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THE NANO-ENVIRONMENT OF A NEUROTRANSMITTER RECEPTOR

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Abstract

The nicotinic acetylcholine receptor (AChR) is the archetype rapid ligand-gated neurotransmitter receptor. It mediates fast intercellular communication in response to the endogenous neurotransmitter acetylcholine, a small organic molecule. The AChR is embedded in and surrounded by lipids, its native membrane milieu. Lipids influence structural and functional properties of the receptor, mainly though the so-called receptor-vicinal ("boundary" or "annular") lipids. From the physicochemical point of view, this receptor-vicinal region constitutes a liquid-ordered phase, as opposed to the more disordered and "fluid" bulk membrane lipids. Changes in Förster's energy transfer (FRET) efficiency induced by fatty acids, phospholipids and cholesterol have led to the identification of discrete sites for these lipids on the AChR protein. Electron-spin resonance spectroscopy has established the stoichiometry and selectivity of the lipid shell surrounding the AChR and disclosed the occurrence of lipid sites. Combined electrophysiological single-channel recordings and site-directed mutagenesis data fostered the identification of such lipid-sensitive residues in the transmembrane region, dissecting their contribution to ligand binding and channel gating, opening and closing. Thus, the interface between the protein moiety and the adjacent lipid shell constitutes the habitat of a variety of pharmacologically relevant processes, including the action of steroids and other lipids.

Keywords: membrane proteins; neurotransmitter receptors; biophysics; lipid-protein interactions.

Resumen

El "nano-ambiente" de un receptor de neurotransmisor. El receptor de acetilcolina nicotínico (AChR) es el arquetípico receptor de un neurotransmisor gatillado por ligando. Media la comunicación intercelular rápida en respuesta a su neurotransmisor, la acetilcolina, una pequeña molécula endógena. El AChR está rodeado por lípidos, que constituyen su ambiente natural en la membrana. Dichos lípidos influyen sobre la estructura y la función del receptor, principalmente a través de los denominados lípidos vecinales (limítrofes, anulares). Desde el punto de vista físico-químico, esta región constituye una fase líquido-ordenada, en contraposición al resto de los lípidos de la membrana, desordenados y más "fluidos". Cambios en la eficiencia de la energía de transferencia de Förster's (FRET) han conducido a la identificación de sitios discretos para ácidos grasos, fosfolípidos y colesterol sobre la superficie del AChR. Mediante espectroscopía de espin-electrónico se pudo establecer la estequeometría y selectividad de la capa de lípidos que rodea al AChR y se descubrieron sitios de unión de tales lípidos. La combinación de mediciones electrofisiológicas de canal unitario con mutagénesis dirigida ha llevado a la identificación de tales sitios en la región transmembrana del AChR, disecando sus relativas contribuciones a la unión del ligando y al gatillado, apertura y cierre del canal, respectivamente. La interfaz entre el AChR y los lípidos constituye el hábitat de una gran variedad de procesos farmacológicamente relevantes, incluyendo la acción de esteroides y otros lípidos.

Palabras clave: proteínas de membrana; receptores de neurotransmisores; biofísica; interacciones lípido-proteicas.

Introduction

Neurotransmitter receptors and their associated signaling components in the synapse are crucial elements in the supracellular assemblies known as neural circuits in the brain. A synaptic complex, i.e. the neuromuscular junction (NMJ), made up of equivalent macromolecular building blocks, has similar signaling functions at the periphery. A key component of this complex machinery is the neurotransmitter receptor, acting as transducer of the chemical signal. Throughout evolution, the basic principle of rapid chemical neurotransmission has proved to be an efficient mechanism, as evidenced by its adoption by a large number of species. The ligand-gated ion channel (LGIC) superfamily comprises several families of evolutionarily related neurotransmitter receptor proteins coded by a few hundred genes so far identified. Of these, the nicotinic acetylcholine receptor (AChR) is one of the best-characterized.

Several genes coding for AChR subunits have been characterized in central and peripheral nervous systems. They exhibit amino acid sequence homology and presumably higher-order structural motifs [1-3]. Within the LGIC superfamily, the AChR and subtype 3 of the 5-hydroxytryptamine (serotonin, 5-HT3) receptor comprise two families of cation-selective channels, whereas glycine and gamma aminobutyric acid type A (GABAA) receptors are anion-selective channels. Members of this superfamily are also known as Cys-loop receptors because in their amino-terminal all their subunits contain extracellular halves of a pair of disulphide-bonded cysteines separated by only 13 residues. Glutamate and histidine receptors are also Cys-looped receptors, but their structure does not conform to the canonical LGIC superfamily. Until recently, the Cys-loop family was thought to comprise only ion channels produced by eukaryotic genes, but a prokaryotic proton-gated ion channel from the AChR family has recently been discovered [4].

The basic mechanism of signal transduction is common to all members of the LGIC and involves relatively fast and similarly simple steps: binding of the neurotransmitter followed by conformational transitions in the receptor proteins that lead to changes in the ionic permeability of the postsynaptic membrane. In the specific case of the AChR, upon binding, acetylcholine initiates a conformational change in this protein that triggers the transient opening of its intrinsic cation-specific channel across the postsynaptic membrane. At the molecular level, this is accomplished by the concerted action of four different but highly homologous AChR subunits in the stoichiometry $\alpha_2\beta\varepsilon\delta$ [5-6] in adult skeletal muscle.

