ELSEVIER

Biochimica et Biophysica Acta 1370 (1998) 299–309

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Where does cholesterol act during activation of the nicotinic acetylcholine receptor?

George H. Addona [a,b,c], Heinrich Sandermann Jr [d], Marek A. Kloczewiak [b], S. Shaukat Husain [b,c], Keith W. Miller [a,b,c,*]

[a] *Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115, USA*
[b] *Department of Anesthesia and Critical Care, Massachusetts General Hospital, Boston, MA 02114, USA*
[c] *Harvard Medical School, Boston, MA 02115, USA*
[d] *GSF-Forschungszentrum fuer Umwelt und Gesundheit, Institut fuer Biochemische Pflanzenpathologie, D-85764 Oberschleissheim, Germany*

Received 17 September 1997; accepted 5 December 1997

Abstract

Why agonist-induced activation of the nicotinic acetylcholine receptor (nAcChoR) fails completely in the absence of cholesterol is unknown. Affinity-purified nAcChoRs from *Torpedo* reconstituted into 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine/1,2-dioleoyl-*sn*-glycero-3-phosphate/steroid bilayers at mole ratios of 58:12:30 were used to distinguish between three regions of the membrane where cholesterol might act: the lipid bilayer, the lipid–protein interface, or sites within the protein itself. In the bilayer, the role of fluidity has been ruled out and certain neutral lipids can substitute for cholesterol [C. Sunshine, M.G. McNamee, Biochim. Biophys. Acta 1191 (1994) 59–64]; therefore, we first tested the hypothesis that flip-flop of cholesterol across the membrane is important; a plausible mechanism might be the relief of mechanical bending strain induced by a conformation change that expands the two leaflets of the bilayer asymmetrically. Cholesterol analogs prevented from flipping by charged groups attached to the 3-position's hydroxyl supported channel opening, contrary to this hypothesis. The second hypothesis is that interstitial cholesterol binding sites exist deep within the nAcChoR that must be occupied for channel opening to occur. When cholesterol hemisuccinate was covalently 'tethered' to the glycerol backbone of phosphatidylcholine, channel opening was still supported. Thus, if there are functionally important cholesterol sites, they must be very close to the lipid–protein interface and might be termed periannular. © 1998 Elsevier Science B.V.

Keywords: Acetylcholine receptor; Reconstitution; Gating; Cholesterol

Abbreviations: nAcChoR, Nicotinic acetylcholine receptor; BDH, D-β-Hydroxybutyrate dehydrogenase; DFP, Diisopropyl fluorophosphate; DOPA, 1,2-Dioleoyl-*sn*-glycero-3-phosphate; DOPC, 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine; PA, Phosphatidic acid; DTT, Dithiothreitol; EDTA, Ethylenediamine-tetraacetic acid; EGTA, Ethylene glycol-bis(*b*-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid; MOPS, 4-Morpholinopropanesulfonic acid; PCP, Phencyclidine; PMSF, Phenylmethylsulfonyl fluoride; Tethered-cholesterol, Oleoyl-2-(cholesteryl hemisuccinyl)phosphatidylcholine; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; TPS, *Torpedo* physiologic saline; TLC, Thin-layer chromatography

* Corresponding author. Fax: +1-617-726-5845; E-mail: k_miller@helix.mgh.harvard.edu

PII S0005-2736(97)00280-0

1. Introduction

The effect of cholesterol on lipid bilayer structure is quite well understood, and the structural requirements for action are quite strict [1]. On the other hand, the modulatory role of cholesterol on membrane proteins has not been explored in detail, and the mechanisms involved remain obscure; yet, it is becoming evident that cholesterol may play an important modulatory role in disease processes and in drug action. For example, cholesterol modulates proteolytic cleavage of the amyloid precursor protein and may be involved in the etiology of Alzheimer's disease [2], it modulates the action of general and local anesthetics on acetylcholine receptors [3,4], and of neurosteroids on the $GABA_A$ receptor [5].

A priori, there are three regions of the membrane with which cholesterol might modulate membrane proteins: in the bilayer, the lipid–protein interface, or on the protein itself. In the first, the protein's function depends on some physical property of the bulk lipid bilayer such as fluidity. In the second, lipid properties at the lipid–protein interface, such as acyl chain length or lateral pressure [6], might be important. In the third, allosteric binding sites, specific for cholesterol would exist on the protein, either in the lipid–protein interface (annular) or within the protein itself (interstitial sites). In the case of the acetylcholine receptor, the best understood member of a family of homologous ligand-gated channels that play important roles in the central nervous system, phospholipids alone cannot support agonist-induced channel opening in the absence of cholesterol [7]. The action on the channel does not correlate with modulation of lipid fluidity [8,9], and it has been suggested that interstitial cholesterol sites may be involved in receptor activation [10] as they appear to be in the $(Ca^{2+}–Mg^{2+})$-ATPase [11]. In the case of rhodopsin, it has been shown that cholesterol affects both function and stability. However, it is still unclear whether the mechanism of cholesterol's modulation of rhodopsin is based on a bulk lipid property [12] or a direct interaction with rhodopsin [13].

Until recently, the kinetic step at which cholesterol acts during activation of the nAcChoR remained undefined. Rapid cation flux measurements are the most direct way to determine the role of lipids in receptor activation [14], but flux kinetics are strictly dependent on vesicle morphology, which may vary with lipid composition [15]. There is no rapid and reliable method for controlling for variation in vesicle internal volume and receptor surface density, as well as for inhomogeneities in the vesicle population [16]. Furthermore, vesicles are not formed when AcChoR is reconstituted into some lipid bilayers [17,18]. Therefore, we recently developed an assay using ethidium bromide fluorescence to probe receptor activation on a rapid time scale [18]. When receptors were rapidly mixed with agonist plus ethidium, the earliest fluorescent component was found to report the fraction of channels that opened and their apparent rate of fast desensitization. Cholesterol was shown to modulate the channel-opening probability from zero in the absence of cholesterol, to native values in the presence of > 20 mol% cholesterol, whereas the rate of fast desensitization was unperturbed. Here, we use this methodology in nAcChoRs reconstituted with various cholesterol analogs to locate the site of cholesterol's action, and to test models of its mechanism of action.

