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Cholesterol stabilizes the structure of the nicotinic acetylcholine receptor reconstituted in lipid vesicles

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A technique of heat inactivation of α-bungarotoxin binding sites, has been used to probe structural alteration of the nicotinic acetylcholine receptor when reconstituted into soybean lipid vesicles containing different amounts of added cholesterol. The profiles of heat inactivation of α-bungarotoxin binding sites are gradually shifted to higher temperatures, as the cholesterol/phospholipid molar ratio in the reconstituted vesicles is increased from 0 to 0.4, thus, indicating that presence of cholesterol within the lipid matrix produces a structural stabilization of the reconstituted acetylcholine receptor protein. The observed stabilization of receptor structure induced by cholesterol is such that, depending upon the different conditions used to form the reconstituted vesicles by detergent dialysis procedures, the profiles of heat inactivation for the reconstituted receptor vesicles at a cholesterol/phospholipid molar ratio of 0.4 become undistinguishable from that exhibited by native acetylcholine receptor membranes isolated from the electric organ of *Torpedo*. Increasing the cholesterol concentration in the reconstituted vesicles also induces a decrease in the apparent 'fluidity' of the membrane, which correlates very closely with the observed stabilization of the receptor protein. Such a correlation, however, does not necessarily imply that changes in receptor structure are caused by perturbations of the membrane 'fluidity'. This conclusion is based on experiments using local anesthetics, well known to cause alteration of membrane lipid dynamics, but unable to modify the characteristic heat-inactivation profiles from native acetylcholine receptor membranes. As a possible alternative to the above observations, it is suggested that the effects of cholesterol on receptor structure could be exerted through direct interaction with the receptor protein. Also, since similarly high concentrations of cholesterol have been reported to be required for optimal cation-gating activity of reconstituted acetylcholine receptor, we interpret our data as indicative of a correlation between structural and functional alterations of the acetylcholine receptor induced by the presence of cholesterol within the membrane bilayer.

Introduction

The nicotinic acetylcholine receptor is a transmembrane glycoprotein found at the vertebrate neuromuscular junction and in the electric organ of *Torpedo* and other species of electric fish. Binding of cholinergic agonists to extracellular (synaptic) domains on the receptor protein promotes a large increase in cation permeability through the formation of a transient cation channel, responsible for the indication of postsynaptic membrane depolarization (for a recent review, see Refs. 1–4).

A major advance in the understanding of this cation channel-linked membrane receptor was achieved by the introduction of membrane reconstitution techniques in these studies. Such procedures, allow the reinsertion of detergent-solubilized acetylcholine receptor into lipid vesicles or planar bilayers with retention of functional properties characteristic of native membrane-bound receptor. Among other contributions, reconstitution into liposomes of defined lipid composition [5–11], has provided an excellent model system in which to characterize the lipid dependence of functional aspects of the receptor. In particular, the presence of cholesterol at high concentrations in the reconstituted vesicles is widely accepted as a requirement for optimal receptor cation-gating activity upon agonist binding and also for pro-

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tection of agonist-induced affinity state transitions [5,7–10]. On the other hand, very little is known of whether the presence of cholesterol within the bilayer, determines structural aspects of the reconstituted receptor protein [11]. Furthermore, although cholesterol may be important for the function of membrane proteins, including the acetylcholine receptor, it is not clear whether its effects are exerted through alteration of properties of the lipid bilayer, i.e., the so-called membrane 'fluidity' [12], or to more specific interactions with the membrane protein [13].

We previously reported on the use of thermal perturbation techniques, using native membranes containing acetylcholine receptor, to monitor structural changes in the receptor protein caused by the presence of cholinergic ligands or membrane perturbants [14,15]. In this paper, we are extending our observations to reconstituted membranes containing acetylcholine receptor and different amounts of cholesterol within the lipid matrix. Our goal is to contribute evidence for a possible role of cholesterol as an effector on receptor structure.