Each AChR subunit contains four hydrophobic segments 20-30 amino acids in length, the M1-M4 membrane-spanning segments. Of these, the M2 segment from each subunit contributes structurally to the formation of the ion channel proper. M4 is considered the most likely segment to be exposed to the bilayer lipid. M1 and M3 effectively incorporate membrane-partitioning photoactivatable probes, and are also likely to be exposed, at least partially, to the lipid phase. It is usually accepted that all four hydrophobic segments M1-M4, referred to as transmembrane (TM) domains, correspond to regions of the protein fully embedded in the membrane. We have proposed that in addition to these two main regions of the AChR, a third important domain is defined at the extensive interface between the protein and lipid moieties, comprising both the lipid-exposed TM portions of the AChR protein and the AChR-vicinal lipid [7-8]. The latter corresponds to the lipid belt ("shell", "annulus", "boundary", "AChR-vicinal") region, that is the lipid moiety in the immediate perimeter of the AChR protein, earlier discovered by Derek Marsh and myself using electron spin resonance (ESR) techniques [9] and further characterized in terms of lipid selectivity and stoichiometry [10-15]. In this review I discuss various experimental approaches that have led to a quite detailed description of the AChR lipid microenvironment and of the cross-talk between the receptor protein and its surrounding lipids. The reader is referred to other reviews covering wider aspects of AChR-lipid interactions [8] or more specific facets of this topic, e.g. the effects of cholesterol (Chol) on receptor supramolecular structure, stability and dynamics at the cell surface [16].

The AChR is surrounded by lipids in the liquid-ordered phase

Model membranes are relatively well characterized in terms of their physical properties. At high Chol concentrations, phospholipid-Chol mixtures mimic many aspects of the phase state displayed by biological membranes rich in Chol. These mixtures lack a defined lipid phase transition and instead are characterized by a single phase state, the liquid-ordered phase (lo) [17], with properties between the gel and the fluid lipid phases. At low Chol concentrations, solid-ordered (so) or liquid-disordered (ld) phases are observed, depending on whether the system is above or below its gel-fluid transition temperature (Tm), respectively. When the binary lipid system is at intermediate Chol concentra-

tions, there is phase coexistence of so and lo (liquid-ordered, below), or of ld and lo (above), depending on the temperature relative to Tm. In the particular case of the AChR, early studies from the group of McNamee [18-19] showed that the phase state of the membrane was important: the capacity of reconstituted AChR to translocate ions in vitro was found to be sensitive to the bulk physical properties of the host membrane, such as its "fluidity". Early ESR studies by Marsh and Barrantes [9], Rousselet et al. [20] and Marsh et al. [15] made apparent the occurrence of two distinct signals in ESR experiments with native and reconstituted membranes containing AChR at relatively high or low concentrations: one signal corresponded to the bulk membrane lipid and the other was interpreted as stemming from the protein-immobilized lipid. These direct interactions between protein and lipid moieties were observed with fatty acids, phospholipids and sterols in the native membrane environment. Rousselet et al. [20] found immobilization with fatty acids but not with phospholipids. Ellena et al. [13] confirmed our findings using reconstituted AChR. This series of studies from different laboratories demonstrated that shell or annular protein-vicinal lipids are relatively immobile with respect to the rest of the membrane lipids and pointed to the existence of phase lateral heterogeneity in membrane lipids much earlier than the concept of "rafts" came into use.

Functional studies contributed to understanding the role of lipids in AChR ion permeability. The need to include sterols and certain phospholipids to preserve this property of the AChR in reconstituted systems was subsequently demonstrated [21]. The relative contributions of phospholipid and sterol was established in various studies in vitro [22-24] and the minimal number of lipid molecules (~45) per AChR was ascertained in early ESR experiments [13,25]. AChR-vicinal lipids appeared to be an inherently relevant environmental feature of the AChR native membrane, but the nature of the interaction between protein and lipid moieties was still obscure, as were the possible functional implications proposed in early work [9].

In order to define the physical state of the AChR membrane lipids, we subsequently resorted to fluorescence methods using the so-called Generalized Polarization ("GP") [26-27] of the fluorescent probe Laurdan (6-dodecanoyl-2-dimethylamino naphthalene). This approach was introduced to learn about the dynamics of the AChR and some physical properties of the protein-vicinal lipid [28-30]. We harnessed two hitherto unexploited properties of Laurdan: i) its ability to act as a Förster-type resonance energy transfer (FRET) acceptor of tryptophan emission [30] and ii) the resulting differences in FRET efficiency upon displacement of Laurdan by exogenous lipids [28-29]. Laurdan is a particularly advantageous fluorescent probe; it localizes itself at the hydrophilic-hydrophobic interface of the lipid bilayer, with its lauric acid moiety at the phospholipid acyl chain region and its naphthalene moiety at the level of the phospholipid glycerol backbone. Its spectral properties are extremely sensitive to the polarity and molecular dynamics of dipoles in its environment. This is due to dipolar relaxation processes that are reflected as relatively large spectral shifts. Excitation of Laurdan under FRET conditions using the tryptophan (Trp) residues of the AChR-rich membrane as donors constituted a new tool for learning about the properties of the lipids in the immediate vicinity of the AChR and to compare them with those of the average, bulk lipid in the rest of the bilayer. The first application of this approach was the determination of distances between the AChR protein and adjacent lipid. From the spectral overlap integral for the AChR membrane-Laurdan pair we calculated Ro, the Förster critical distance, to be 29 Å [30]. We modeled the AChR as a cylinder of about 80 Å in diameter with donor Trp residues lying in a ring within the perimeter of its TM portion. From the electron microscope data available at that time [31] this region of the protein was assumed to have a radius of 32.5 Å. The height of the plane of AChR tryptophan residues was set with respect to the plane of acceptors by using the parameter H, the distance between donor-acceptor planes normal to the membrane surface, which was allowed to vary between 0 and 50 Å in view of the long-axis dimensions of the AChR molecule and the width of the lipid bilayer. Using the above model we found differences between the AChR-vicinal lipids within a 14 Å radius of the AChR surface on the one hand, and the bulk lipids on the other. When no relaxation occurred, GP values were high, indicating low water content in the hydrophilic/hydrophobic interface region of the membrane. GP observed under FRET conditions from the intrinsic protein fluorescence exhibited higher absolute values than those obtained by direct excitation of the probe, indicating the lower polarity of the lipid in the protein-vicinal lipid microenvironment of the AChR. The main dipoles sensed by Laurdan in the membrane were water molecules. Thus, this series of studies showed that AChR-vicinal lipids are more rigid and exhibit a lesser degree of water penetration than bulk lipids, and that a single thermotropic phase with

the characteristics of the so-called liquid-ordered phase defines the entire AChR-rich postsynaptic membrane [30].

