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Lipid modulation of nicotinic acetylcholine receptor function: the role of neutral and negatively charged lipids

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The effects of negatively charged and neutral lipids on the function of the reconstituted nicotinic acetylcholine receptor from *Torpedo californica* were determined with two assays using acetylcholine receptor-containing vesicles: the ion flux response and the affinity-state transition. The receptor was reconstituted into three different lipid environments, with and without neutral lipids: (1) phosphatidylcholine/phosphatidylserine; (2) phosphatidylcholine/phosphatidic acid; and (3) phosphatidylcholine/cardiolipin. Analysis of the ion flux responses showed that: (1) all three negatively charged lipid environments gave fully functional acetylcholine receptor ion channels, provided neutral lipids were added; (2) in each lipid environment, the neutral lipids tested were functionally equivalent to cholesterol; and (3) the rate of receptor desensitization depends upon the type of neutral lipid and negatively charged phospholipid reconstituted with the receptor. The functional effects of neutral and negatively charged lipids on the acetylcholine receptor are discussed in terms of protein–lipid interactions and stabilization of protein structure by lipids.

Introduction

The nicotinic acetylcholine receptor (AChR) from *Torpedo californica* has been purified and reconstituted into defined lipid environments [1], providing a model system to study protein–lipid interactions. The AChR is a pentameric integral membrane protein which is necessary for cholinergic synaptic transmission [2]. The binding of acetylcholine to the AChR is coupled to the opening of a cation-specific channel. In the continued presence of an activating ligand, the ionic permeability of the AChR is inhibited. This process, termed 'desensitization', is correlated with the shift of the AChR from a low-affinity state for agonist binding to a high-affinity state [3]. The opening of the ion channel and the affinity-state transition involve conformational changes of the AChR, which are sensitive to the membrane lipid environment [4].

Previously, this laboratory has shown that cholesterol (CH) and negatively charged phospholipids, such as phosphatidic acid (PA), are required for an optimal ion flux response of the reconstituted AChR [5]. These effects on activity are correlated with the affinity of these lipids for the AChR. For example, sterol and phosphatidic acid spin-labels showed a relatively high-affinity for the AChR, compared to other spin-labeled phospholipids [6]. Results from FTIR studies of the AChR have suggested an important role for cholesterol and negatively charged lipids in stabilizing the secondary structure of the protein [7]. Increasing the CH or PA concentration in reconstituted membranes resulted in an increase in the α-helix and β-sheet of the AChR, respectively.

The effects of cholesterol on the structure and function of the AChR appear to be correlated with the binding of cholesterol to the protein. Evidence from fluorescence quenching studies of reconstituted membranes have suggested the existence of two classes of cholesterol binding sites on the AChR [8]. The first class of sites (annular sites) were accessible to phospholipids, while the second class of sites were not (non-annular sites). The non-annular sites were shown to have a 20-fold greater affinity for cholesterol than the annular sites.

Furthermore, photoaffinity labeling experiments [9] have shown that a very closely related cholesterol derivative can directly bind to the AChR in native membranes.

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Abbreviations: AChR, acetylcholine receptor; [^{125}I]BgTx, ^{125}I-iodinated α-bungarotoxin; CH, cholesterol; FTIR, Fourier transform infrared; PA, phosphatidic acid; PC, phosphatidylcholine; PS, phosphatidylserine; TO, α-tocopherol.

A current hypothesis, which correlates all of the above observations, is that cholesterol binding to the non-annular sites on the AChR stabilizes α-helicies, which are necessary to support a functional ion channel. Cholesterol might perform this function by interacting with α-helicies, which span the membrane. To examine the specificity of the putative AChR–cholesterol interactions, we have varied the structure of the sterol and observed the function of the AChR in three different negatively charged lipid environments.

We report here that cholesterol-like sterols, such as cholestanol, and other neutral lipids, such as α-tocopherol and squalene, which are unrelated to cholesterol in structure, can effectively substitute for cholesterol in providing a membrane lipid environment to support an optimal ion flux response. The results suggest that the AChR-cholesterol effects may depend, predominantly, on the association of the neutral lipids with a hydrophobic pocket on the AChR.