Materials and Methods

Cholesterol, tetracaine hydrochloride, sodium cholate and crude extracts of phosphatidylcholine from soybean (type 2-S, asolectin lipids) were purchased from Sigma and β-D-octylglucoside was from Pharmacia. Neutral lipids present in asolectin, were eliminated by passing the lipid extracts, dissolved in chloroform, through a silica gel 60 (230–400 mesh, Merck) column, followed by elution with chloroform. Phospholipids in asolectin were then eluted from the column by using chloroform/methanol mixtures. [^{125}I]-α-Bungarotoxin was purchased from New England Nuclear and the fluorescence probe 1,6-diphenyl-1,3,5-hexatriene was from Molecular Probes.

The electric organ of live *Torpedo marmorata* was used to prepare membranes highly enriched in acetylcholine receptor, as previously described, including alkaline extraction of peripheral membrane proteins [16]. These alkaline-treated membranes were suspended in 10 mM Hepes buffer (pH 7.4) containing 100 mM $NaNO_3$ (Hepes buffer) and had specific activities of approx. 4 nmol of α-bungarotoxin bound/mg of membrane protein.

Reconstitution of acetylcholine receptor into vesicles containing soybean phospholipids and cholesterol in different proportions, was achieved through a modification of a previously reported detergent-dialysis procedure [17]. When octylglucoside was used as the detergent, mixtures containing phospholipid, cholesterol and octylglucoside, dissolved in chloroform, were dried under vacuum as thin films in glass tubes. A volume of hydration buffer (10 mM Tris, pH 7.4, containing 0.1 mM EDTA and 100 mM NaCl) was then added, and the lipid/detergent mixtures were suspended by sonication using a Soniprep 150 apparatus. When cholate was used as the detergent, it was included in the hydration buffer and, therefore, the dried lipid films were prepared in the absence of detergent. For either detergent, the final concentration of phospholipids (in terms of lipid phosphorus) and detergent in these mixtures were of 40 mM and 4% (w/v), respectively. Alkaline-treated membranes containing acetylcholine receptor (1.0–1.5 ml at 2 mg protein/ml) were solubilized by mixing (1 : 1, v/v) with the detergent/lipid mixtures from above, so that final concentrations in all solubilized mixtures were 20 mM lipid phosphorus, 2% (w/v) detergent and 1 mg protein/ml, plus an additional amount of cholesterol present only in those samples designed to contain it. Under these solubilization conditions, the 'effective' (micellar) concentration of detergent [18], is maintained much lower than those causing loss of ionic channel activity [4]. Insoluble material remaining upon approx. 30 min incubation at room temperature, was removed by centrifugation and the supernatants were dialyzed against 3×0.5 l changes of hydration buffer, followed by 2×0.5 l changes of Hepes buffer. Efficiency of reconstitution under these conditions was 80–90% [17]. The reconstituted vesicles were frozen in liquid N_2 prior to use.

'Heat inactivation' refers to monitoring the characteristic loss of α-bungarotoxin binding by acetylcholine receptor membranes as heat is supplied to the samples. A detailed description of the procedures used in 'heat inactivation' has been given elsewhere [14,15].

Steady-state fluorescence polarization was measured on a Perkin-Elmer LS-5 spectrofluorimeter equipped with an automatic polarization accessory and a thermostated cell holder. Membrane samples were diluted to a final lipid phosphorus concentration of 20–40 μM to secure constant 'dilute' polarization values [19] and diphenylhexatriene (1 mM), dissolved in ultraviolet grade tetrahydrofuran, was added to the membranes at a fluorophore to phospholipid molar ratio of 1/1000. After 2 h incubation at room temperature, the probe was excited at 365 nm with slit widths set at 4 nm. A Corning 3–73 filter was used in the emission beam to eliminate scattered light.