AChR-lipid interactions as viewed from the protein moiety

Electron microscopy experiments over twenty years ago already indicated that about half the mass of the AChR protein protrudes into the extracellular space, about 30% corresponds to TM domains, and the remainder is in the cytoplasmic compartment [32-35]. The agonist-recognition domains of the AChR were also shown to be located in the extracellular portion of the macromolecule [33] (and see review in [36]) at a distance of about 25 Å from the apex of the AChR [37] and about 30 Å from the membrane surface [31,38-39].

Site-directed mutagenesis of the AChR combined with patch-clamp electrophysiological and photoaffinity labeling experiments with non-competitive channel blockers support the notion that one of the TM AChR regions, the M2 domain, lines the walls of the pore. The data are also indicative of α-helical periodicity in the residues exposed to the lumen of the AChR channel [40]. Nuclear magnetic resonance (NMR) studies of the M2 segment of the receptor's α subunit [41] indicated that this domain is inserted in the bilayer at an angle of 12º relative to the membrane normal, in a totally α-helical configuration. Analogously, a synthetic peptide corresponding to the Torpedo αM2 segment in organic solvents also adopts a totally α-helical configuration [42]. Cryoelectron microscope data confirmed that M2 forms the innermost ring of membrane-spanning segments, isolated from membrane lipids [43-44].

During the 90's, cryoelectron microscopy revealed the relatively featureless appearance of the other putative TM domains (M1, M3 and M4). A large portion of this AChR region was postulated to be arranged in the form of a β-barrel outside the central rim of M2 channel-forming rods [45]. This interpretation contrasted with photoaffinity labeling studies, in which the observed periodicity of the lipid-exposed residues in M4 and M3 was consistent with an α-helical pattern [46-48], and with deuterium-exchange Fourier transform infrared spectroscopy studies indicating a predominantly α-helical structure in the AChR TM region [49]. In addition, secondary structure analysis (CD and Fourier transform infrared spectroscopy) of isolated and lipid-reconstituted TM AChR peptides indicated an α-helical structure for M2, M3, and M4 segments [50]. Furthermore, a synthetic peptide corresponding to the αM3 segment of Torpedo AChR exhibited a totally α-helical structure by 2-dimensional 1H-NMR spectroscopy [51]; NMR studies of a synthetic γM4 peptide were also found to be compatible with an α-helical secondary structure [52].

A considerable advance in defining the structure of the AChR at atomic resolution53 was achieved by crystallographic studies of a water-soluble acetylcholine-binding protein from a snail. The structure of this protein, highly homologous to the water-soluble extracellular domain of the AChR protein proper [53], provided the first truly high-resolution data of the region of the AChR putatively involved in agonist recognition, the first step in the cascade leading to channel opening. More recent work has resulted in the crystallization of the actual water-soluble extracellular domain of the mouse AChR α-subunit bound to α-bungarotoxin. The crystal structure was solved at 1.94 Å resolution [54].

The cryo-electron microscopy data of Unwin and coworkers [43-45] at 4 Å resolution provided inspiring insights into the structure of the AChR, and particularly relevant to the subject of this review, the electron microscopy data revealed interesting features of the membrane-embedded domains of the AChR protein. I described the occurrence of three concentric rings in the AChR region [7]: a) an inner ring exclusively formed by five M2 segments, corresponding to the walls of the AChR ion pore, which have no contact with the membrane lipid; b) a middle ring, formed by ten helices corresponding to the M1 and M3 TM segments. This middle ring is separated from the inner five-member ring of M2s, and its outer face is exposed to lipids and also to c) the outermost ring, consisting of five M4 segments (Figs. 1-3). The proton-gated ion channel protein recently found in the cyanobacterium Gloeobacter violaceus [4], termed "Glvi" or "GLIC", shares only 20% amino acid identity with one of its homologues in Homo sapiens, the α7 neuronal AChR. However, some key regions contributing to the gating properties of AChRs are conserved, such as the Cys-loop, the proline in M1, the equatorial ring of hydrophobic residues in M2, and the four TM segments. The latter feature is reinforced by the recently available X-ray structure of an ortholog of the Gloeobacter channel, the pentameric protein "ELIC" from Erwinia chrysanthemi at 3.3 Å resolution, which exhibits only 16% sequence identity with the AChR and lacks the cytoplasmic region [55]. The crystal structures of GLIC and ELIC clearly show the three concentric rings in the TM region which I described for the AChR [7], and suggests that its equivalent segments M1, M2 and M3 in the two inner rings of AChRs are involved in interactions at subunit interfaces, whereas M4, located at the

peripheral, outer ring, only loosely interacts with M1 and M3 of the same bundle and is less likely to be involved in subunit-subunit interactions [55]. If one were to ascribe a functional role to M4, this TM region probably conveys the signal from the lipid microenvironment to the rest of the TM helix bundle.

Influence of the lipid environment on AChR secondary structure

The lipid environment exerts a modulatory effect on AChR secondary structure. Early studies reported that Chol increased the α-helix content of the AChR. The sterol was postulated to stabilize AChR structure by packing its rigid planar ring into grooves of TM helices [56]. CD spectroscopy [57], Raman spectroscopy [58] and 1H/3H exchange studies [59] detected no large differences in structure or solvent accessibility between resting and desensitized AChR. One study reported no changes in secondary structure in the presence of Chol [60]. In contrast, evidence from cryoelectron microscopy [39] indicated differences between resting and desensitized and resting and activated AChR. Furthermore, the accessibility of AChR fluorophores to membrane probes between resting and desensitized forms of the AChR is different [61], as is the accessibility of residues near the ligand-recognition site [62] and the TM regions [63]. Castresana et al. [64] reported that the helical content of the AChR was not affected by addition of agonist, whereas the proportion of β-structure decreased to 24% concomitantly with an increase in disordered structure. Prolonged exposure to the agonist, leading to desensitization, resulted in significant rearrangement of the AChR structure. Lack of Chol in an asolectin reconstitution system produced an increase in disordered structure in T. marmorata AChR [65]. Addition of exogenous Chol resulted in restoration of the proportion of AChR ordered structure in asolectin liposomes but not in liposomes prepared from egg PCs, leading these authors to suggest that a component other than PC is needed for the restoration of AChR structure in the presence of Chol. Fernández-Ballester et al. [65] also suggested that the desensitization phenomenon does not depend on the presence of Chol or other lipids. Methot et al. [66] found 39% α-helix, 35% β-sheet, 20% random coil and 6% β-turn in T. californica AChR reconstituted in DOPC:DOPA: Chol (3:1:1) by FTIR, in other words sufficient α-helical content to form four-helical TM segments and a substantial portion of the extracellular region. It is interesting to note that much earlier, Finer-Moore and Stroud [67] had predicted a high helical content (44%) and 27% β-sheet in a theoretical analysis using a Fourier transformation of the AChR primary structure. Methot et al. [66,68] found no significant agonist-induced changes in the secondary structure of the AChR upon exposure to the agonist carbamoylcholine or to the local anesthetic tetracaine, regardless of the presence or absence of Chol in the reconstitution system.

