THE LIPID ENVIRONMENT OF THE NICOTINIC ACETYLCHOLINE RECEPTOR IN NATIVE AND RECONSTITUTED MEMBRANES

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I. INTRODUCTION

The nicotinic acetylcholine receptor (AChR) transduces the binding of the agonist into a local depolarization of the postsynaptic membrane through the transient opening of a cation-selective channel. The AChR is still the best characterized cell surface neurotransmitter receptor, owing to the inherent properties of the AChR itself and the membrane where it occurs, the existence of appropriate biological sources, its abundance in the plasma membrane, and other reasons discussed in earlier reviews.[1-4] It is a glycoprotein of more than 250,000 mol wt composed of two quasi-identical subunits (α) and three additional subunits (β, γ, and δ) in a stoichiometry of α_2, β, γ, and δ.[5-7] Apparent molecular weights of the α, β, and δ chains determined by SDS gel electrophoresis are 39,000, 48,000, 58,000, and 64,000, respectively. The amino acid sequence of all subunits has been deduced from the corresponding nucleotide sequences of the four genes by cDNA recombinant techniques (see below). The molecular weights predicted from the nucleotide sequences are 50,116 (α), 53,681 (β), 56,279 (γ), and 57,565 (δ) (Mishina et al.[8] and references therein). The α subunits carry the recognition site for agonists and competitive antagonists. All subunits are glyco-polypeptides[7] and transverse the membrane at least once.

The interactions between the lipids surrounding the AChR and the receptor protein itself, and between these lipids and the bulk bilayer lipids, are complex in nature and certainly occur in different time regimes. Little is known about their consequences for receptor function, and still less about the nature of such interactions. Only indirect evidence is available on the alterations of AChR function upon disruption of the bilayer by phospholipase A_2 action on the peripheral[9,10] and neuronal[11] AChRs. The details of the lipid bilayer structure and the constraints it exerts on the dynamics of the AChR in the membrane are also of importance for understanding the function of the receptor protein. This review deals with some structural and dynamic properties of the membrane in which the nicotinic AChR is inserted.

II. STRUCTURAL RELATIONSHIP BETWEEN AChR AND THE LIPID BILAYER

A. CONTACT REGIONS BETWEEN AChR AND THE MEMBRANE LIPID

Even before the complete amino acid sequence of all AChR subunits was known, experiments were designed to test which portions of the receptor were in contact with the membrane lipid bilayer. Photoreactive probes that partition favorably into the lipid phase were found to