2. Materials and methods

2.1. Materials

Torpedo nobiliana were obtained live from Biofish Associates (Georgetown, MA). All phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL), and cholesterol from Calbiochem (La Jolla, CA). Cholesteryl hemisuccinate and cholesterol 3-sulfate were purchased from Sigma (St. Louis, MO). Cholesteryl phosphorylcholine was purchased from Fluka (Ronkokoma, New York). Affigel 102, acrylamide, bis-acrylamide, and Coomassie brilliant blue were purchased from Biorad (Richmond, CA). Spectra/Por tubing for dialysis was obtained from Spectrum Medical Industries (Houston, TX). [^{3}H]Acetic anhydride was obtained from New England Nuclear (Billerica, MA) and dithiothreitol (DTT) and bromoacetyl bromide were purchased from Fisher Biotech (Pittsburgh, PA). Choline bromide, ethylenediamine-tetraacetic acid (EDTA), ethylene glycol-bis(*b*-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid

(EGTA), phenylmethylsulfonyl fluoride (PMSF), and 1-(5-dimethylamino naphthalene-1-sulfonamido)ethane-2-trimethylammonium, were purchased from Sigma (St. Louis, MO) and all other chemicals were obtained from Aldrich (Milwaukee, WI) unless otherwise stated.

Bromoacetylcholine bromide was synthesized from choline bromide and bromoacetyl bromide [19]. [^{3}H]Acetylcholine iodide was synthesized from [^{3}H]acetic anhydride and β-dimethylaminoethanol [20]. Dns-C_6-Cho was synthesized as previously described [21]. (1-oleoyl-2-(cholesteryl hemisuccinyl) phosphatidylcholine was synthesized as previously described [11].The compound was purified by preparative TLC usingchloroform/methanol/concentrated ammonia/water (5:3:0.3:0.15 v/v) as the solvent. The purified compound ran as a single spot on TLC with an R_f of approximately 0.25. Mass spectrometry of the purified compound showed the correct molecular weight of 990 g/mol.

2.2. Preparation of nAcChoR-rich membranes from Torpedo nobiliana

The nAcChoR-rich membranes were prepared from *T. nobiliana* following published procedures [22,23]. The electroplaques were excised from a freshly euthanized *T. nobiliana* that was packed in ice for 1 h. Each electric organ was cut into small pieces and approximately 400 g of tissue was puréed in a Cuisinart with an equal amount of homogenization buffer containing 0.1 mM PMSF and 10 mM in iodoacetamide. The PMSF and iodoacetamide were added to the buffer just before use. The puréed tissue was then homogenized by pulsing for 2×60 s in a Waring blender. The homogenate was spun in a Sorvall RC 5B/GSA centrifuge for 10 min at 5250 rpm, 4°C to remove connective tissue. The supernatant was filtered through gauze before being spun for 2 h at 9750 rpm, 4°C. This supernatant was discarded and the pellets containing nAcChoR-rich membranes were combined and resuspended in an equal amount of buffer A (100 mM NaCl, 10 mM MOPS, 0.1 mM EDTA and 0.02% sodium azide, pH 7.4). The resuspended pellets were rapidly frozen in liquid nitrogen and then stored at −80°C.

2.3. Purification and reconstitution of the nAcChoR using affinity chromatography

Briefly, receptor-rich membranes isolated as described above were suspended in buffer A to a final concentration of 2 mg/ml, treated with the acetylcholine esterase inhibitor DFP (0.05 ml in 0.95 ml ethanol) and stirred on ice for 30 min. The suspension was then pelleted by centrifugation for 1 h at 4°C at 41,000 rpm in a Dupont A-641 rotor. The diisopropyl fluorophosphate-treated crude membranes were homogenized in buffer A, made to a protein concentration of 20 mg/ml, rapidly frozen in liquid nitrogen and stored at −80°C until needed. The nAcChoR was purified and reconstituted into lipid vesicles using a modification of a previously described procedure [24]. Affi-gel 102 (in place of Affi-gel 401) was derivatized with DL-*N*-acetyl homocysteine thiolactone and then modified with bromoacetylcholine bromide. The affinity column was pre-equilibrated with 100 ml of buffer A containing 1 mg/ml DOPC and 1% cholate. The crude membranes were solubilized in buffer A containing 2% sodium cholate at a final protein concentration of 2 mg/ml and stirred at 4°C for 1 h. The solution was then centrifuged for 1 h at 41,000 rpm at 4°C in a Dupont A-641 rotor, and the supernatant applied to the affinity column at a flow rate of 2 ml/min. The column was washed with 150 ml of buffer A containing 1 mg/ml DOPC and 1% cholate, then 100 ml of buffer A containing 2.5 mg/ml DOPC and 1% cholate and allowed to equilibrate overnight to ensure complete exchange of lipids. The next day, the column was washed again with 100 ml of buffer A containing 2.5 mg/ml DOPC and 1% cholate, and then 100 ml of buffer A containing 0.3 mg/ml DOPC and 1% cholate. The nAcChoR was eluted in 100 ml of buffer A containing 1% cholate, 0.3 mg/ml DOPC and 15 mM carbachol. Fractions absorbing at 280 nm were pooled and dialyzed for 48–60 h at 4°C against 2 l of buffer A under a constant nitrogen purge with a total of 6 buffer changes. After dialysis, the reconstituted membranes were harvested by centrifugation and rapidly frozen in liquid nitrogen and stored at −80°C.

Re-reconstitution was performed by solubilizing 3 mg (1 ml) of purified AcChoR (previously reconstituted in DOPC membranes via detergent dialysis) in

1 ml of buffer A containing 2% cholate (1% final concentration) and 2 mg of desired lipid, with the lipid usually in the mole ratio of 58:12:30 DOPC/DOPA/steroid. The solubilized mixture was then dialyzed as above.