The canonical 4-helix TM models [69-70], increasingly validated by cryoelectron microscopy data [43-44] and more recently by X-ray diffraction studies [55], placed between 17% and 20% of the AChR mass inside the membrane bilayer. Earlier studies [71-72] had suggested that each of the membrane-spanning segments was independently stable in the lipid bilayer. Corbin et al. [50] reported that in a membrane environment the AChR TM segments have the intrinsic propensity to adopt an α-helical secondary structure. The discussion about the secondary structure of the AChR TM region has further implications for current models of the other regions of the AChR molecule. Thus, compatibilizing the most recent experimental work with the theoretical models that assume four TM helices implies placing an important proportion of β-sheet structure in the extramembranous regions (extracellular and cytoplasmic-facing) of the AChR, a feature that appears to find validation at least in the case of the AChBP, the structural homológue of the AChR extracellular moiety [53]. The AChBP has extensive β-sheet structure. Görne-Tschelnokow et al. [73] concluded from their FTIR data that the TM region of the AChR contains 40% β-sheet plus turn structure. Using the same technique, Baezinger and Methot [49] produced experimental evidence supporting an all-helical model of the AChR TM region. Additional FTIR studies [49,68] indicate that 1H/2H exchange kinetics is slower in the presence of DOPA or Chol, suggesting that the lipid environment modulates the conformational dynamics of the membrane-embedded peptide hydrogen atoms that exchange with deuterium.

Physical contact between lipids and the AChR surface: fluorescence studies

The vicinity of AChR protein to lipids in the membrane bilayer was apparent in early fluorescence quenching studies (AChR intrinsic fluorescence) performed on native membranes [61] and in reconstituted systems [19]. The fluorescence emission of the AChR is typical of that of other integral membrane proteins. Fifty-one Trp and 80 Tyr residues are present in T. californica AChR [70], but the spectrum appears to be dominated by the Trp emission [61]. Fluorescence studies [74] and sequence topology [75] indicate

that only Trp453 in the γ subunit is present in the membrane-embedded region in Torpedo californica, with the addition of one more Trp residue in the α subunit in the case of Torpedo marmorata. These intrinsic fluorophores of the AChR are accessible to quenching by lipid analogs from the bilayer region; the heterogeneous nature of the fluorophore population is reflected in the occurrence of various quenching constants. From the study of other systems such as the sarcoplasmic reticulum Ca2+-ATPase [76] paramagnetic quenching of membrane-bound fluorophores is assumed to be predominantly static in nature. This is due to the fact that the measured phospholipid lateral diffusion in membranes, D, is about 10-8 cm2 s-1, i.e. relatively slow with respect to the time window of these measurements and thus the distance between fluorophore and quencher does not change appreciably during the lifetime of the former. In the case of the quenching of AChR intrinsic fluorescence, the mechanism is more complex, probably mixed in nature, because of the dynamic components arising from rotational motions and peptide chain wobbling. Paramagnetic quenching requires a minimum distance between quencher and fluorophore of about 5 Å. The occurrence of effective paramagnetic quenching by spin-labeled lipids thus constitutes strong evidence that quenching occurs in the TM regions of the AChR, and that the latter contain discrete sets of intrinsic fluorophores accessible to the nitroxide probe. The KQ's and the apparent fraction of available fluorophores (fa) can be obtained from the modified Stern-Volmer equation:

$$Fo/(Fo - F) = 1/(fa\ KQ\ [Q]) + 1/fa \qquad \text{(Eq. 1)}$$

where Fo is the initial AChR intrinsic fluorescence intensity and F is the fluorescence intensity of AChR in the presence of a given concentration, [Q], of nitroxide-labeled lipids. The value of 1/KQ, the spin-labeled lipid concentration at which 50% of the initial intensity is quenched assuming that all fluorophores are fully accessible to quencher, provides a quantitative estimate of the efficiency of the spin-labeled probes as quenchers of the fluorescence of membrane-embedded Trp residues. In the case of T. marmorata AChR in its native membrane this follows the sequence [14]:

CSL > ASL > 16-SASL > 12-SASL > 5-SASL

The higher efficiency of the steroid analog spin-labeled cholestane (CSL) than spin-labeled androstane (ASL) in quenching AChR intrinsic fluorescence can be explained in terms of the different location of the paramagnetic nitroxide group relative to the membrane surface. CSL is located close to the lipid/water interface, which most probably enables this spin label to quench Trp residues either in the extramembranous domain of the AChR, or in shallow regions of the TM region.