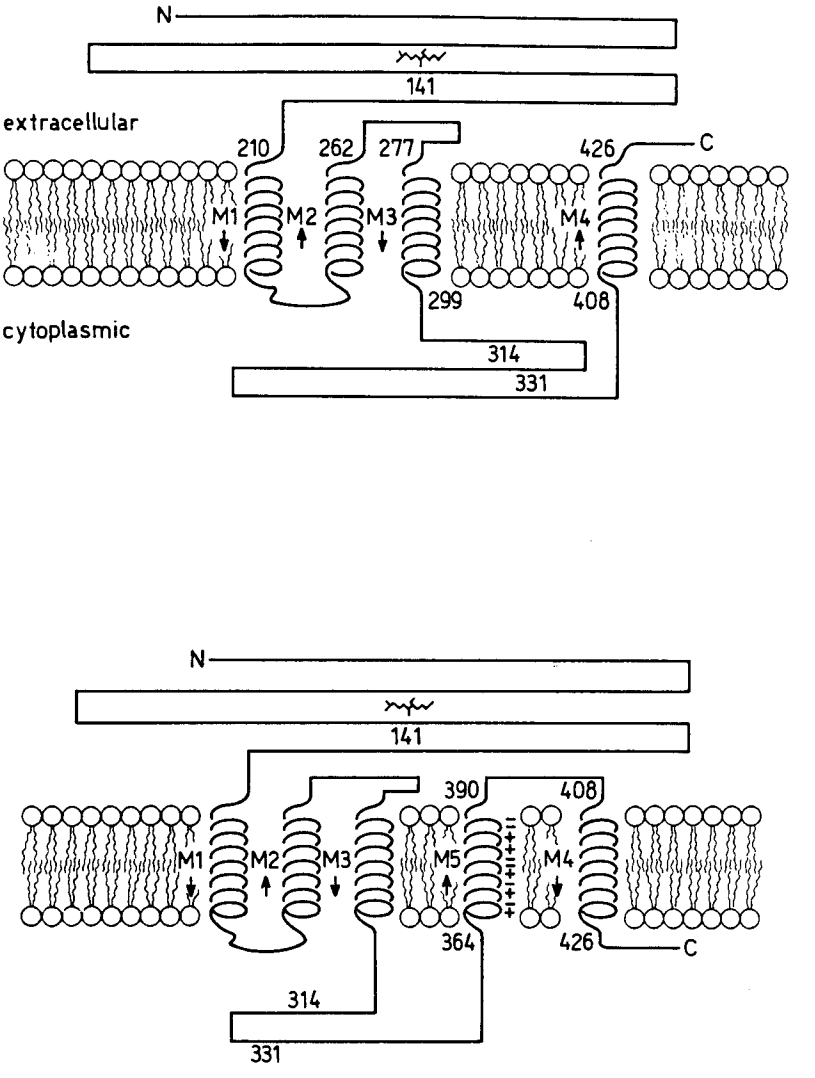
covalently label the α chain after irradiation.[12] The photolabeled portion of the α chain, a M_r 13,000 hydrophobic polypeptide, was subsequently isolated.[13] Crosslinking of other photoreactive probes with the β and γ subunits[14] or all four subunits[15] has also been reported. Arylazido photoreactive phosphatidylcholine (PC) analoges also result in the labeling of all subunits[16] from the lipid phase. Quenching by nitromethane of pyrene-1-sulfonyl azide, a hydrophobic probe tagged on the β and γ subunits, was shown to decrease upon desensitizing the AChR with high concentrations of carbamoylcholine,[17] suggesting that exposure of the probe — and by inference of the tagged polypeptide segment in the AChR — to the lipid phase varies with conformational transitions of the protein. The same conclusions were reached in studies of AChR in its native membrane in the presence and absence of the potent agonist suberyldicholine, using nitroxide spin labels as quenchers with different degrees of penetration in the lipid bilayer.[18]

B. TRANSMEMBRANE TOPOLOGY OF AChR CHAINS

In an earlier review,[2] the various experimental approaches that have been applied to demonstrate the transmembrane nature of all AChR subunits were discussed. In short, they are based on the use of controlled proteolysis,[19,20] antisubunit antibodies in intact and permeabilized membranes,[21,22] immunocytochemistry,[23-25] lactoperoxidase labeling,[26] and the various photoaffinity labeling techniques listed in the previous section.

In more recent years, application of cDNA recombinant techniques to the study of the AChR has had a profound impact on our understanding of this protein. Knowledge of the complete amino acid sequences obtained therewith has prompted comparison of the four distinct AChR subunits and the discovery of extensive homologies between the five polypeptide chains that form an AChR monomer. The homologies are not merely compositional: upon introduction of "spacer" segments at appropriate stretches and by bringing into register hydrophobic and hydrophilic regions of all subunits, extensive topological homologies become apparent. The occurrence of such homologous regions along the AChR chains led Noda et al.,[27-29] Claudio et al.,[30] and Devilliers-Thiery et al.[31] to postulate models of the subunit arrangement with respect to the lipid bilayer in which four hydrophobic domains in each chain transverse the membrane back and forth and display α-helical configuration. These hypotheses were also based on (1) the observed length of the hydrophobic segments, (2) the analogy with some transmembrane proteins like bacteriorhodopsin and glycophorin A, and (3) favorable length of the observed stretches in α-helix as opposed to other polypeptide conformations (e.g., β-sheets) to span the bilayer thickness. These models predicted that both the amino- and carboxyl-termini of the subunits face the extracellular milieu. This contrasts with views on some other integral membrane proteins studied to date: the N-terminus is usually expected to remain on the extracellular space after cleavage of the leader sequence, and the C-terminus to be exposed to the intracellular face of the membrane.[32] In fact, available experimental evidence points to (1) the existence of leader sequences that are cleaved after subunit synthesis and (2) the location of the N-terminus of the δ-subunit on the extracellular face of the membrane.[33] This type of model — the so-called four-helix model — places about 30% of the AChR protein on the cytoplasmic face of the membrane (Figure 1).

