9]. It is possible that all these compounds act by a similar mechanism.

These results provide additional evidence that AChR ion permeability control is very sensitive to the lipid environment as observed in [13]. Reconstituted membranes will be an important system to systematically study lipid:protein interactions. In addition, the reconstituted membranes may make it possible to define the role of individual subunits of the AChR complex and to establish the kinetic and structural linkage between ligand binding and ion flux.

Acknowledgements

The technical assistance of Pamela Badger is greatly appreciated. This research was supported by USPHS grant no. NS 13050.

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