Stoichiometry and selectivity of the protein-vicínal lipid

ESR is an excellent biophysical method for studying the mobility, stoichiometry and selectivity of lipids at the intramembranous surface of integral membrane proteins, because its dynamic sensitivity is optimally matched to the time-scale of lipid rotational motions in biological membranes, in the order of nanoseconds. ESR spectra of spin-labeled lipids sense molecular motions corresponding to rotational frequencies of about 108-105 s-1 [77]. Thus, spectra of lipids motionally restricted by interactions with the surface of, or bound to, integral membrane proteins are suitably resolved from those corresponding to the fluid bilayer regions of the membrane. We have reported that lipid mobility at the lipid shell surrounding the AChR protein (the AChR-vicinal lipid) is reduced relative to that of the bulk membrane lipid, giving rise to a two-component ESR spectrum from which the number and selectivity of the lipids at the lipid-protein interface may be quantitated (see e.g. [15]). In this way we were first able to demonstrate protein-induced restrictions on the mobility of spin-labeled fatty acids and ASL [9] and of spin-labeled phospholipids [15] in AChR-rich membranes from T. marmorata. Subsequent work demonstrated the preferential association of AChR with spin-labeled steroids, phosphatidic acid (PA), and fatty acids, rather than with other kinds of lipid [13,20]. The possible functional implications of this topographical relationship between lipid and AChR protein became apparent in studies showing that Chol and negatively charged phospholipids were required to support the ion-gating activity of the AChR [22-23,78] whereas fatty acids blocked the ion-flux response [79]. The latter was interpreted as the perturbation of the functionally significant interaction between AChR and Chol or negatively charged phospholipids.

FRET studies using the SM fluorescent derivative also yielded information on the relative affinity of the SM derivative for the AChR protein. Thus, Py-SM exhibited a moderate-to-low selectivity for the protein-vicinal lipid domain, with a calculated relative affinity Kr ≅ 0.55 [ref. 80]. This figure should be added to the list of known selectivities of other lipids for the AChR calculated from early ESR experiments: PS (0.7),

PC (1.0), PE (1.1), PA (2.7) and stearic acid (4.1) by Ellena et al. [13], and to those determined by us more recently, also using ESR techniques [81], allowing a classification of lipids according to their selectivity for the AChR protein. The study by Mantipragada et al. [81] provided the first detailed description of the dynamic composition of "first-shell" lipids in the belt region surrounding a receptor protein. AChR-vicinal lipids fall into three categories: a) a high specificity group made up of fatty acids like stearic acid, cardiolipin and phosphatidylinositols [81-82], androstanol [15,82], and phosphatidic acid [13]; b) an intermediate group, made up of SM [80]; PS and PG [81], and c) a moderate-to-low specificity group, where we find PC, PE, and the gangliosides GD1b, GM1, GM2 and GM3 [81].

It is interesting to note that owing to the high packing density of AChR molecules in the postsynaptic membrane, only three to four phospholipid layers of "bulk" lipid separate the protein-vicinal first layer from the nearest-neighbor first-layer lipid surrounding an adjacent AChR protein [8]. In Torpedo receptor-rich native membranes prepared from electrocytes, all lipids in the membrane are in the liquid-ordered (lo) phase, with decreasing polarity towards the AChR protein molecules [30]. This is because the protein-vicinal lipid-belt region is more rigid and ordered than the bulk bilayer lipid, as determined by Laurdan GP measurements using FRET [29-30]. However, the receptor-vicinal and the bulk lipid regions form a single, liquid-ordered phase from the physico-chemical point of view.

The protein-vicinal lipid is relatively immobilized with respect to motions both around and perpendicular to the long molecular axes of the lipid molecules, i.e. with rotational correlation times ~50-100 times longer than is typically found with fluid bilayer lipid [9]. The protein-vicinal lipid also exhibits a lower degree of penetration of water molecules, thus rendering it less polar than the bulk bilayer lipid [29-30]. Another dynamic aspect that characterizes the two lipid regions is the relatively high exchange between the two moieties: although the AChR-vicinal lipid is expected to have a lateral diffusion coefficient 50-100 times slower than that of the fluid bilayer lipid, i.e. ~105 s-1 [ref. 9], the lipid exchange process between the AChR-vicinal lipid and the bulk lipid exhibits rates in the order of 1-5 x 108 s-1. Lipid species displaying selectivity for the AChR protein spend on average a longer period in the immediate vicinity of the protein; they are concentrated relative to those lipids exhibiting little or no selectivity. In systems where the selectivity of the lipid for the protein is changed by varying the pH or the ionic strength, it has been shown that the on-rate remains constant, whereas the off-rates reflect the specificity of a given lipid, which is independent of the lipid/protein ratio [83].

We have also found that some local anesthetics, noncompetitive antagonists of the AChR, exert their action at the AChR-lipid interface [11,14,81]. The effect of local anesthetics contrasts with that of general anesthetics: both reduce the motionally restricted boundary or shell-lipid, but the latter also fluidize the bulk lipid, an effect that we have not observed with local anesthetics at the concentrations used [81]. It is also interesting to note that local anesthetics compete more effectively with the phospholipid PI than with the fatty acid analogue, although both lipids display similar relative association constants for the AChR. Unwin and colleagues suggested the possibility that alcohols and local anesthetics bind to the AChR in water-filled cavities in between the M2 channel-lining ring and the middle M1-M3 TM ring [43].

Changes in the physical state of the native AChR membrane induced by exogenous lipids

When Laurdan GP was measured in Torpedo native AChR membrane (either by direct excitation or under FRET conditions) in the presence of exogenous lipids, GP and by inference the "fluidity" and order of the membrane were found to diminish upon addition of oleic acid and DOPC, and not to vary significantly upon addition of Chol hemisuccinate, indicating an increase in the polarity of the single, ordered-liquid lipid phase in the two former cases [28].

Complementary information about the bulk lipid order was obtained from measurements of fluorescence anisotropy of DPH and two of its derivatives. The membrane order diminished in the presence of oleic acid and DOPC. The location of Laurdan was determined using the parallax method of Chattopadhyay and London [84], based on the relative position of a fluorescence probe embedded in the membrane and its quenching by probes having nitroxide spin labels at different positions along their acyl chains. The parallax determination is accomplished by pairwise comparison of quenching parameters with different pairwise combinations of the PC analogs with spin labels in carbons 7, 10 and 12.

When the temperature dependence of Laurdan fluorescence was studied in the native AChR-rich membrane, dipolar relaxation was found to gradually increase with increasing temperature [30], probably reflecting an increment in

water content and disorder in the shallow membrane region sensed by the probe, both in AChR-vicinal and bulk lipid regions. Similar conclusions were reached from a study of fluorescence anisotropy of DPH and derivatives, which sense the thermally-induced disorder in other regions of the membrane [28].

