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Biochimica et Biophysica Acta 1609 (2003) 177–182
BIOCHIMICA ET BIOPHYSICA ACTA
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Low chemical specificity of the nicotinic acetylcholine receptor sterol activation site

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Received 6 September 2002; received in revised form 22 November 2002; accepted 25 November 2002

Abstract

The nicotinic acetylcholine receptor (nAcChoR) has an absolute requirement for cholesterol if agonist-stimulated channel opening is to occur [Biochemistry 25 (1986) 830]. Certain non-polar analogs could replace cholesterol in vectorial vesicle permeability assays. Using a stopped-flow fluorescence assay to avoid the limitations of permeability assays imposed by vesicle morphology, it was shown that polar conjugates of cholesterol could also satisfy the sterol requirement [Biochim. Biophys. Acta 1370 (1998) 299]. Here this assay is used to explore the chemical specificity of sterols. Affinity-purified nAcChoRs from *Torpedo* were reconstituted into bilayers at mole ratios of 58:12:30 [1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC)/1,2-dioleoyl-*sn*-glycero-3-phosphate (DOPA)/steroid]. When the enantiomer of cholesterol was used, or when the stereochemistry at the 3-hydroxy group was changed from β to α by substituting epicholesterol for cholesterol, activation was still supported. The importance of cholesterol's planar ring structure was tested by comparing planar cholestanol (5α-cholestan-3β-ol) with nonplanar coprostanol (5β-cholestan-3β-ol). Both supported activation. Thus, these steroids support activation independent of structural features known to be important for modulation of lipid bilayer properties. This provides indirect support for a steroid binding site possessing very lax structural requirements.

Keywords: Acetylcholine receptor; Reconstitution; Gating; Cholesterol; Steroid specificity

1. Introduction

The nicotinic acetylcholine receptor (nAcChoR) is a ligand-gated postsynaptic ion channel whose function is modulated by lipids. Besides a requirement for a fluid phospholipid bilayer phase, agonist-induced ion flux is well known to have an obligatory, although low-specificity requirement for cholesterol [1–8]. In ion-flux assays, the response depends on the morphology of the vesicles and membrane leakiness, but a morphology-independent non-vectorial fluorescence assay has confirmed the cholesterol requirement [9]. Upon rapid mixing of receptors with agonist and the reporter molecule ethidium bromide, the earliest fluorescent component reported 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 [9]. The rate of fast desensitization remained unperturbed. Upon establishing the threshold of required cholesterol, we further probed the role of cholesterol in channel activation by restricting the location of cholesterol in the bilayer by covalently attaching polar moieties to its 3-hydroxy group [10]. These conjugates were fully active and the functionally important cholesterol sites appeared to be located close to the lipid–protein interface.

Abbreviations: nAcChoR, nicotinic acetylcholine receptor; DOPA, 1,2-dioleoyl-*sn*-glycero-3-phosphate; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; Tethered-cholesterol, oleoyl-2-(cholesteryl hemisuccinyl)phosphatidylcholine; *nat*-cholesterol, natural cholesterol; *ent*-cholesterol, enantiomer of *nat*-cholesterol; TLC, thin-layer chromatography

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doi:10.1016/S0005-2736(02)00685-5

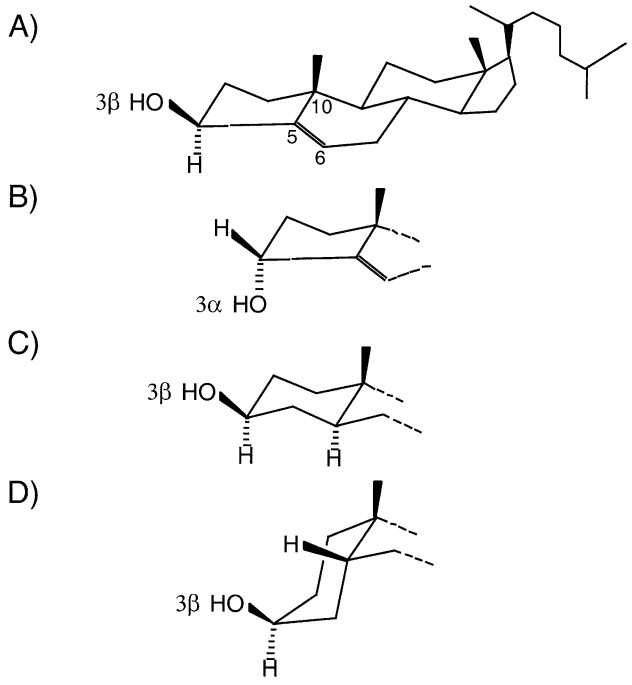

Fig. 1. The structures of some cholesterol analogs used in this study. (A) Cholesterol; (B) epicholesterol; (C) cholestanol; and (D) coprostanol.