2.4. Characterization of reconstituted nAcChoR

Protein concentration [25], phospholipid content [26], and cholesterol content [27] were determined as previously described. All bilayer compositions are given in mol%. SDS-PAGE [28] was performed under denaturing conditions to verify the identity and purity of the receptor. Two methods were used to determine the concentration of agonist binding sites. First, by competition of the fluorescent cholinergic ligand, (dansylaminoethyl)trimethylammonium perchlorate, by acetylcholine [29] and second by a [^{3}H]AcCho binding assay [22]. Receptor concentration refers to protein unless agonist sites are specified which would be two agonist sites per oligomer.

2.5. Determination of cholesterol and cholesterol derivatives in re-reconstituted AcChoR samples

The semi-quantitative determination of cholesterol and cholesterol analogs in reconstituted and re-reconstituted membrane preparations was achieved by extraction and TLC identification of the extracted lipids on silica gel G plates (Analtech, Newark, DE, USA) in the following solvent system: isopropanol:chloroform:methanol:ammonia, 40:40:20:8 (v/v) [30]. Briefly, 0.2 ml samples of reconstituted or re-reconstituted membranes were extracted with 0.2 ml of the TLC solvent for 30 min under constant mixing at room temperature and then centrifuged for 2 min in an Eppendorf centrifuge. The liquid phase was transferred to another tube, evaporated under a stream of nitrogen, and dissolved in 0.1 ml of the TLC solvent. Samples of 0.03 to 0.05 ml were spotted on the TLC plate and the compounds visualized by charring the plates at 120°C after spraying with 5% potassium dichromate in 40% sulfuric acid. The R_f values found for cholesterol, cholesteryl hemisuccinate and cholesterol 3-sulfate in isopropanol:chloroform:methanol:ammonia, 40:40:20:8, v/v were 0.98, 0.57 and 0.66, respectively. Cholesteryl phosphorylcholine and (1-oleoyl-2-(cholesteryl hemisuccinyl)phosphatidylcholine had R_f values of 0.55 and 0.25, respectively, in chloroform/methanol/concentrated ammonia/water (5:3:0.3:0.15 v/v).

2.6. Stopped-flow fluorescence energy transfer assay

The stopped-flow fluorescence energy transfer experiments were performed with a BioSX-17 MV spectrofluorimeter with a 150-W xenon arc lamp and a SpectraKinectic monochromator fitted with a diffraction grating (Applied Photophysics, Leatherhead, England). All experiments were carried out at 20°C in *Torpedo* physiologic saline (TPS) buffer (250 mM NaCl, 5 mM KCl, 3 mM CaCl, 2 mM $MgCl_2$, 5 mM NaH_2PO_4, and 0.02% NaN_3 at pH 7.0). Equal volumes of ethidium (plus ligands where appropriate) and nAcChoR vesicle suspension were rapidly mixed to give a final receptor concentration of 0.2 μM, equivalent to 0.4 μM in acetylcholine binding sites and 0.8 μM ethidium. When a high agonist concentration was used, 10 mM carbamylcholine was always the final concentration. Tryptophan residues were excited at 290 nm (0.5 mm slit), and ethidium fluorescence was recorded with a 530-nm high bandpass cutoff filter. The electronic filter was set to 150 μs. Four to eight traces were acquired digitally for each set of conditions and then signal-averaged. Each trace (2000 data points) was acquired for 100 s with the instrument in a logarithmic time-base mode. Data collection was continued if the reaction did not reach equilibrium within this time. A nonspecific binding trace was always obtained for each experiment by mixing 0.8 μM ethidium, 10 mM carbamylcholine and 500 μM phencyclidine with receptor.

3. Results

3.1. Characterization of the nAcChoR

The lipid content of the affinity-purified AcChoR reconstituted into DOPC was modulated by resolubilizing AcChoR membranes with 1% cholate containing the desired lipid mixture and then achieving reconstitution by detergent dialysis. The procedure is referred to as re-reconstitution. All re-reconstituted samples were prepared with lipid mole ratios of

58:12:30 (DOPC/DOPA/steroid). We chose 30 mol% steroid because receptor activation is maximal above 20–25% cholesterol [18]. The molar proportion of DOPA was kept constant at 12% of the total lipid, because this reproduces the average charge density due to phospholipids in native membranes, assuming that the average negative charge is 1.5 per molecule of DOPA [31]. No attempt was made to compensate for surface charge when using charged steroids, but this did not seem to affect the results. This is consistent with our previous observation that the receptor's behavior was identical in DOPC/cholesterol and DOPC/DOPA/cholesterol bilayers [18]. The total lipid-to-protein ratios in all of the samples were approximately 300:1. The structures of the cholesterol analogs used in this study are shown in Fig. 1.

Aliquots of all samples containing cholesterol, cholesteryl hemisuccinate, cholesterol 3-sulfate, cholesteryl phosphorylcholine and 1-oleoyl-2-(cholesteryl hemisuccinyl)phosphatidylcholine were extracted from the re-reconstituted membranes as described in Section 2 and analyzed by thin layer chromatography. The only cholesterol analog detected by the cholesterol assay of Rudel and Morris [27] was cholesteryl hemisuccinate; the assay was thus useful for ruling out the presence of trace amounts of cholesterol where appropriate. Each of the samples showed the presence of the expected quantities of the respective compounds with no traces of cholesterol. It proved critical to assay for the presence of native cholesterol in the reconstituted DOPC membrane preparation because occasionally, native cholesterol (10–15 mol%) was found to carry over into the purified membranes. All experiments reported here used receptor reconstituted into DOPC membranes that were verified to have no residual cholesterol.