Energetics of channel gating in living cells measured by Laurdan fluorescence

The characterization of Laurdan thermotropic behavior was subsequently extended to living mammalian cells expressing endogenous or heterologous AChR. Interestingly, the differences in physical properties of cell membranes measured by Laurdan GP in a variety of cells, reflecting the molecular dynamics of water molecules, could be correlated with the energetic changes in the AChR ion channel occurring as a function of temperature, as measured in single-channel recordings [85]. Laurdan expends energy in solvent (water) reorientation, as evidenced in the red shift of its emission spectrum. The decrease in Laurdan GP upon increasing the temperature reflects an increase in water diffusion into the membrane. Water penetration into the membrane is facilitated by the increased thermal-induced disorder in the bilayer lipid. The higher AChR channel conductance upon increasing the temperature is a manifestation of the augmented ion and water permeability in the AChR channel or "pore" region as we observed in single-channel recordings with the patch-clamp technique. Our study [85] further indicated that AChR channel kinetics depends not only on intrinsic properties of the AChR protein but also on the physical state of the membrane in which the receptor is embedded.

Topology and lipid selectivity of individual AChR membrane-embedded domains

The topography of the pyrene-labeled cysteine residues in TM regions of the AChR with respect to the membrane was experimentally determined by differential fluorescence quenching with spin-labeled derivatives of fatty acids, PC, and the steroids cholestane and androstane [82]. TM peptides were obtained by controlled enzymatic digestion from purified Torpedo californica AChR, derivatized with N-(1-pyrenyl)maleimide (PM), purified, and reconstituted into asolectin liposomes. For the intact AChR, PM fluorescence mapped to proteolytic fragments consistent with labeling of cysteine residues in αM1, αM4, γM1 and γM4. Stern-Volmer plots of whole AChR quenching by spin-labeled lipid analogs showed no deviation from linearity. Stearic acid and androstane spin label derivatives were the most effective quenchers of the pyrene fluorescence of whole AChR and derived TM peptides. In the case of spin-labeled stearic acid derivatives, the 5-SASL isomer quenched more effectively than the 7-SASL and the 12-SASL analogs, indicating a shallow location of the pyrene-labelled Cys residues [86]. In fact, the quenching studies indicated that all labeled Cys residues are located in shallow positions with respect to the membrane bilayer in the AChR and derived TM peptides. In the case of γM4, and by inference in other M4 segments of the AChR, this is compatible with a linear α-helical structure (αCys^{412} and γCys^{451} are located near the N-terminus of the TM segment). In the case of αM1, "classical" models locate αCys^{222} and Cys^{230} at the center of the hydrophobic segment in an extended α-helical structure and therefore alternative structures/topologies for the M1 domain must be considered. One possibility is that the M1 segment contains non-helical structure, and/or kinks. In a mixed α-helix/β-sheet model of the AChR [87], αM1 was constructed as a three-strand β-sheet interrupted by short loops generated by searching in the database of known structures for an appropriate backbone conformation. The proline residues themselves could not lie within a β-strand, so they were positioned in the loops. The same model can be extended to γM1, having a proline residue (Pro229) immediately adjacent to Cys^{230} and two other (Pro222 and Pro244) at the end of the TM region. Thus, the conserved proline residues in the M1 segments of the AChR might introduce "curls" or kinks in a manner analogous to that reported for one of the TM segments of a K+ channel [88-90]. The occurrence of proline residues –potential helix-disrupting residues- is a striking feature of M1 in the AChR and all members of the rapid LGIC superfamily [91]. Another possibility is that a portion of the N-terminus of the M1 TM α-helix extends beyond the lipid bilayer, therefore placing Cys^{222}/Cys^{230} near the lipid/water interface. This interpretation now appears more likely on the basis of electron microscopy data [43-44].

We studied the γM4 AChR domain incorporated in liposomes made of 60% POPC-40% Chol by means of fluorescence spectroscopy [92]. In a pure phospholipid (POPC) system in the ld phase, the lipid-peptide interactions predominated over interactions between helices, and γM4 occurred as an isolated peptide, matching the hydrophobic region of the bilayer. In contrast, in a mixed 60% POPC-40% Chol system in the lo phase, peptide-peptide interactions prevailed, and peptide aggregation occurred. These Chol-

dependent properties of a representative membrane-embedded segment of the AChR may bear relevance to the organization of the γ-subunit α-helical bundle motif and the AChR membrane-spanning region at large. The tendency of the hydrophobic γM4 peptide to maximize peptide-peptide interactions in the presence but not in the absence of Chol may be related to the ability of this sterol to stabilize the α-helix content of the native AChR [78-93]. Diffusion coefficients of D = 14 ×10-8 cm^2 s-1 (for 7% peptide) and D = 12 ×10-8 cm^2 s-1 (for 3% peptide) were obtained for the γM4 peptide. These values have the same order of magnitude or are slightly higher than those typically found for diffusion in an ld phase (D = 1.1×10-8 cm^2 s-1) [ref. 94], D = 1-3 ×10-8 cm2 s-1 for transmembrane proteins and D = 9-14 ×10-8 cm^2 s-1 for a fluorescent lipid derivative [95] and the fluorescent lipid analogue NBD-phosphatidylethanolamine (D = 8 x 10-8 cm^2 s-1) [ref. 96]. The lateral diffusion of the AChR reconstituted in pure DMPC bilayers was studied with fluorescence recovery after photobleaching techniques. D values in the range of 10-8 cm2 s-1 were observed for both the AChR monomer and dimer in the lo phase [96]. Additional multiple-component recoveries with D values of less than 5 x 10-11 cm^2 s-1 were found below the lipid phase transition. Thus the translational diffusion of the γM4 peptide is not significantly different from that of the 9S AChR monomer or the 13S dimer in the low concentration limit; neither the peptide nor the whole AChR encounter hindrance to lateral diffusion on the part of the lipid bilayer itself, whereas in native membranes, the densely packed AChR molecules are rather immobile (see review [91]).