Here we expand the above studies to probe the structural features of cholesterol that are required for channel activation. Specifically, we test the hypothesis that cholesterol's mechanism for support of activation is related to its well-known abilities to modulate lipid fluidity [11–13]. We used nAcChoRs from *Torpedo* that were affinity purified and reconstituted into bilayers containing different cholesterol analogs. We modulated the stereochemistry and the ring structure. The structures of the main cholesterol analogs used are shown in Fig. 1.

2. Materials and methods

2.1. Materials

Torpedo nobiliana were obtained live from Biofish Associates (Georgetown, MA). Phospholipids and other materials were obtained commercially as previously described [9,10]. Steroids were obtained from Steraloids, Inc., Newport, RI, except for the enantiomer of natural cholesterol (*ent*-cholesterol) which was a kind gift from Dr. Scott R. Rychnovsky, UC, Irvine.

2.2. Purification and reconstitution of the nAcChoR using affinity chromatography

Crude nAcChoR-rich membranes were prepared from *T. nobiliana* and reconstituted into 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) lipid vesicles via detergent dialysis as described [9]. They were kept at −80 °C until needed. Re-reconstitution (lipid exchange) was performed by solubilizing 3 mg (1 ml) of these nAcChoR/DOPC membranes in 1 ml of buffer containing 2% cholate (1% final concentration) and 2 mg of the desired lipid mixture, with the lipid usually in the mole ratio of 58:12:30 DOPC/1,2-dioleoyl-*sn*-glycero-3-phosphate (DOPA)/steroid. The solubilized mixture was then dialyzed. The amount of steroid in the lipids of the affinity-purified nAcChoR membranes was generally set at 30 mol% because receptor activation is maximal above 20–25% cholesterol [9].

2.3. Characterization of reconstituted nAcChoR

Protein, phospholipid and cholesterol were determined as previously described [9,10]. All bilayer compositions are given in mole percentage. SDS-PAGE was performed to verify the identity and purity of the receptor, and two methods were used to determine the concentration of agonist binding sites [9]. First, by competition of the fluorescent cholinergic ligand, (dansylaminoethyl) trimethylammonium perchlorate, by acetylcholine and second by [^{3}H]AcCho binding. Receptor concentration refers to protein unless agonist sites are specified which would be two agonist sites per oligomer.

2.4. Determination of cholesterol and cholesterol derivatives

Semiquantitative determination of cholesterol and cholesterol analogs in reconstituted and re-reconstituted membrane preparations was achieved by extraction and thin-layer chromatography (TLC) identification [10].

2.5. 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 SpectraKinetic monochromator fitted with a diffraction grating (Applied Photophysics, Leatherhead, England). All experiments were carried out at 20 °C in *Torpedo* physiologic saline buffer (250 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$ 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. Intrinsic tryptophan residues were excited at 290 nm (0.5 mm slit) causing resonance energy transfer to the extrinsic probe ethidium bromide. Fluorescence was recorded with a 530-nm-high band-pass cutoff filter. The electronic filter was set to 150 μs. Four to eight traces were acquired digitally for each set of conditions and then