Results

Figs. 1 and 2 show 'heat-inactivation' profiles corresponding to reconstituted membranes prepared by detergent dialysis procedures, in the presence of different amounts of added cholesterol. Cholate and octylglucoside were selected as the detergents to be used in these experiments, because of a previous study [14] in which it was shown that the destabilizing effects on receptor structure induced by treatment with either of these detergents, can be reversed upon elimination of the

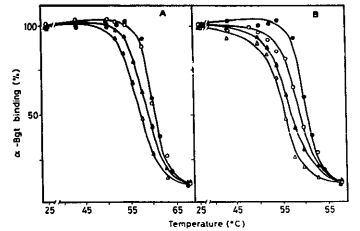

Fig. 1. Heat inactivation of α-bungarotoxin (α-Bgt) binding sites in acetylcholine receptor membranes (approx. 1 mg protein/ml) reconstituted in whole asolectin/cholesterol lipid mixtures, from octylglucoside (panel A) or cholate (panel B) -solubilized extracts. Cholesterol/lipid phosphorus molar ratios in the reconstituted vesicles were 0 (△), 0.2 (▲) and 0.4 (○). The profile of heat inactivation of α-bungarotoxin binding sites in native acetylcholine receptor membranes (●) is also included for a better comparison with the reconstituted samples.

detergent by dialysis. Qualitatively, all the results shown in Figs. 1 and 2 suggest that increasing the cholesterol presence in the reconstituted membranes, causes a thermal stabilization of the neurotoxin binding sites on the acetylcholine receptor. Moreover, when an added cholesterol/phospholipid molar ratio of 0.4 is used to prepare the reconstituted membranes, the corresponding 'heat-inactivation' profiles are identical (Fig. 1A), or closely resemble that exhibited by native acetylcholine receptor membranes.

Reconstituted acetylcholine receptor membranes used in these studies, were formed either from whole asolectin lipids (Fig 1A and B) or from neutral lipid-depleted asolectin (Fig. 2A and B). A comparison between Figs. 1 and 2 shows that, while addition of cholesterol has

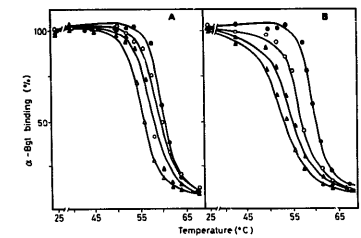

Fig. 2. Experimental conditions and symbols used here are the same as those described in Fig. 1 except that purified asolectin phospholipids (see Materials and Methods) instead of whole asolectin, were used for reconstitution.

TABLE I

Effects of cholesterol on the T_{50} values from heat inactivation of toxin binding sites in reconstituted acetylcholine receptor membranes differing in cholesterol content

Reconstituted membranes used in these studies were made from either octylglucoside or cholate-solubilized extracts, using whole asolectin or purified asolectin phospholipids. Reported T_{50} values (°C) are the average from 3–6 different experiments. Standard deviation of heat inactivation data is within 0.3°C.

Type of membrane	Cholesterol/phospholipid (mol/mol)		
	0	0.2	0.4
Octylglucoside, whole asolectin	57	58.5	60
Octylglucoside, asolectin phospholipids	55	57	59
Cholate, whole asolectin	56	57	59
Cholate, asolectin phospholipids	54	56	58

similar effects in stabilizing the acetylcholine receptor structure in both systems, those using whole asolectin lipids are more efficient than those containing only the phospholipids from asolectin, in allowing the reconstituted acetylcholine receptor to recover 'heat-inactivation' profiles resembling more closely that exhibited by native receptor membranes. These effects of whole asolectin might be related to the presence of additional neutral lipids in these extracts which, in the absence of added cholesterol, were shown to be critical for functional reconstitution of acetylcholine receptor in asolectin vesicles [20]. Also, the use of cholate or octylglucoside in the detergent-dialysis procedure does not appear to cause qualitative differences in the effects of added cholesterol. However, it can be observed that, under our experimental conditions, reconstitution from octylglucoside extracts (Figs. 1A and 2A) is slightly more efficient than that from cholate (Figs. 1B and 2B) in allowing the reconstituted receptor to regain 'heat-inactivation' profiles similar to that of native membranes.