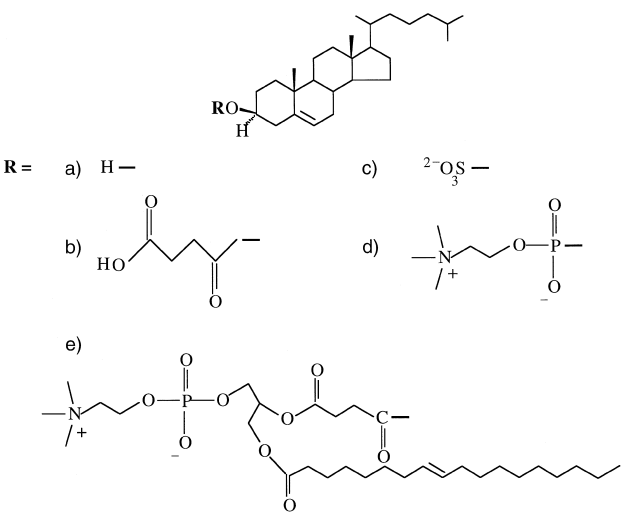

Fig. 1. The structures of cholesterol analogs used in this study. All substituents were attached to the 3-position hydroxyl on cholesterol where (a) is cholesterol, (b) cholesteryl hemisuccinate, (c) cholesterol sulfate, (d) cholesteryl phosphocholine and (e) (1-oleoyl-2-(cholesteryl hemisuccinyl)phosphatidylcholine or tethered-cholesterol.

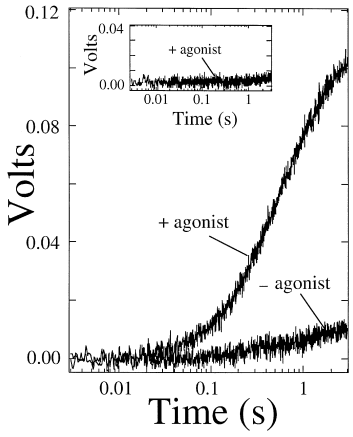

Fig. 2. The effect of carbachol on the time-resolved fluorescence of ethidium rapidly mixed with AcChoR reconstituted in DOPC/DOPA/cholesterol at a mole ratio of 58:12:30. The final concentration of ethidium, reconstituted AcChoR and carbachol when used was 0.8 μM, 0.2 μM and 10 mM, respectively. AcChoR re-reconstituted into membranes containing 30 mol% cholesterol rapidly mixed with ethidium alone (lower trace) or with 10 mM carbachol (upper trace). The inset is AcChoR re-reconstituted in DOPC/DOPA (88:12) rapidly mixed with ethidium and 10 mM carbachol. All traces were corrected for nonspecific binding.

3.2. Effects of cholesterol analogs on AcChoR activation

Fig. 2 shows the time dependence of ethidium fluorescence corrected for nonspecific binding following rapid mixing of re-reconstituted AcChoR membranes containing 30 mol% cholesterol with a solution of ethidium and carbamylcholine to give a final concentration of 0.8 μM ethidium and 0 or 10 mM carbamylcholine. The early component is appar-

ent in the first second at 10 but not 0 mM carbamylcholine and has a rate of 2.1 s^{-1} in this single experiment compared to an average value of 4.7 ± 1.2 s^{-1} reported previously in the presence of 10 mM carbamylcholine [18]. As expected, the early component was not observed in the presence of 10 mM carbamylcholine for AcChoR reconstituted into DOPC/DOPA (inset, Fig. 2), nor in the stock AcChoR reconstituted into DOPC from which all re-reconstituted AcChoR was prepared (data not shown). These latter samples serve as controls for the re-reconstitution procedures, providing the baseline against which the effects of added steroid can be assessed.

3.3. Negatively charged cholesterol analogs

Fig. 3a shows the time dependence of ethidium fluorescence corrected for nonspecific binding following rapid mixing of carbamylcholine plus ethidium with re-reconstituted AcChoR membranes containing 30 mol% cholesteryl hemisuccinate at the concentrations indicated. The corrected trace with no agonist present shows no early component, but that with 10 mM agonist has a clear early component with a rate of 2.3 ± 0.12 s^{-1}. Thus, 30 mol% cholesteryl hemisuccinate supports rapid agonist-induced conformational changes, a result that is consistent with the cation flux work [32]. When AcChoRs were re-reconstituted with 30 mol% cholesterol 3-sulfate, they underwent agonist-induced structural changes in a similar manner (Fig. 3b) and with a comparable rate of fast desensitization of 2.2 ± 0.1 s^{-1}.

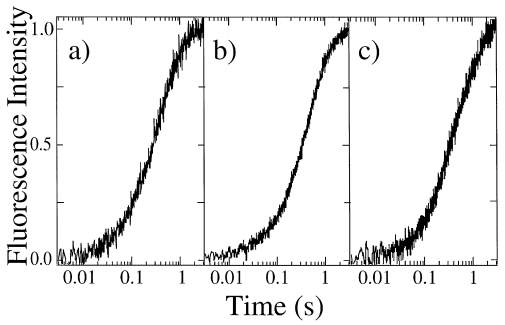

Fig. 3. The effect of cholesterol analogs on state transitions of the AcChoR induced by a saturating concentration of agonist (10 mM carbachol) detected using 0.8 μM ethidium. The final AcChoR concentration was 0.2 μM. All traces have been normalized to AcChoR re-reconstituted into membranes containing 30 mol% cholesteryl hemisuccinate, which had a fluorescence maximum of 120 mV at 3.0 s. Trace (a): AcChoR re-reconstituted into membranes containing 30 mol% cholesteryl hemisuccinate, (b): AcChoR re-reconstituted into membranes containing 30 mol% cholesterol 3-sulfate (fluorescence maximum of 131 mV at 3.0 s) and (c): AcChoR re-reconstituted into membranes containing 30 mol% cholesteryl phosphorylcholine (fluorescence maximum of 51 mV at 3.0 s). All traces were corrected for nonspecific binding.