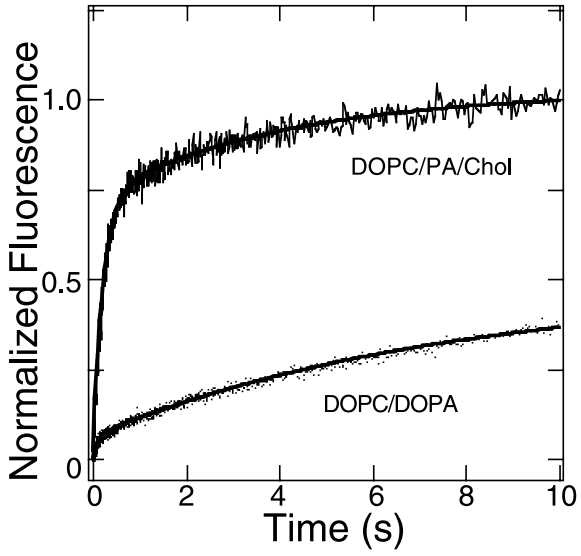

Fig. 2. The effect of agonist (carbachol) on the time-resolved fluorescence of ethidium rapidly mixed with nAcChoR reconstituted into membranes with various lipid compositions. The final concentrations of ethidium, reconstituted nAcChoR and carbachol were 0.8 μM, 0.2 μM and 10 mM, respectively, in each trace. The lower traces are nAcChoR reconstituted into DOPC/DOPA at a mole ratio of 88:12. The upper traces are nAcChoR re-reconstituted into membranes containing in addition 30 mol% cholesterol at a mole ratio of 58:12:30 DOPC/DOPA/cholesterol.

signal averaged. Each trace (2000 data points) was acquired for 100 s with the instrument in a logarithmic timebase mode, and traces were fitted to two exponentials corresponding to the early and fast components of reference [9]. This method was developed based on pioneering work by a number of groups as detailed in our earlier manuscripts [9,10].

3. Results

3.1. Characterization of the nAcChoR

Total lipid-to-protein mole ratios in all samples were ~ 300:1. Aliquots of all samples containing cholesterol, epicholesterol, cholestanol and coprostanol were extracted from the re-reconstituted membranes and analyzed by TLC with every sample showing expected quantities of steroid (30%). Most importantly, no traces of residual cholesterol from native membrane preparations were observed in the stock nAcChoR reconstituted into DOPC.

3.2. Effects of cholesterol stereoisomers on nAcChoR activation

Fig. 2 shows the time dependence of ethidium fluorescence after rapid mixing of re-reconstituted nAcChoR membranes containing 30 mol% cholesterol and a solution of ethidium and carbamylcholine yielding a final concentration of 0.8 μM ethidium and 10 mM carbamylcholine. An early component, reporting the fraction of channels that opened and their apparent rate of fast desensitization (see Introduction), is observed within the first second in the presence but not the absence (not shown here) of 10 mM carbamylcholine [9,10]. The early component had a rate of $4.7 \pm 0.54\ s^{-1}$ that is consistent with steroids that support activation induced by 10 mM carbamylcholine. This and all early component rates below fell within the range previously reported for cholesterol [9]. The early component was not observed in the presence of 10 mM carbamylcholine for nAcChoR reconstituted into DOPC/DOPA (lower trace Fig. 2).

When nAcChoR membranes were reconstituted with 30 mol% *ent*-cholesterol, in which every chiral center in choles-