Materials and Methods

Materials

Chloroform solutions of all the synthetic phospholipids were purchased from Avanti Polar Lipids (Birmingham, AL) with a dioleoyl (18:1) fatty acid composition. Cholesterol was obtained from Calbiochem (La Jolla, CA). All other sterols, neutral lipids and cholic acid were obtained from Sigma (St. Louis, MO). Dowex 50W-X8 cation exchange resin was obtained from Sigma and DE81 filters were obtained from Whatman (Maidstone, UK). [^{125}I]Iodinated α-bungarotoxin ([^{125}I]BgTx, >200 mCi/mmol) was obtained from Amersham (Arlington Heights, IL) and $^{86}Rb^+$ (10 mCi) was obtained from New England Nuclear (Boston, MA).

Acetylcholine receptor purification and reconstitution of AChR

Receptor was purified from frozen *T. californica* electric organ by affinity chromatography, as described by Ochoa et al. [10] and then dialyzed to remove the cholate detergent. The receptor was either used immediately or frozen in liquid nitrogen and then thawed immediately before use.

Chloroform solutions of phospholipids and neutral lipids were mixed in the amounts necessary to obtain a mole ratio of 60:20:20 for PC/negatively charged lipid/neutral lipid and a final lipid/protein mole ratio of 10000:1. The lipids were then dried down under nitrogen and lyophilized. Subsequently, the lipids were sonicated in the presence of 6% (w/v) cholate for 3 min to give homogeneous suspensions. Purified AChR was mixed with the lipid suspension in a ratio of 2:1 (v/v). The final cholate and protein concentrations were 2% and 1 mg/ml, respectively. The solubilized lipid-protein micellar suspension was then extensively dialyzed against buffer A (100 mM NaCl, 10 mM Mops, 0.1 mM EDTA, 0.02% NaN_3, pH 7.4) to remove the cholate and allow the formation of vesicles.

Ion flux assays

Carbamylcholine-stimulated influx of $^{86}Rb^+$ into AChR-containing vesicles was measured according to Fong and McNamee [4]. Flux assays were performed at 4°C in triplicate on each sample. Typically, ion flux was allowed to occur for 30 s in the presence or absence of activating ligand, prior to separation of the external solution from the vesicles by cation exchange chromatography. The total internal volume of the vesicles was determined by allowing influx to occur for at least 48 h.

Determination of toxin binding sites on AChR

Equilibrium binding of [^{125}I]BgTx to AChR in Triton X-100 was measured by trapping negatively charged AChR-toxin complexes on DEAE (DE81) filters, according to Fong and McNamee [4], with one modification. DE81 filters were placed in Packard Pony Vials, 4 ml of CytoScint liquid scintillation fluid added, the filters were incubated overnight at room temperature and the vials were then counted for 1 min on a Packard Tri-Carb 1500 Liquid Scintillation Analyzer set to count ^{125}I.

Rate of toxin binding assays

The rate of [^{125}I]BgTx was measured in the absence of detergent, as described by Fong and McNamee [4]. Three different types of binding rate constants were determined at 20°C: (1) k, the pseudo first-order rate constant in the absence of Carb; (2) k_{co}, the pseudo first-order rate constant when 10 μM Carb was added at the same time as toxin; and (3) k_{pre}, the pseudo first-order rate constant determined after exposure of the vesicles to 10 μM Carb for 30–60 min at 20°C. For each toxin binding reaction, samples were taken at 30, 60, 90, 120, 150 s and 1 h and filtered through DE81 filters. The filters were treated and counted as described above.

Time course of AChR desensitization

The effect of lipid composition on desensitization was determined by preincubating AChR-containing vesicles with 1 mM Carb at 4°C for a defined period of time and then performing the manual ion flux assay on the sample. The cpm value measured in the absence of Carb preincubation was used as a basis for the calculation of the fraction of remaining cpm. Semilogarithmic plots of the fraction of remaining $^{86}Rb^+$ cpm trapped in the vesicles vs. Carb preincubation time were constructed, and a least-squares linear regression was determined for each graph, using the Sigma-Plot program

on an IBM-PC. The slopes of such graphs give the rates for AChR desensitization.