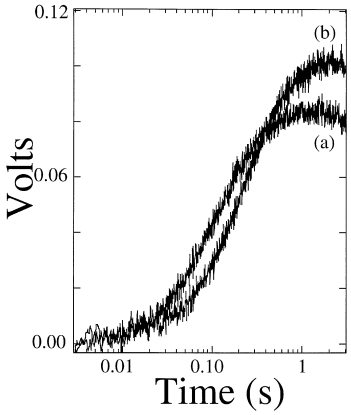

Fig. 4. The effect of the tethered-cholesterol, (1-oleoyl-2-(cholesteryl hemisuccinyl)phosphatidylcholine, on agonist-induced conformation changes detected using 0.8 μM ethidium. The final AcChoR concentration was 0.2 μM and carbachol concentration 10 mM. Trace (a): AcChoR re-reconstituted into membranes containing 30 mol% tethered-cholesterol and (b): AcChoR re-reconstituted into membranes containing 30 mol% tethered-cholesterol and 30 mol% cholesterol. All traces were corrected for nonspecific binding.

3.4. Zwitterionic cholesterol analogs

Fig. 3c shows that when 30 mol% of the zwitterionic steroid, cholesteryl phosphorylcholine, is included in the re-reconstituted AcChoR membranes, high agonist concentrations induce an early component of ethidium fluorescence that has an apparent rate of 2.4 ± 0.08 s^{-1}, confirming the suggestion from the section above that the nature of the substituent at the 3-position of cholesterol is of little importance for receptor activation.

A more dramatic confirmation of the latter conclusion is provided by the analog in which cholesterol is tethered to a phospholipid, (1-oleoyl-2-(cholesteryl hemisuccinyl)phosphatidylcholine. When this tethered-cholesterol was re-reconstituted into membranes

at 30 mol%, it also supported rapid agonist-induced conformation changes; the early component having an apparent rate of $3.4 \pm 0.2\ s^{-1}$ very similar to that of cholesterol and its analogs above (Fig. 4a). Furthermore, when an additional 30 mol% of cholesterol was incorporated into this membrane preparation (DOPC/DOPA/Chol/tethered-Chol 28:12:30:30), very similar fluorescence traces were obtained (Fig. 4b).

3.5. A spin-labeled cholesterol analog

The spin-labeled steroid, 3-DOXYL-17β-hydroxy-5α-androstane, could be incorporated by re-reconstitution at up to 12 mol% in DOPC/DOPA nAcChoR membranes. These membranes supported rapid agonist-induced conformation changes; the early component having an apparent rate of $2.4 \pm 1.4\ s^{-1}$. Addition of 30 mol% cholesterol roughly doubled the amplitude of the early phase without significantly affecting its rate.

4. Discussion

In this study, we aimed to distinguish between three regions of the membrane where cholesterol might have a role in the activation of the nAcChoR: the lipid bilayer, the lipid–protein interface, and the protein itself. In each case, we chose the most plausible mechanistic model consistent with the available data, and selected cholesterol analogs to distinguish between these models. We used a morphology-independent fluorescence assay based on the channel blocker ethidium bromide, which reports channel activation [18]. Use of such assays is in line with other studies of the role of cholesterol in reconstituted systems such as rhodopsin [12] and the (Ca^{2+}–Mg^{2+})-ATPase from sarcoplasmic reticulum [11].

4.1. Does cholesterol act in the bilayer?

First, we considered the lipid bilayer. The role of fluidity has already been discounted [9,32]. Any model needs to be able to account first for the fact that in the absence of cholesterol, activation does not occur, but that it occurs with just a few percent cholesterol [18], and second for the remarkable observation that many other neutral 'lipids', such as α-tocopherol, coenzyme Q_{10} and vitamins D_3 and K_1, can be substituted for cholesterol [32,33]. This suggested to us that cholesterol's unique ability to rapidly flip-flop in the membrane from one leaflet to the other might be the key to its action [18], because this ability would be shared by the other activation permissive 'lipids', but not by phospholipids. This hypothesis is only plausible if a mechanism coupling such a transbilayer motion to the receptor's function can be devised. If an agonist-induced conformation change results in the cross-sectional area of the receptor changing asymmetrically in the bilayer, causing a differential change in the surface area of each leaflet of the bilayer, a mechanical bending strain would be introduced, which would tend to oppose the conformation change. In the absence of cholesterol, the activation energy might be prohibitive, but the strain would be readily relieved by a net flux of cholesterol from the convex to the concave side of the bilayer as indicated in Fig. 5a. Such a model requires that cholesterol be able to flip-flop on the submillisecond time scale. It is well established that cholesterol flips in the bilayer within the dead time of conventional techniques [35,36], and more recent NMR measurements of the flip-flop rates of un-ionized bile salts allowed an estimate of cholesterol's flip-flop rate as $> 10^3\ s^{-1}$ [37], which is fast enough to be involved in channel activation [38].

An argument against this model is that cryoelectron microscopy [39] reveals no structural change in the lipid–protein interface upon activation. However, the diameter of the oligomeric receptor in the membrane is approximately 60 Å, with a resolution of $\sim$ 8.7 Å [39], giving a cross-sectional area of about 2800 Å. The partial molar area in the phospholipid bilayer may be as small as 18 $Å^2$ [40]. Each cholesterol that flips, therefore, causes a differential surface area change equivalent to approximately 1.25% of the oligomer's cross-sectional area.