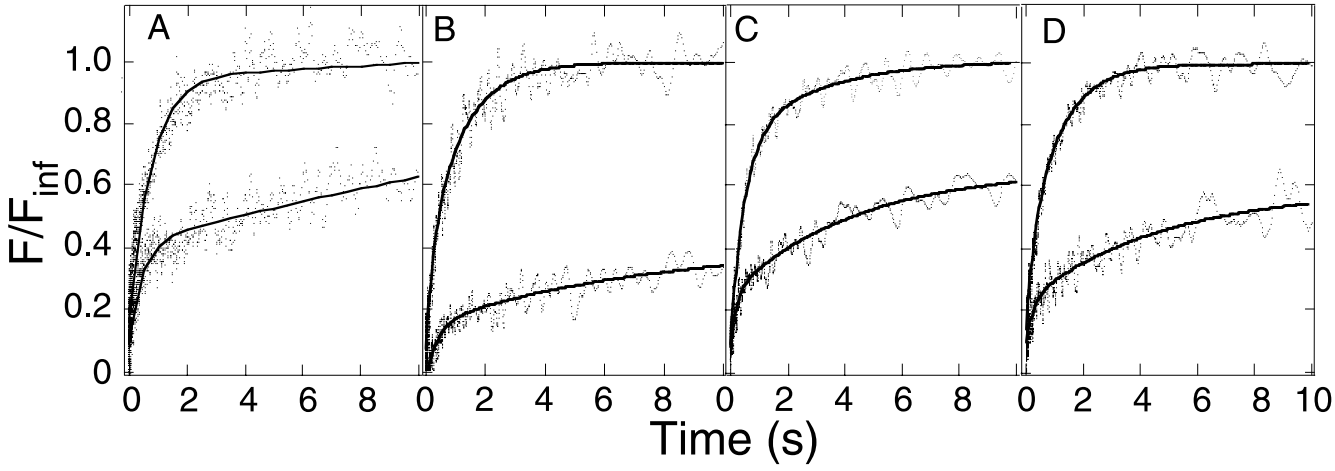

Fig. 3. The effect of cholesterol analogs on state transitions of the nAcChoR (0.2 μM) induced by 10 mM carbachol and detected using 0.8 μM ethidium. In each panel, the lower trace has no carbachol and the upper trace has 10 mM carbachol. An early phase was observed for each steroid, but only in the presence of carbachol (see Results). The mole ratio of steroid was always 30%, to give a composition of 58:12:30 DOPC/DOPA/sterol. (A) *Ent*-cholesterol; (B) epicholesterol; (C) coprostanol; and (D) cholestanol.

terol is reversed [14], the agonist-dependent early component was observed with a rate of $1.3 \pm 0.043\ s^{-1}$ (Fig. 3A).

To examine the role of the 3-hydroxyl group, nAcChoR membranes were reconstituted with 30 mol% epicholesterol, the 3α-enantiomer of cholesterol. Fig. 3B shows the time dependence of ethidium fluorescence following rapid mixing of carbamylcholine plus ethidium with the epicholesterol-containing receptor membranes. With no agonist present, no early component was observed but at 10 mM carbachol, there was an early component with a rate of $3.9 \pm 0.49\ s^{-1}$. Thus, 30 mol% epicholesterol supports rapid agonist-induced conformational changes.

3.3. Modulation of the ring structure

When the 5–6 double bond is reduced, the effect on the steroid ring geometry depends on whether the hydrogen on carbon 5 is *cis* (coprostanol) or *trans* (cholestanol) to the methyl on carbon 10 (Fig. 1). When the nAcChoR was reconstituted with 30 mol% of either of these two steroids, an agonist-induced early component was observed. With coprostanol and cholestanol, the early components had rates of 2.04 ± 0.067 and $3.8 \pm 0.42\ s^{-1}$, respectively (Fig. 3C and D).

4. Discussion

Previously we have characterized the early phase of agonist-induced action on ethidium fluorescence to be associated with gating [9,10]. Such gating depended on the presence of cholesterol, and cholesterol tethered to lysophosphatidylcholine via a 2-succinyl spacer [oleoyl-2-(cholesteryl hemisuccinyl)phosphatidylcholine (tethered-cholesterol) [15]] also supported gating. This suggested that if any cholesterol sites were involved, they would be close to the lipid–protein interface (periannular sites [10]) rather than deep within the protein (interstitial sites [16]). Although some studies rule out a role for lipid fluidity [4,15], others suggest the importance of sterols for stabilizing resting states of the nAcChoR [8]. Indeed, previous studies do not rule out a role in gating for annular lipid (that lipid which is nearest neighbor to the nAcChoR). Our present study compares the ability of a structurally diverse set of steroids to support nAcChoR activation (Fig. 3) with their known effects on lipid bilayer structure and function [17–20]). Our results are best appreciated by considering two important structural features of the steroids employed and how they influence lipid bilayers (Fig. 1).

First, only when the hydroxy group at the 3-position on ring A is in the equatorial β position do steroids order bilayers, increase monolayer packing and decrease bilayer permeability [17,18,21]. Switching the hydroxy group from the β to the α position (cholesterol to epicholesterol) ablates these actions, yet it did not alter the ability to support gating of the nAcChoR (Fig. 2 vs. Fig. 3B).

Secondly, a planar steroid ring system is critical for the above mentioned sterol actions on lipid bilayers [18,19]. Cholesterol has a tetracyclic ring structure, consisting of three six-membered rings, classically labeled from the left (Fig. 1) as A, B and C, and one five-membered ring labeled D. The B–C and C–D ring fusion occurs at single bonds whose substituents are *trans*, allowing the ring system to adopt a planar conformation. These structural features are thought to have been optimized during biochemical evolution [11]. In cholesterol, the presence of the unsaturated double bond at the AB ring junction prevents the A- and B-rings from adopting full chair conformations.