Results

Ion flux activity of AChR in vesicles of different lipid composition

Sealed vesicles are required to measure the ion flux activity of the AChR. Therefore, receptor samples with a lipid to protein mole ratio of 10000:1 were reconstituted to allow the formation of appropriate vesicles. Table I lists the results of the rubidium ion influx assays for AChR vesicle samples containing different negatively charged and neutral lipids. The structures for the phospholipids and many of the neutral lipids used here are depicted in Fig. 1. Each $^{86}Rb^+$ influx reaction was allowed to occur for 30 s at 4°C in the presence or absence of Carb. Because the $^{86}Rb^+$ influx is affected by the vesicle size and AChR concentration, a parameter, termed 'response' was calculated by normalizing the internal volume (IV) of trapped $^{86}Rb^+$ to both the equilibrium internal volume (EIV) and the number of [^{125}I]BgTx sites.

Comparison of the different samples suggests that an ion flux response of less than 0.03 μM^{-1} corresponds to an inactive sample. We shall consider 0.03 μM^{-1} (ion flux response in PC/PA vesicles) as the basal level of AChR activity. Table I indicates that only the PC/PS/AChR vesicles were inactive. The flux responses of PC/PS/CH and of PC/PA/CH were typically enhanced 3–4-fold and 10-fold, respectively, compared to PC/PS and PC/PA vesicles. Comparison of samples containing cholesterol to other sterols, such as cholestanol and vitamin D-3, and other neutral lipids, such as squalene and α-tocopherol, shows that these hydrophobic molecules are functionally equiva-

TABLE I

Ion flux responses of AChR-containing vesicles [a]

Lipids [b]	+Carb (cpm)	−Carb (cpm)	EIV (cpm)	IV (μl/ml)	EIV (μl/ml)	Response [c] (μM^{-1})(×10^2)
PC/PA	3791	1335	30317	0.52	6.43	3.3±0.5
PC/PA/CH	7725	508	26407	1.53	5.60	14.3±1.5
PC/PA/CHS	8440	2568	36836	1.24	7.81	7.2±0.7
PC/PA/CHL	6191	504	23243	1.21	4.93	10.8±1.5
PC/PA/VD	5916	415	20779	1.17	4.40	14.2±1.4
PC/PA/TO	9574	535	16794	1.92	3.56	30.±4.4
PC/PA/AN	6153	460	18186	1.21	3.85	13.0±1.7
PC/PA/SQ	9847	1124	44362	1.85	9.40	9.1±0.9
PC/CA	2369	654	29391	0.37	6.42	3.1±0.8
PC/CA/CH	9195	562	40405	1.89	8.83	10.0±1.0
PC/CA/CHS	4505	944	43632	0.78	9.53	4.0±0.5
PC/CA/CHL	7479	377	29992	1.55	6.55	11.3±1.1
PC/CA/VD	6493	354	25362	1.34	5.54	9.2±1.5
PC/CA/TO	10388	448	33968	2.17	7.42	11.6±0.8
PC/CA/AN	10064	479	27732	2.09	6.06	10.3±1.6
PC/CA/SQ	17017	2558	47938	3.16	10.48	11.7±1.9
PC/PS	1032	841	16350	0.04	3.45	0.5±0.1
PC/PS/CH	1919	317	13462	0.34	2.84	5.7±0.4
PC/PS/CHS	3707	1305	32857	0.51	6.93	3.0±0.5
PC/PS/CHL	1815	368	12788	0.31	2.70	4.5±0.6
PC/PS/VD	1887	299	12339	0.34	2.60	5.9±0.8
PC/PS/TO	5426	301	14348	1.08	3.03	17.6±2.6
PC/PS/AN	1983	368	9934	0.34	2.10	6.8±1.1
PC/PS/SQ	7723	648	27228	1.49	5.75	12.6±0.8

[a] Vesicle internal volume (IV) is defined as the internal volume filled in response to 30 s exposure to Carb, subtracting the internal volume filled in the absence of Carb, per total volume after addition of $^{86}Rb^+$ to the external medium. Equilibrium internal volume (EIV) was measured by allowing the influx reaction to proceed for 48 h at 4°C.

[b] Abbreviations: PC, dioleoylphosphatidylcholine; PA, dioleoylphosphatidic acid; CA, cardiolipin (bovine brain); PS, dioleoylphosphatidylserine; CH, cholesterol; CHL, cholestanol; CHS, cholesterol hemisuccinate; VD, vitamin D-3 (cholecaliferol); TO, (±)-α-tocopherol; AN, androstanol; SQ, squalene. Most of the vesicle samples contain PC, negatively charged lipid and neutral lipid in the mole ratio 60:20:20. Samples containing only PC and negatively charged lipid have a lipid mole ratio of 80:20.