Functional effects of the lipid environment on the AChR

Several channels and receptors, the AChR protein included, are embedded in the postsynaptic membrane. Given the extensive contacts between membrane lipids and the AChR, the physicochemical properties of the constituent lipids are likely to influence the physicochemical and functional properties of the protein moiety and likewise, the latter is bound to modify the corresponding properties of the membrane lipids. This prediction is experimentally validated by the fact that various types of lipid have been found to affect the function of channels and receptors, as analyzed in this section. One functionally relevant example is the phospholipid regulation exerted on the ATP-sensitive potassium channel (KATP channel), the channel that i) controls the movement of K ions in and out of the pancreatic β cells, thus linking blood glucose concentration with insulin secretion, ii) modulates the tone of smooth muscle in blood vessels and iii) regulates the length of the action potential in cardiac muscle. KATP channel has been shown to be modulated by phosphatidylinositol-4,5-bisphosphate (PIP2) [97-98]. Exogenous, synthetic steroidal substances of therapeutic use, as well as endogenous steroids, have been shown to bind to and affect muscle and neuronal-type AChR [99-100].

Lipids and agonist-induced state transitions: Cholesterol and negatively charged phospholipids

When AChRs were reconstituted into lipid vesicles made up of only PC they were found not to be functional [22-24,101]. The presence of Chol and anionic lipids such as PA or PS restored the capacity to undergo agonist-induced state transitions [102]. However, contradictory interpretations still remain. Thus Rankin et al. [103] concluded that AChR reconstituted in PA/PC mixtures was unable to undergo agonist-induced state transitions, whereas Baezinger et al. [102] used AChR preparations reconstituted in Chol and found that they are able to undergo agonist-induced structural transitions, though anionic lipids were needed for full activity. Recent studies stress the importance of acidic phospholipids like PA (e.g. POPC liposomes containing POPA) in the reconstitution mixture to preserve almost complete functionality of the AChR. Addition of Chol (POPC/POPA/Chol, 3:1:1) had a positive synergistic effect [104]. These results further suggest that this lipid composition stabilizes the receptor in the resting state and allows agonist-induced state transitions, as previously observed with FTIR spectroscopy and radioactive ion-flux experiments [105].

Lipid effects on AChR agonist-induced conformational states

The requisite lipid composition for maintenance of agonist-induced affinity transitions between conformational states is not necessarily optimal for adequate receptor-controlled ion translocation [22-23]. The latter property has been reported to depend on the presence of neutral and anionic phospholipids [24]. In one of our studies, unsaturated PE in combination with approximately 30% of a cholesteryl ester proved the best lipid mixture for the reconstitution of the AChR-mediated ion-permeation in artificial membranes [23]. The less saturated the acyl chains of PE, the higher the observed response.

Other lipids and hydrophobic molecules are also known to modulate AChR activity [8,106-

107]. Fatty acids and sterols are of particular significance. Their selectivity for the AChR microenvironment was demonstrated in early experiments [9,106]. The major lipid components of biological membranes in general are phosphoglycerides, Chol, and sphingolipids. In the case of the AChR-rich membranes prepared from the electric organ of Torpedinidae species, the major phospholipid constituents are choline- (40%), ethanolamine- (35%), and serine- (13%) glycerophospholipids. The effect of phosphoglyceride composition on AChR function has been the focus of several studies [22-24]. Chol and steroids have also been shown to play a major role in AChR function [82,106,108]. Sphingomyelins (N-acylsphingosine-1-phosphorylcholine or ceramide-1-phosphorylcholine, "SM"), the simplest class of the sphingolipids, amount to about 10% of cellular lipids in mammalian cell membranes. They are more abundant in the plasmalemma than in intracellular membranes and like the other choline-containing phospholipid, PC, are enriched in the outer, exoplasmic leaflet of the bilayer [109-110]. Sphingolipids are important lipid constituents, essential for cell growth [111]. Aminophospholipids such as PS or PE, on the other hand, are enriched in the inner, cytoplasmic leaflet of the membrane [112]. SM has also been implied to play a key role in the so-called "SM cycle", in which SM-derived ceramide acts as a lipid second messenger [113]. In the case of the AChR-rich membrane from Torpedinidae, SM accounts for about 5% of the total phospholipid content [114-115]. We have established its topography in the Torpedo AChR-rich membrane and its moderate-to-low affinity for the receptor protein [80]. More recently, we found that inhibition of sphingolipid biosynthesis resulted in the accumulation of unassembled AChR oligomers in the endoplasmic reticulum, leading us to suggest a "chaperone-like" effect of sphingolipids on the AChR biosynthetic pathway, affecting both the efficiency of the assembly process and subsequent receptor trafficking to the cell surface [116].

A correlation between the structural characteristics of some lipids and AChR conformation could be established in fluorescence spectroscopy studies using the fluorescent probe crystal violet. Interestingly, the probe also exerts pharmacological action on the AChR: it is an open channel blocker, exhibiting a higher affinity for the desensitized state than for the resting state of the receptor. Fatty acids were found to decrease, albeit to different extents, the KD of crystal violet in the absence of agonist. Only cis-fatty acids, which increase membrane fluidity, caused an increase in KD in the presence of agonist. Steroids produced a concentration-dependent diminution of the KD of the resting AChR state, which approached that of the KD of the desensitized state. However, the presence of steroids did not alter the desensitized state of the AChR, a result that concurs with the fact that the steroids tested did not change the polarity of the membrane sensed by Laurdan GP [117].