An alternative type of model (the five-helix model) predicts a much smaller (ca. 20%) portion of the AChR on the cytoplasmic compartment; in addition to the four transmembrane helices per chain, it postulates a fifth, amphipathic transmembrane helix (M5, MA, or A[34,35]). A basic difference between the two models becomes immediately apparent: the 5-helix model places the C-terminus on the cytoplasmic compartment (Figure 1). The other essential difference resides in the occurrence of the amphipathic MA helix, which is characterized by a continuous hydrophobic face on one side and a hydrophilic face on the other, the latter thus providing an appropriate substratum for the lining of the internal walls of the AChR ionic channel. The continuous hydrophilic face is about 3.8 nm long, i.e., sufficient to span the thickness of the bilayer, and has a distribution of charges (21 positive, 19 negative, 10 uncharged) in its residues[36]



A

FIGURE 1. Models of AChR topography with respect to the membrane bilayer. Common to early models is the long hydrophilic extracellular domain at the N-terminus, three closely spaced α-helical transmembrane portions (M1 to M3), a hydrophylic cytoplasmic domain, and a fourth transmembrane helix (M4). Dissimilarities comprise the postulation of a fifth amphipatic helix (M5 in b), two additional transmembrane domains (M6 and M7 in c), and the extracellular (a) versus cytoplasmic (b,c) location of the C-terminus. Minor differences are the fully exposed (a,b) or hidden (c) location of the N-terminus. The 4-helical model (a) was proposed by Noda et al.,[27] Claudio et al.,[30] and Devilliers-Thiery et al.[31] The 5-helical model was postulated by Fairclough et al.,[36] Guy,[35] and Young et al.[43] The 7-transmembrane domains were proposed by Criado et al.[42] A significant departure from all these models is that of Ratnam et al.[47] which is referred to as the "bulky cytoplasmic domain" in the figure (see text).