[c] Response = IV/EIV/[AChR]. AChR concentration is expressed as the molar concentration of [^{125}I]BgTx binding sites. Errors are given as the standard deviation of the mean of at least triplicate samples.

lent to cholesterol in eliciting an optimal ion flux response. Similar results were obtained for each of the three negatively charged lipid compositions studied.

Table I shows that in lipid environments containing PA or PS, vesicles with tocopherol showed 2- and 3-fold increases in ion flux response with respect to cholesterol, respectively. Comparison of the three negatively charged phospholipid environments shows that the responses in PS are about 2-fold lower than those in PA or cardiolipin. We do not attach great significance to the quantitative differences listed above because the ion flux response includes contributions from the rates of fast and slow desensitization, in addition to the rate of channel activation.

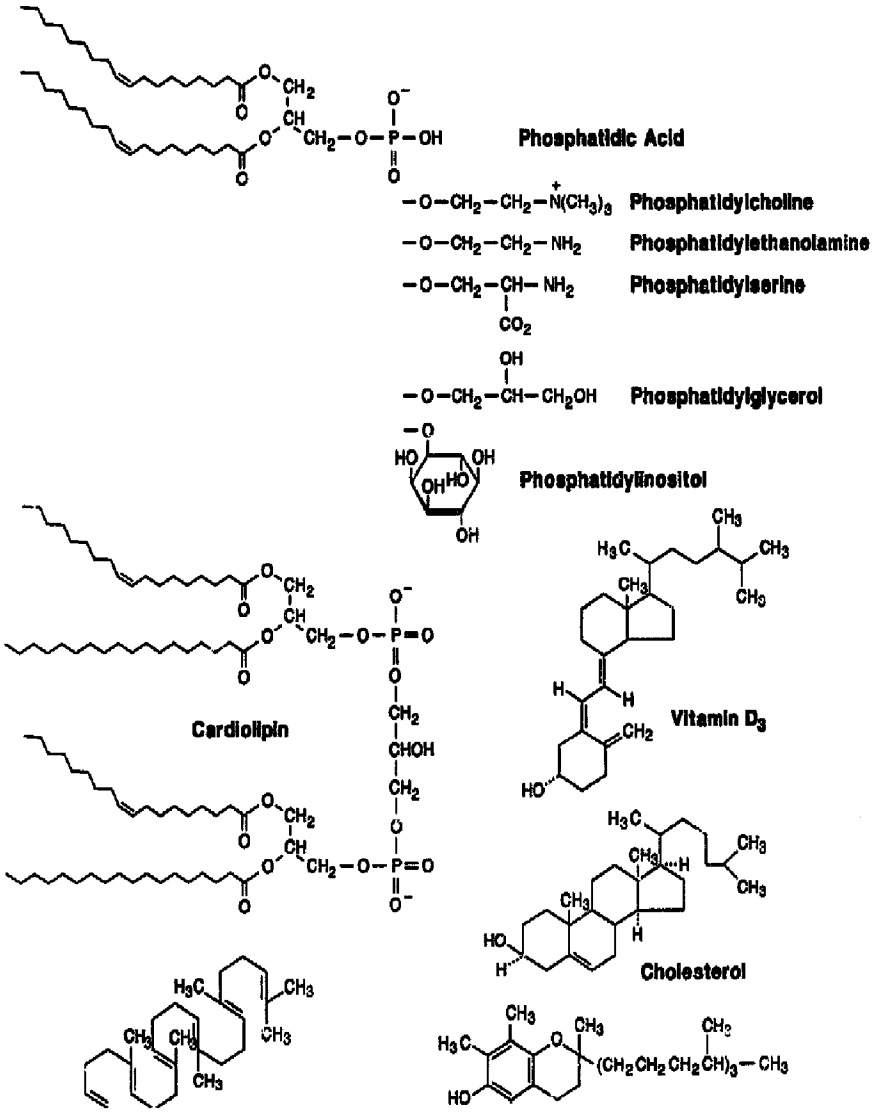

Fig. 1. Structures of representative phospholipids and neutral lipids.