The sigmoid-like patterns of 'heat-inactivation' curves can be more easily expressed in terms of their T_{50} values, i.e., the temperatures at which 50% of the initial capacity of the membranes to bind α-bungarotoxin remains (Table I). Depending upon the concentration of added cholesterol, T_{50} values ranged from 54°C for the less stable reconstituted system (asolectin phospholipids in the absence of added cholesterol, using cholate as the detergent) to 60°C (whole asolectin and cholesterol at a cholesterol/phospholipid molar ratio of 0.4, using octyl glucoside as the detergent), which is also the T_{50} value characteristic of native acetylcholine receptor membranes [14].

The effects of cholesterol on bulk properties of the lipid matrix has been investigated using diphenyl-

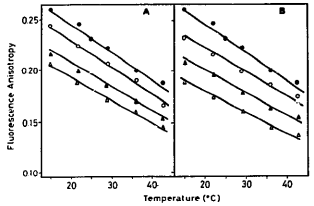

Fig. 3. Temperature dependence of the fluorescence anisotropy of diphenylhexatriene, incorporated into acetylcholine receptor membranes reconstituted in whole asolectin/cholesterol lipid mixtures, from octylglucoside (panel A) or cholate (panel B) -solubilized extracts. Cholesterol/lipid phosphorus molar ratios in the reconstituted samples were 0 (△), 0.2 (▲) and 0.4 (○). The temperature dependence of the fluorescence anisotropy of diphenylhexatriene, incorporated into native acetylcholine receptor membranes (●), is also included for comparison with the reconstituted samples.

hexatriene to probe the apparent 'fluidity' of the reconstituted membranes. Fig. 3 shows representative results obtained from several of the reconstituted membranes used in the above 'heat-inactivation' studies. At any given temperature, none of the samples investigated exhibited apparent 'fluidity' values identical to those from native membranes. This is not surprising because of the differences in lipid composition between the native and the asolectin-reconstituted receptor membranes. Nonetheless, as expected from the behaviour of cholesterol in lipid bilayers [21,22], there is a progressive decrease in the membrane's apparent fluidity (increase in steady-state anisotropy of the fluorescence probe), as the cholesterol molar fraction increases. These observations are similar to those reported by others [10,23] based on the order parameter obtained from electron spin resonance spectroscopy to assess apparent 'fluidity' of receptor membranes.

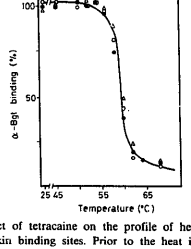

Fig. 4. Effect of tetracaine on the profile of heat inactivation of α-bungarotoxin binding sites. Prior to the heat inactivation assay, native acetylcholine receptor membranes at approx. 2 mg protein/ml, were preincubated overnight, at 4°C, in the presence of added aliquots from a concentrated solution of tetracaine in buffer. Final concentrations of tetracaine in the incubation mixture were zero (control, ●), 1 (○) and 10 (△) mM.

For either the cholate or the octylglucoside-reconstituted membrane series differing in the contents of added cholesterol, plots of the steady-state anisotropy values measured at a given temperature versus the T_{50} values from 'heat-inactivation' curves, yield straight lines with excellent linear regression coefficients (plots not shown), thus suggesting that the stabilizing effects on the receptor protein structure induced by cholesterol could be mediated by modification of 'fluidity' properties of the lipid matrix. Such a conclusion, however, is not supported by the results in Fig. 4 in which it is shown that high concentrations of tetracaine, a quaternary amine local anesthetic well known to produce large changes in the lipid order parameter [24], has no effects on the thermal stability of the neurotoxin binding sites on the receptor protein. Similar observations were made using mepivacaine, instead of tetracaine (data not shown).