To test this hypothesis, we used charged cholesterol analogs, which would not flip across the membrane on the time scale required for activation. First, as a control for the effects of surface charge, we used cholesteryl hemisuccinate, a fraction of which should be uncharged in the lipid–water interface, and therefore still able to support activation via the cholesterol flipping mechanism. When AcChoR was re-recon-

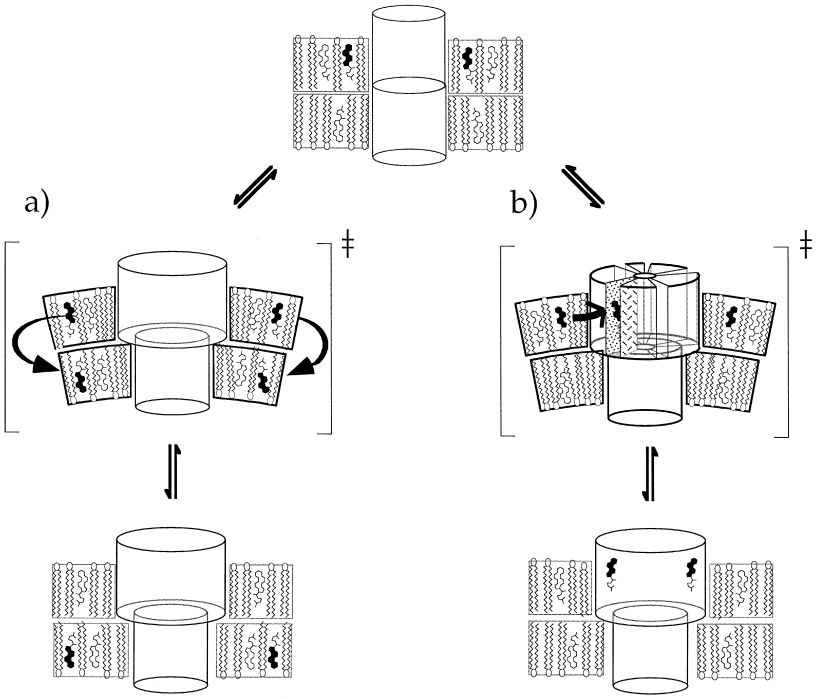

Fig. 5. Models in which conformation-induced bending strain in the lipid bilayer is relieved by cholesterol. This class of model assumes that activation of the channel involves a conformational change in which the cross-sectional area of the channel increases in a nonuniform fashion. This results in a net asymmetric expansion in the two leaflets of the membrane's bilayer, causing an increase in the mechanical strain of the bilayer. Relief of the mechanical strain, which can be achieved via two mechanisms, allows the conformation change to proceed and the channels to open. In both models, the receptor is initially in the resting state with the necessary amount of cholesterol in the bilayer. Upon activation, the induced bilayer stress is relieved in model (a) by cholesterol molecules rapidly flipping from the upper leaflet to the lower leaflet of the bilayer. In model (b), cholesterol binding sites on the receptor became exposed during the conformation change. The induced bilayer stress is then relieved by cholesterol diffusing laterally to bind to the exposed sites on the receptor, stabilizing the open state. The cholesterol binding sites would have to rapidly equilibrate with cholesterol, thus be readily accessible from the lipid–protein interface. For simplicity, only two bound cholesterol molecules are shown, but one within each subunit interface is more likely [34].

stituted into DOPC/DOPA/cholesteryl hemisuccinate at a mole ratio of 58:12:30, the preparation was found to support agonist-induced conformational changes (Fig. 2a), behaving exactly like re-reconstituted receptor containing cholesterol as the steroid. This result is consistent with the slow flux studies that showed cholesteryl hemisuccinate supported agonist-induced cation flux [32].

The second analog tested was cholesterol-3-sulfate, which bears a fixed negative charge of two and should be unable to flip-flop rapidly. Receptor re-reconstituted with 30 mol% cholesterol 3-sulfate was found to support receptor activation, exactly like cholesterol itself (Fig. 2b). Finally, to eliminate any unexpected effects of net negative charge, a zwitterionic cholesterol analog, cholesteryl–phosphorylcholine, was also found to support receptor activation (Fig. 2c). Thus, the ability of these two charged cholesterol analogs to support channel activation effectively rules out any model based on cholesterol flipping from one leaflet of the bilayer to the other.

4.2. Nonannular or interstitial sites

The second region of the membrane we considered was the receptor protein itself. The importance of nonannular, or interstitial, sites, sequestered within the protein, perhaps between subunits, has been emphasized by Ding et al. [11]. That such sites exist on the nAcChoR is inferred from studies with brominated cholesteryl hemisuccinate, which, upon titration into receptors reconstituted into brominated phospho-

lipid, causes the intrinsic fluorescence to be further quenched [10,34]. Supporting evidence is: that it is not possible to remove all the cholesterol from native membranes when it is repeatedly extracted into lipid vesicles [41], that receptors have a high affinity for cholesterol in lipid monolayers [42], and that photoactivatable cholesterol analogs incorporate in the protein, in some cases in an agonist-sensitive manner [14,43].

Our titration curve of the role of cholesterol in channel activation correlates with the above intrinsic fluorescence quenching curves within the rather large experimental errors [18]. To test the interstitial site model more rigorously, we synthesized a cholesterol-containing phospholipid [11], which resides at the lipid–protein interface (Fig. 1e). This tethered-cholesterol analog was then re-reconstituted into inactive DOPC/DOPA nAcChoR membranes with and without the addition of free cholesterol. The analog alone restored activity to the inactive preparation (Fig. 4), and, furthermore, the addition of free cholesterol did not further enhance activity, indicating that the lipid-linked cholesterol itself had restored full function. The succinamide arm on the tethered-cholesterol would allow some access of the steroid backbone to a binding site, but based on the diameter of the receptor in the membrane region (~ 60 Å) and the length of the spacer arm (~ 6 Å), the outer end of this site must be close to the lipid–protein interface. Further, the insensitivity to the size of the substituent on the 3-position suggests that it does not contribute to the binding site, and the tolerance for charge also suggests the head group remains hydrated at the lipid–protein–water interface. Therefore, occupation of deeply buried interstitial sites is in no way required for activation.

This finding for the nAcChoR contrasts with that for $(Ca^{2+}–Mg^{2+})$-ATPase, where this tethered-cholesterol analog was first used to establish the presence of interstitial sites in $(Ca^{2+}–Mg^{2+})$-ATPase [11]. In this protein, tethered-cholesterol did not support activity, which was only restored in the presence of free cholesterol.