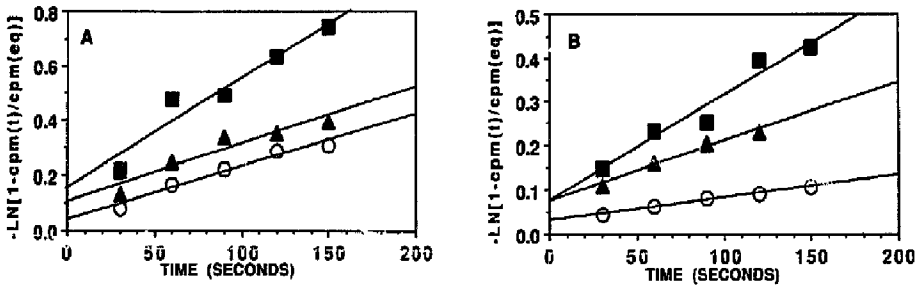

Fig. 2. Time course of [^{125}I]BgTx binding to reconstituted AChR-containing membranes for two representative lipid compositions in the presence and absence of Carb. Toxin binding was initiated by the addition of AChR-containing membranes (lipid/protein mole ratio of 10000:1, 5–10 nM in toxin binding sites) to Buffer A containing 30 nM [^{125}I]BgTx. Aliquots were filtered and counted as described in Methods. The lipid compositions were: (A) PC/PS and (B) PC/PA/TO. ■, No Carb added; ▲, co-incubation with 10 μM Carb; ○, membranes preincubated with 10 μM Carb for 30 min and then binding measured in the presence of 10 μM Carb.

Affinity-state transition of AChR in different lipid environments

Fig. 2 shows plots of $-\ln(1-\text{cpm}(t)/\text{cpm}(\text{eq}))$ vs. time (s) for membranes containing PC/PS and PC/PA/TO, where cpm(t) is the counts of [^{125}I]BgTx bound to AChR at time t, after the initiation of the reaction and cpm(eq) is the equilibrium value of the counts bound to AChR, measured after 1 h. The slopes of these plots directly give the pseudo first-order toxin binding rate constants, which are given in Table II. The plots illustrate the difference between an inactive sample (PC/PS), where k_{co} and k_{pre} are very close in value, and an active one, in which there is a relatively large difference between k_{co} and k_{pre}.

Table II lists the values of [^{125}I]BgTx binding rate constants to AChR in different membranes. We will define the AchR as undergoing the affinity-state transition if k_{pre} is at least 2-fold lower than k_{co}. It is apparent that only PC/PS vesicles do not undergo the affinity-state transition. The remaining vesicle samples show a significantly smaller value of k_{pre} than of k_{co}. Fong and McNamee [11] have previously shown that the AChR can undergo the transition in PC/PA/CH and PC/PS/CH (lipid mole ratio 56:19:25) membranes.

TABLE II

Pseudo-first-order [^{125}I]BgTx binding rate constants for AChR reconstituted in six different lipid environments (s^{-1}) ($\times 10^{-1}$) [a]

Lipids	k_{max} [b]	k_{co}	k_{pre}
PC/PS	4.08	2.07	1.89
PC/PA	3.24	2.41	0.79
PC/PS, CH	5.58	2.97	0.77
PC/PA/CH	2.94	1.66	0.30
PC/PS/TO	3.55	2.39	0.48
PC/PA/TO	2.40	1.34	0.51

[a] [^{125}I]BgTx binding to AChR was measured in the presence and absence of Carb as described in Materials and Methods and Results.

[b] Rate constants were determined from the slope of plots of $-\ln[1-\text{cpm}(t)/\text{cmp}(\text{eq})]$ vs. time (s), as detailed under Results.