Discussion

Information on structural changes of the acetylcholine receptor can be obtained from calorimetric procedures including differential scanning calorimetry [25] as well as other thermal perturbation techniques [14,15, 26]. These techniques have the advantage of being highly selective to monitor overall structural alteration of the acetylcholine receptor protein, thus, overcoming the contributions of lipid or other proteins to the observed signal and enabling its use to study complex samples such as native membranes. In particular, 'heat-inactivation' of the neurotoxin binding sites on the receptor protein constitutes a direct and simple approach to assess changes in receptor conformation which might have occurred as a consequence of ligand binding or membrane perturbations [14,15].

We report here 'heat-inactivation' studies on reconstituted acetylcholine receptor membranes to test whether cholesterol has a role in altering structural features of the membrane-bound receptor. For either cholate or octylglucoside-reconstituted acetylcholine receptor membranes, our results consistently indicate that, as the cholesterol concentration in the membranes increases, there is a progressive structural stabilization of the reconstituted receptor protein which, at sufficiently high cholesterol/phospholipid ratios (approx. 0.4, mol/mol), closely resembles or equals that corresponding to the acetylcholine receptor in its native membrane environment. In relation to this, it was previously shown that increasing the cholesterol concentration in reconstituted membranes, gradually improves the ability of the reconstituted protein to recover its

characteristic cation-gating activity [7–9]. Furthermore, a cholesterol/phospholipid molar ratio of approx. 0.4, was also reported to be optimal for recovery of cation-gating activity in functional studies of reconstituted acetylcholine receptor [9].

The effects of cholesterol in stabilizing the receptor protein structure correlate very closely with the alteration by cholesterol of the apparent membrane 'fluidity', a term which includes the occurrence of all molecular motions throughout the lipid bilayer. From such a correlation it is unclear whether the effects of cholesterol on receptor structure are exerted directly or as a consequence of a modification of the membrane's physical properties. In an attempt to distinguish between these two alternatives, we studied the effects of local anesthetics in our system. At high concentrations, local anesthetics are well known for their ability to perturb membrane 'fluidity'. In fact, the potency of these compounds as anesthetics correlates with their efficiency in altering membrane 'fluidity' [27–29]. In spite of this, we found that tetracaine or mepivacaine did not modify the profiles of 'heat inactivation' in native receptor membranes. Furthermore, local anesthetics were shown not to have any effects on the transition endotherm characteristic of the acetylcholine receptor protein in differential scanning calorimetry experiments [25]. From this, we conclude that a change in membrane 'fluidity' does not necessarily imply an effect on the receptor protein structure and, therefore, it could be hypothesized that such effects could perhaps be exerted through direct interaction with the receptor protein. Such an hypothesis is consistent with previous observations in which spin label derivatives of cholesterol were suggested to interact strongly with the acetylcholine receptor [23]. Also, it was reported that a photoreactive cholesterol analogue binds to the receptor protein [30] and that brominated cholesterol quenches the receptor's intrinsic fluorescence [31]. Furthermore, Fourier transform-infrared studies of erythrocyte membrane proteins [32] and reconstituted acetylcholine receptor [11], indicate that cholesterol stabilizes transmembrane α-helical segments of the proteins. Receptor protein sites presumably involved in such an interaction could perhaps be adscribed to the hydrophobic M4 membrane spanning α-helical segments, which are common to all acetylcholine receptor subunits and become covalently labeled by photoactivable phospholipid analogues [33].

Finally, it should be noticed that in our 'heat-inactivation' assay, the observed effects of cholesterol on the acetylcholine receptor imply that perturbations at the transmembrane portion of the protein caused by interaction with lipids, elicits an alteration on the neurotoxin binding sites, which are located well above the membrane surface [34]. The occurrence of similar long-range perturbations transmitted through the receptor protein have been suggested previously [9,15,35].

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