Lipid sites on the AChR molecule

The presence of binding sites for hydrophobic molecules distinct from the annular ones, on the surface of the (Ca2+-Mg2+)-ATPase, has been deduced from FRET studies between Trp residues and dansyl-undecanoic acid, and quenching of the intrinsic protein fluorescence by brominated lipids [118-119]. The simple addition of Chol had no effect on the intrinsic fluorescence of the enzyme ATPase reconstituted in dioleoylphosphatidylcholine (DOPC) liposomes containing brominated PC. However, reconstitution of the ATPase with mixtures of DOPC and dibromo-Chol resulted in fluorescence quenching. From these data a model was proposed in which Chol is excluded from the lipid-protein interface, i.e. from the annular binding sites, but is still able to bind at a second set of sites from which phospholipids are excluded, namely non-annular binding sites. The same explanation was furnished in the case of the AChR by McNamee's group [25] and non-annular binding sites for fatty acids, Chol and its analog cholesteryl hemisuccinate were postulated to occur on the surface of the receptor, but distant from the protein-lipid interface or "annular" lipids. In an early modeling study, we "docked" Chol molecules on the lipid-facing surface of the AChR TM region in crevices between M1, M3 and M4 from adjacent subunits [120]. The crevices were apparent even in low-resolution electron microscope images of the AChR protein [39]. Five such sites were located in each leaflet of the membrane, making a total of ten steroid sites, in general agreement with the discrete number of sites postulated by McNamee's group [19, 25]. A second outcome of this early model was the finding that only a few lipid molecules can gain simultaneous access to more than two TM segments. This concept was further elaborated in a subsequent model based on Unwin's suggestion [38] of similarities between the tertiary structure of the B5 pentamer of E. coli enterotoxin and the AChR. In their description of the Chol sites on the AChR protein, Jones and McNamee [19] postulated that these sites were at interstitial regions of the AChR. Miller and coworkers [121] further elaborated on the topology of the Chol sites, which they located at a distance of about 0.6 nm from the first shell or annular lipids.

Early work on AChR-rich membranes demonstrated the natural abundance of Chol [114,122] and the protein-induced restriction in mobility of spin-labeled androstanol [9]. The latter biophysical work suggested the occurrence of sites for steroid-like molecules in the AChR microenvironment. The search for such sites involved the use of other spin-labeled sterols [13] and photoaffinity labeling techniques [123] which demonstrated the close proximity of a Chol analog and the AChR. The radioactive photoaffinity label [3H]cholesteryl diazoacetate, a carbene-generating probe, was incorporated into all AChR subunits. Spin-labeled cholestane was also shown to be incorporated into AChR-rich regions of the membrane [11]. González-Ros and coworkers [124] showed that a photoactivatable analog of Chol can be displaced from the AChR by unlabeled Chol and that the labeling is sensitive to the desensitization phenomenon.

Jones and McNamee [19] used brominated sterol to define annular (about 45 per AChR monomer) and non-annular (about 5-10 per AChR) sites, the latter with ~20-fold higher affinity for Chol. Ellena et al. [13] showed that another sterol, androstanol, exhibited a higher selectivity relative to PC, occupying about 38 sites on the AChR. Subsequent ESR work showed preference of the AChR for a PS analogue (~60% perturbation by the membrane protein) over other lipids (~30%) [12]. After proteolytic removal of the extramembrane portions of the membrane-bound receptor, binding sites for the spin-labeled androstane and stearic acid disappeared, but those for phospholipids and sphingomyelin analogues did not. The occurrence of androstane sites in extracellular AChR domains has been challenged by subsequent work [121]. Photoaffinity labeling studies with azido-Chol also do not support the occurrence of extracellular sites for the sterol [125] as found with the GABAA receptor.

Fernández-Ballester et al. [65] studied the effect of Chol on the secondary structure and cation permeability of reconstituted Torpedo AChR. They found that the presence of phospholipids was necessary in the reconstitution mixture to support the modulatory effect exerted by Chol on AChR ion permeability in vitro. Blanton et al. [126] used the steroid promegestone to photolabel T. californica AChR. The probe was incorporated into each of the AChR subunits in approximately equal amounts both in the presence and in the absence of Carb. While no evidence of [3H]promegestone incorporation was detected in the inner ring M2 segment, residues reacting with the steroid in the outer ring M4 domains were identified and found to be identical to those previously shown with other ligands reported to be in contact with the lipid bilayer. The outer ring of M4 TM segments occupies the outermost, peripheral region of the receptor macromolecule, in direct contact with membrane lipids –essentially embedded in the bilayer. The steroid promegestone was further found to be a noncompetitive antagonist of the AChR, allosterically affecting the receptor by interacting with residues situated at the lipid-protein interface [126]. Corbin et al. [125] used the photoactivatable Chol analog 3α-(4-azido-3-[125I]iodosalicylic)-cholest-5-ene (azido-Chol) to label the TM regions of T. californica AChR. In αM4, residues Cys412, Val413, Phe414 and Met415 were labeled. This stretch corresponds to outer ring residues embedded in the cytoplasmic-facing hemilayer of the membrane.

Another strategy for determining the occurrence of lipid sites in the AChR exploited the decrease in FRET efficiency (E) between the intrinsic fluorescence of AChR-rich membranes and Laurdan, induced by different lipids. E between AChR (donor) and Laurdan (acceptor) was calculated using equations 7 and 8 below. E is given by:

$$E = Ro6/(Ro6 + r6) \qquad \text{(Eq. 7)}$$

where r is the intermolecular distance and Ro is a constant parameter for each donor-acceptor pair, defined as the distance at which E is 50%. E can also be calculated as:

$$E = 1 - (f/\, f\, D) \approx 1 - (I/ID) \qquad \text{(Eq.8)}$$

where ϕ and ϕD are the fluorescence quantum yields of donor in the presence and absence of the acceptor, respectively, and I and ID are the corresponding emission intensities. FRET efficiency was found to decrease upon addition of exogenous lipids, which displace Laurdan molecules from the AChR-microenvironment. The maximal decrease in E resulting from the addition of a fatty acid (18:1) amounted to about 60%, whereas Chol or phospholipid reduced E by 35% and 25%, respectively. The sum of the decreases caused by DOPC and colesteryl hemisuccinate equaled that obtained in the presence of 18:1 alone. From this series of experiments we reached the conclusions that i) there are independent sites for phospholipid and sterol, both accessible to fatty acid, in the vicinity of the AChR [28], in agreement with previous ESR work [12,19] and ii) fatty acids of different chain length and saturation share a common site(s) but produce different effects on the physical properties of AChR-associated lipid belt region and bulk lipids, respectively [62].

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