Rate of AChR desensitization in different lipid environments

Fig. 3 shows semilogarithmic plots of the percentage remaining cpm vs. Carb preincubation time (s) in four different lipid environments. The slopes of these plots directly give the rate constants for desensitization listed in Table III. Table III gives the rates for AChR desensitization and the calculated half-lives for this process. The rate of desensitization is dependent on the type of

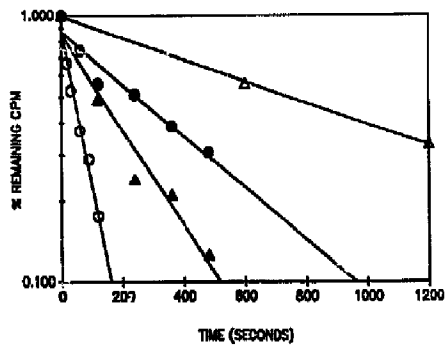

Fig. 3. Rates of AChR desensitization measured using the ion flux assay. 50 μl of reconstituted AChR-containing membranes (lipid/protein mole ratio 10000:1 and approx. 50 μM in AChR) were preincubated with 1 mM Carb for the indicated times. 15 μl of 0.5–1 μCi/ml $^{86}Rb^+$ containing 4.33 mM Carb was then added and flux was allowed to occur for 30 s at 4°C. A 50 μl aliquot was then applied to a 2 ml Dowex 50W-X8 column and eluted with 3 ml of 175 mM sucrose. The eluate was counted in a Packard liquid scintillation counter without added scintillant. Lipid compositions of the AChR-containing membranes were: (○) PC/PA/TO; (▲) PC/PS/TO; (●) PC/PA/CH; and (△) PC/PS/CH.

TABLE III

Rates of AChR desensitization in four different lipid environments [a]

Lipids	k (s^{-1} ($\times 10^3$)) [b]	$t_{1/2}$ (s) [c]
PC/PS/CH	0.92	749
PC/PA/CH	2.25	308
PC/PS/TO	4.15	167
PC/PA/TO	13.40	52

[a] Desensitization of $^{86}Rb^+$ ion flux response was measured by preincubating AChR-containing membranes with 1 mM Carb prior to addition of $^{86}Rb^+$ to the external medium.

[b] First-order rate constants were obtained from the slopes of semilogarithmic plots of fraction of remaining cpm vs. time, as detailed under Materials and Methods.

[c] The half-life for desensitization was calculated as follows, $t_{1/2} = 0.693/k$.

neutral and negatively charged lipid reconstituted along with the AChR. Membranes containing tocopherol have a significantly larger rate than those with cholesterol. Also, membranes containing PA have higher rates than those reconstituted with PS. The rates measured using the ion flux assay predominantly represent the slow phase of AChR desensitization, since fast desensitization is probably complete after 1 s of exposure to Carb [12].

Discussion

The work presented here was begun in an attempt to define the features of the cholesterol molecule which are necessary to support an optimal ion flux response of the reconstituted AChR. We have found that cholesterol can be replaced by several sterols, some of which are closely related (cholestanol), and other neutral lipids, such as squalene and α-tocopherol, which are unrelated in structure to cholesterol, to give functional ion channels. This was true in three different negatively charged phospholipid environments. These results suggest that it is not the specific molecular structure of cholesterol that is important to support AChR function. The hydrophobicity of the neutral lipids seems to be the major factor in supporting the ion flux activity of the AChR.

The stimulatory effect of neutral lipids was first demonstrated by Kilian et al. [13] who showed that the neutral lipid fraction of asolectin (crude soybean lipids) could be replaced by pure α-tocopherol to yield an ion flux response which was considerably higher than that observed with AChR-containing vesicles reconstituted with asolecthin.

The results reported here extend those of Fong and McNamee [4], which confirmed that cholesterol and PA were necessary to support optimal ion flux activity of the reconstituted AChR. Fong [11] also showed that PA could be replaced by phosphatidylglycerol, phosphatidylinositol, or PS to give functional AChR ion channels. We have shown here that PA can be replaced by PS or cardiolipin to give functional ion channels.

Fong and McNamee [4] also showed that the ability of a specific lipid composition to support an optimal ion flux response was positively correlated with the ability of the AChR to undergo the affinity-state transition. This transition is thought to be due to conformational changes in the presence of agonist (Carb), which results in AChR desensitization [3]. They showed that only lipid compositions that gave functional ion channels could undergo the transition. The results presented here confirm this interpretation. We found that five of the lipid compositions which yielded functional ion channels (Table II) also underwent the affinity-state transition. The one sample which was inactive in the ion flux assay (PC/PS), did not undergo the affinity-state transition.