Cholestanol and coprostanol are reduced derivatives of cholesterol with the 5–6 double bond saturated. In cholestanol, the 5-hydrogen is *trans* to the 10-methyl group, allowing the A and B cyclohexyl rings to adopt coplanar chair conformations. In coprostanol, on the other hand, the 5-hydrogen is *cis* to the 10-methyl, twisting the A ring system out of the plan of the steroid backbone (Fig. 1) and removing its ability to exert cholesterol-like actions on lipid properties. Reduction of the 5–6 double bond without disruption of the planar ring system (cholesterol to cholestanol) caused no change in the ability to support gating. In spite of the dramatic difference in ring structure between cholestanol and coprostanol, the ability of carbachol to cause gating remained intact (Fig. 3D vs. C).

Thus, two important determinants of the sterol structure that are well known to optimize phospholipid–sterol interactions in lipid bilayers do not influence the ability of the nAcChoR to gate. Although, annular lipid packing in the lipid–protein interface might be significantly different from that in bilayers, the gross structural difference between cholestanol and coprostanol makes this unlikely. Thus, lipid bilayer physical properties may not be the key factor in permitting agonist-induced nAcChoR activation, although they may be in slow conformational changes such as desensitization [7,8]. Our data confirm the nonspecificity observed previously in vectorial nAcChoR-assay systems [1,3,4], and they extend the lack of specificity previously observed with the present fluorescence assay with regard to 3-β esters. For example, cholesterol 3-phosphocholine, cholesterol 3-sulfate and tethered-cholesterol all supported agonist-induced activation [10].

Cholesterol has several chiral carbons and the mirror image of natural cholesterol (*nat*-cholesterol), *ent*-cholesterol, has been synthesized [14,22]. The physical properties of *ent*-cholesterol and *nat*-cholesterol are therefore identical unless they are interacting with a chiral target to form diastereomeric complexes. *Ent*-cholesterol does not interact diastereomerically with phospholipids including DOPC [23]. However, it does not support life in the nematode *Caenorhabditis elegans*, which requires exogenous sterols for viability [22], and in cholesterol–amphotericin B complexes, *ent*-cholesterol interacts with lower affinity but forms channels of higher conductance than *nat*-cholesterol, providing a clear example of diastereomeric interactions

distinguishing between nonspecific and specific models of action [24]. We found that *ent*-cholesterol supported agonist-induced gating in the nAcChoR, providing no evidence in favor of enantiospecific interactions with the nAcChoR and confirming that the stereochemistry of the 3-β position is unimportant.

Even compared to other sterol-requiring receptors, the lax structural specificity of the nAcChoR is unique. Thus, in a recent review of the role of cholesterol as an obligatory activator of receptor proteins, two G-protein-coupled receptors with seven putative membrane-spanning domains were considered, namely, the oxytocin and the cholecystokinin receptors [25]. Cholesterol analogs were added as cyclodextrin complexes to cholesterol-depleted receptor membranes. Both receptors could be reactivated with cholesterol, coprostanol and certain other modified sterols, but the oxytocin receptor was much more specific in its sterol activator requirement. Thus, the cholecystokinin receptor, but not the oxytocin receptor, could be reactivated with epicholesterol or 3-keto-cholesterol. It was concluded that activation of the cholecystokinin but not the oxytocin receptor was related to lipid fluidity [25].

Previous work has suggested that there might be cholesterol binding sites on the nAcChoR [10,16]. Although the structure of the nAcChoR's transmembrane domains is as yet unknown [26], certain inferences can be drawn about these sites. They are thought to be located near the lipid–protein interface, possibly in the receptor's interior (interstitial sites [16]) but with the polar end of the site within 6 Å of the lipid bilayer (periannular sites [10]). Furthermore, large substituents are tolerated on the 3 position of the steroid ring [10], and neither the 3-β hydroxyl nor the planarity of the steroid ring system now seems to be required.

In conclusion, our results are in agreement with previous work that the effect of cholesterol on the physical properties of the lipid bilayer is not the primary determinant of the action of steroids in permitting agonist-induced nAcChoR activation.

Acknowledgements

This work has been supported by grants from the National Institute of General Medical Sciences (GM58448 to K.W.M.) and from the Bayrisches Staatsministerium fuer Landesentwicklung und Umweltfragen (H.S.). We thank Dr. Scott R. Rychnovsky for a kind gift of *ent*-cholesterol.

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