4.3. Sites in the lipid–protein interface

Although the above evidence rules out interstitial sites, it is quite consistent with cholesterol sites located in the lipid–protein interface. Agonist binding induces its conformation change over tens of angstroms, probably causing an extensive conformation change in the receptor. Recent work points to the participation in gating of at least the M1, M2 and M4 helices and probably other regions [38,44]. Thus, it is plausible that there are functional cholesterol sites close to the lipid–protein interface. Indeed, androstanol shows a small statistical preference for the lipid–protein interface relative to phosphatidylcholine, where it occupies some ~ 38 sites [24]. Putative cholesterol sites on the nAcChoR have been explored in homology modeling studies based on the structure of membrane permeablizing toxins whose structures are known in their soluble form. In one model based on the structure of myohemerythrin, which has four sequentially connected antiparallel α-helices, the known structure was built into a pentamer around a central pore. The overlapping subunits left five grooves in the lipid–protein interface of the correct size to accommodate cholesterol [45]. A similar conclusion was reached in a more recent study, based on a suggestion of Unwin [46], that the tertiary structure of the nAcChoR might be similar to that of the B5 pentamer of the heat-labile enterotoxin of *E. coli* [47]. Both these models suggest that the putative cholesterol binding sites are in the lipid–protein interface and are exposed to the bilayer on one face, a conclusion that is quite consistent with the results obtained with our analogs. Thus, the hypothetical cholesterol sites on the nAcChoR must be more accessible to the bilayer than those in the $(Ca^{2+}–Mg^{2+})$-ATPase studied by Ding et al. [11].

Recently, it has been suggested that extramembraneous regions of the receptor are involved in the binding of the spin-labeled androstane because the motionally restricted component of the spectra is lost when the extramembraneous portion of the nAcChoR are digested [48]. If this hypothesis is true, our results would suggest that such cholesterol sites must be very close to the bilayer. However, such a vigorous digestion is likely to have perturbed subunit–subunit interactions and alternative interpretations are possible.

A precedent for low affinity but specific lipid modulation of a multisubunit protein is provided by D-β-hydroxybutyrate dehydrogenase (BDH) [49]. Upon reconstitution, activity was only restored if

phosphatidylcholine was present in the bilayer. Half maximal activation occurred at 18 mol% phosphatidylcholine with a Hill coefficient of 2.4. These figures may be compared to those for cholesterol's action on the nAcChoR of 10 mol% and 1.9. BDH is thought to have one phosphatidylcholine per subunit, and the similarity of the activation isotherms suggests that something similar might be the case for the nAcChoR.

4.4. Possible models of cholesterol's action

The kinetics of activation cannot be studied in detail because they are too rapid for stopped flow techniques; therefore, it is not possible to distinguish between a model where cholesterol must be bound to its sites before activation can occur, and a model where cholesterol plays a more dynamic role such as reducing the activation energy of channel opening. The exchange rate of androstanol between the bilayer and the lipid–protein interface is likely to be comparable to that of other spin-labeled lipids on the nAcChoR ($> 10^6$ s^{-1}) [50]. Thus, it should be able to exchange during gating, making a dynamic role plausible. An interesting new hypothesis invokes the mechanical properties of the membrane in much the same way as the flip-flop model did; only here, the bending strain might be relieved by cholesterol diffusing laterally into its binding sites that are only exposed during the conformation change (Fig. 5b). This model demands that the annular sites be asymmetrical with respect to the bilayer, and that they be in rapid exchange with the bilayer, concepts that are open to experimental test.

4.5. Conclusions

Our data are consistent with the hypothesis that cholesterol sites exist on the nAcChoR, and that they must be involved in agonist-induced activation. The new experimental conclusion is that these sites must be in contact with the lipid bilayer, a conclusion that is in accord with modeling studies noted above. In this respect, the acetylcholine receptor differs from the (Ca^{2+}–Mg^{2+})-ATPase [11]. In the latter case, the tethered cholesterol analog failed to act like cholesterol, supporting the notion of interstitial sites well separated from the bilayer. The terminology now needs to be modified to allow a classification of nonannular sites into interstitial and periannular, where the latter are binding sites in crevices on the surface of the protein that are open to the lipid bilayer. The term annular sites still applies to the remaining area on the lipid–protein interface, where lipid motion is restricted on the EPR time scale relative to that in the bilayer, and where only moderate lipid selectivity may be observed.

The data all tend to point to the existence of cholesterol sites, and the challenge is now to design experiments capable of providing definitive proof of their existence. In the meantime, it remains possible that some unidentified property of the lipid–protein interface is important. However, it is difficult to see how any such model could account for the complete lack of activation in the absence of cholesterol.

Acknowledgements

We thank Profs. S. Svetina and B. Zeks for stimulating discussions on possible mechanisms for cholesterol's actions. This research was supported in part by grants from the National Institute on Alcohol Abuse and Alcoholism (AA-07040) and from the National Institute of General Medical Sciences (GM15904) to K.W.M. and from the Bundesministerium fuer Forschung und Technologie, Bonn, from Fondation Limagrain, Chappes, France and from the Fonds der Chemischen Industrie, Frankfurt, to H.S.

References

[1] R.B. Gennis, Biomembranes: molecular structure and function, in: C.R. Cantor (Ed.), Springer Advanced Texts in Chemistry, Springer, New York, 1989, 533.
[2] S. Bodovitz, W.L. Klein, J. Biol. Chem. 271 (1996) 4436–4440.
[3] D.E. Raines, S.E. Rankin, K.W. Miller, Anesthesiology 81 (1994) A884.
[4] H.R. Arias, M.B. Sankaram, D. Marsh, F.J. Barrantes, Biochim. Biophys. Acta 1027 (1990) 287–294.
[5] P.J. Bennett, M.A. Simmonds, Br. J. Pharmacol. 117 (1996) 87–92.
[6] R.S. Cantor, Biochemistry 36 (1997) 2339–2344.
[7] T.M. Fong, M.G. McNamee, Biochemistry 25 (1986) 830–840.