The general effect of neutral lipid stimulation of the ion flux response of the reconstituted AChR contrasts with cholesterol stimulation of the Na-Ca exchanger from cardiac plasma membrane [14]. The reconstituted exchanger in PC/PS or PC/PA vesicles required high levels of cholesterol for activity. The sterol requirement was specific for cholesterol, since analogues with only minor structural changes, such as epicholesterol, did not support exchange activity. However, the cholesterol requirement was lost when the protein was reconstituted into PC/cardiolipin vesicles. In this case, high exchange activity could be obtained in the presence or absence of cholesterol or in the presence of several sterol analogues. Thus, for the Na-Ca exchange protein, specific cholesterol stimulation was critically dependent on the negatively charged phospholipid environment. Our results suggest that neutral lipid stimulation of the AChR ion flux response is not dependent on the type of negatively charged phospholipid reconstituted along with the protein.

In contrast to the AChR ion flux response, a marked lipid dependence of the rate of agonist-induced desensitization was found (Table III). Membranes containing tocopherol showed a higher rate of densensitization than those with cholesterol. Also, AChR-containing membranes reconstituted with PA showed higher rates than those with PS. These results suggest that the membrane lipid environment can directly affect the conformational changes responsible for desensitization. This is consistent with the results of Fong and McNamee [4] who showed that the fraction of AChR in the low-affinity state for ligand binding (desensitized) was dependent on the phospholipid environment. They also showed that the Carb dissociation constants were not sensitive to the lipid environment.

Our Carb titrations of the AChR ion flux response are consistent with no dependence of K_d for Carb

binding on the membrane lipid environment (data not shown). We have observed values of ED_{50}, the Carb concentration at which the ion flux response is reduced to half its maximal value, in the range $(3–10) \cdot 10^{-6}$ M Carb for the lipid compositions listed in Table II. Values for ED_{50} have previously been used to estimate K_d for Carb binding to the AChR. Because the amount of trapped $^{86}Rb^+$ in AChR-containing vesicles is dependent upon the rates of slow and fast desensitization, in addition to the rate of channel activation, we consider this ED_{50} range insignificant. Our data support the idea that the membrane lipid environment has little or no effect on the binding of agonist (Carb) to the AChR.

Relationship between neutral and negatively charged lipid binding and AChR structure and function

Previous results obtained in this laboratory [6,8] and photoaffinity labeling experiments [9], suggest a direct correlation between cholesterol binding to the AChR and activation of the ion channel. Fong and McNamee [7] hypothesized that the AChR-cholesterol interaction stabilized α-helical structures necessary for the function of the ion channel. The results reported here suggest that the putative AChR-cholesterol interaction is based predominantly on hydrophobic interactions, since neutral lipids with structures unrelated to cholesterol supported an optimal ion flux response.

A clue to the location of the putative cholesterol binding site(s) has come from the fluorescence quenching experiments of Arias et al. [15]. They showed in native membranes that local anesthetics, such as procaine and tetracaine, competed for sites on the AChR with a cholesterol-like spin label. Local anesthetics have been shown to bind to the AChR at two classes of sites [16], a specific site, at which histrionicotoxin can also bind, and several non-specific sites, located at the lipid/protein interface. Cholesterol and other neutral lipids may also possibly bind to the sites for local anesthetics.

We speculate that the binding of neutral lipids to the AChR at the putative cholesterol binding site(s) stabilizes α-helical structures necessary for the function of the ion channel. One prediction of this hypothesis is that if the concentration of a neutral lipid, such as α-tocopherol, were increased in reconstituted AChR-containing membranes, there would be an increase in the α-helical content of the protein, which could be measured by FTIR methods.

Based on the results reported here and additional experiments in our laboratory, the neutral lipids and negatively charged phospholipids act independently to stimulate the AChR ion flux response. For example, Bushan and McNamee (unpublished observations) have inferred a correlation between the binding of PA to the AChR, as measured using FTIR, and ion flux response, measured as a function of pH. In related reports, the location of the binding site for a PS photoaffinity label was shown to be on the M4 transmembrane domain of the AChR [17]. Previously, it was shown that a PC photoaffinity label was bound on the M4 transmembrane domain of the receptor [18].

These results suggest that there are distinct binding sites for negatively charged and zwitterionic phospholipids on the AChR. We can speculate that the putative AChR-negatively charged lipid interaction is based predominantly on charge, since the binding of many different types of lipid can activate the ion channel.

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