[8] C. Sunshine, M.G. McNamee, Biochim. Biophys. Acta 1191 (1994) 59–64.
[9] G. Fernandez-Ballester, J. Castresana, A.M. Fernandez, J.L. Arrondo, J.A. Ferragut, J.M. Gonzalez-Ros, Biochemistry 33 (1994) 4065–4071.
[10] O.T. Jones, M.G. McNamee, Biochemistry 27 (1988) 2364–2374.
[11] J. Ding, A.P. Starling, J.M. East, A.G. Lee, Biochemistry 33 (1994) 4974–4979.
[12] D.C. Mitchell, M. Straume, J.L. Miller, B.J. Litman, Biochemistry 29 (1990) 9143–9149.
[13] A.D. Albert, J.E. Yung, P.L. Yeagle, Biochim. Biophys. Acta 1285 (1996) 47–55.
[14] A.M. Fernandez, B.G. Fernandez, J.A. Ferragut, R.J.M. Gonzalez, Biochim. Biophys. Acta 1149 (1993) 135–144.
[15] R. Anholt, D.R. Fredkin, T. Deerinck, M. Ellisman, M. Montal, J. Lindstrom, J. Biol. Chem. 257 (1982) 7122–7134.
[16] J.B. Udgaonkar, G.P. Hess, J. Membr. Biol. 93 (1986) 93–109.
[17] L. Pradier, M.G. McNamee, The nicotinic acetylcholine receptor, in: P. Yeagle (Ed.), The Structure of Biological Membranes, CRC Press, Boca Raton, FL, 1992, pp. 1047–1106.
[18] S.E. Rankin, G.H. Addona, M.A. Kloczewiak, B. Bugge, K.W. Miller, Biophys. J. 73 (1997) 2446–2455.
[19] V.N. Damle, M. McLaughlin, A. Karlin, Biochem. Biophys. Res. Commun. 84 (1978) 845–851.
[20] A. Anjaneyulu, J. Label. Compd. Radiopharm. 22 (1985) 745–750.
[21] G. Waksman, Z.M.C. Fournie, B. Roques, FEBS Lett. 67 (1976) 335–342.
[22] L.M. Braswell, K.W. Miller, J.F. Sauter, Br. J. Pharmacol. 83 (1984) 305–311.
[23] M.P. McCarthy, M.A. Moore, J. Biol. Chem. 267 (1992) 7655–7663.
[24] J.F. Ellena, M.A. Blazing, M.G. McNamee, Biochemistry 22 (1983) 5523–5535.
[25] M.A.K. Markwell, S.M. Haas, L.L. Beiber, N.E. Tolbert, Anal. Biochem. 87 (1978) 206–210.
[26] C.W.F. McClare, Anal. Biochem. 39 (1971) 527–530.
[27] L.L. Rudel, M.D. Morris, J. Lipid Res. 14 (1973) 364–366.
[28] U.K. Laemmli, Nature (London) 227 (1970) 680–685.
[29] R.R. Neubig, J.B. Cohen, Biochemistry 18 (1979) 5464–5475.
[30] R. Neher, TLC of steroids and related compounds, in: E. Stahl (Ed.), Thin Layer Chromatography, Springer, New York, 1969, pp. 311–362.
[31] T. Pott, J.C. Maillet, E.J. Dufourc, Biophys. J. 69 (1995) 1897–1908.
[32] C. Sunshine, M.G. McNamee, Biochim. Biophys. Acta 1108 (1992) 240–246.
[33] P.L. Kilian, C.R. Dunlap, P. Mueller, M.A. Schell, R.L. Huganir, E. Racker, Biochem. Biophys. Res. Commun. 93 (1980) 409–414.
[34] V. Narayanaswami, M.G. McNamee, Biochemistry 32 (1993) 12420–12427.
[35] J.M. Backer, E.A. Dawidowicz, Biochim. Biophys. Acta 551 (1979) 260–270.
[36] Y. Lange, J. Dolde, T.L. Steck, J. Biol. Chem. 256 (1981) 5321–5323.
[37] D.J. Cabral, D.M. Small, H.S. Lilly, J.A. Hamilton, Biochemistry 26 (1987) 1801–1804.
[38] C.J. Lingle, D. Maconochie, J.H. Steinbach, J. Membr. Biol. 126 (1992) 195–217.
[39] N. Unwin, Nature 373 (1995) 37–43.
[40] S. Carnie, J.N. Israelachvili, B.A. Pailthorpe, Biochim. Biophys. Acta 554 (1979) 340–357.
[41] W.S. Leibel, L.L. Firestone, D.C. Legler, L.M. Braswell, K.W. Miller, Biochim. Biophys. Acta 897 (1987) 249–260.
[42] J.L. Popot, R.A. Demel, A. Sobel, D.L.L. Van, J.P. Changeux, Eur. J. Biochem. 85 (1978) 27–42.
[43] D.S. Middlemas, M.A. Raftery, Biochemistry 26 (1987) 1219–1223.
[44] A. Karlin, M.H. Akabas, Neuron 15 (1995) 1231–1244.
[45] M.O. Ortells, V.B. Cockcroft, G.G. Lunt, D. Marsh, F.J. Barrantes, The nicotinic acetylcholine receptor and its lipid microenvironment, in: A. Pullman (Ed.), Membrane Proteins: Structures, Interactions and Models, Kluwer Academic Publishers, The Netherlands, 1992, pp. 185–198.
[46] N. Unwin, J. Mol. Biol. 229 (1993) 1101–1124.
[47] M. Ortells, G.G. Lunt, Protein Eng. 9 (1996) 51–59.
[48] M. Dreger, M. Krauss, A. Herrmann, F. Hucho, Biochemistry 36 (1997) 839–847.
[49] H. Sandermann, T.M. Duncan, J.O. McIntyre, S. Fleischer, Cooperative regulation of membrane enzymes by lipids, in: A. Watts (Ed.), Protein–Lipid Interactions, Elsevier, Oxford, 1993, pp. 67–82.
[50] D.M. Fraser, S.R. Louro, L.I. Horváth, K.W. Miller, A. Watts, Biochemistry 29 (1990) 2664–2669.