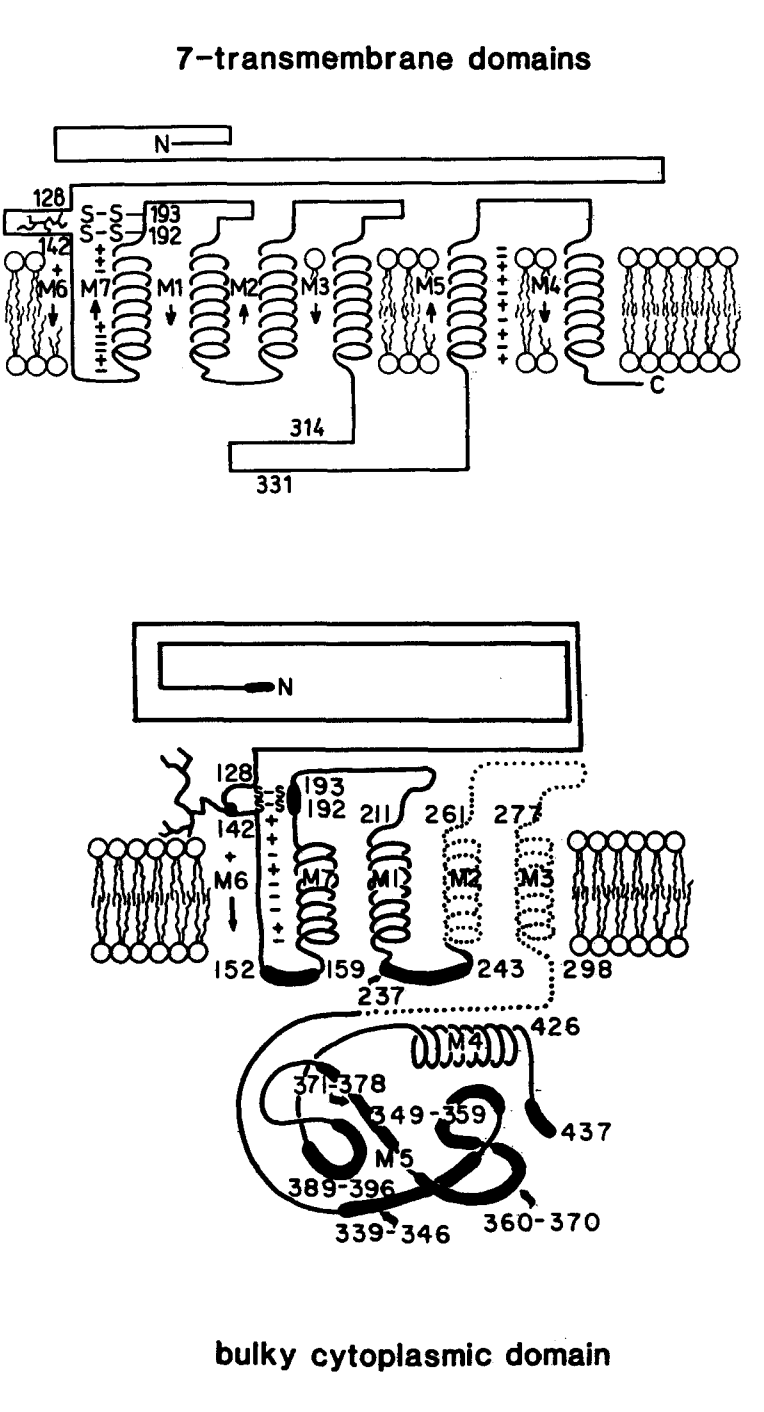


FIGURE 1B

that makes it optimally suited for intersubunit ion-pairing. Current views on this point, based on point mutation and patch-clamp data on the one hand and immunochemical evidence on the other, have made the hypothesis that the MA amphipatic segment is a transmembrane helix less tenable (see below). Other models specifically addressing the structure of the channel-lining portion of the subunits have been postulated.[37-39] Kosower[40] proposed the sequences α 372 to 395, β 401 to 424, γ 395 to 413, and δ 419 to 442 as the constituents of the ion channel walls.

In addition to the above-mentioned information gained through the use of photolabeling probes, theoretical models have raised new questions concerning the topography of the AChR with respect to the membrane. The need to resort to independent tests of the current models becomes imperative. Immunochemical techniques are particularly appropriate for this purpose, since both poly- or monoclonal antibodies can be raised against synthetic peptides with amino acid sequences matching those of defined segments in the AChR chains. Such antibodies have been recently produced and employed, sometimes in conjunction with immunocytochemical techniques, to test available models. Lindstrom et al.[41] and Criado et al.[42] produced evidence that residues 159 to 169 and 152 to 159, respectively, in the α chain C-terminus face the cytoplasmic side of the membrane. In order to account for their findings, they proposed a model with seven transmembrane domains (two additional segments, M6 and M7). M6 and M7 are amphipatic polypeptide chains, M6 in particular nonhelical (β-sheets), and were thus considered possible domains for lining the walls of the channel. Young et al.[43] developed polyclonal antibodies against a synthetic hexadecapeptide corresponding to residues 501 to 576 at the COOH-terminus of the δ subunit and against the 350 to 358 region of the β subunit. The C-termini of these two subunits were also shown to be exposed to the cytoplasmic side of the membrane. LaRochelle et al.[44] have postulated on similar grounds that the δ 360 to 370 stretch is located on the cytoplasmic face. In conjunction all these pieces of evidence support the view that the COOH-terminus of all AChR subunits faces the cytoplasmic compartment, thus favoring the odd number of crossings type of model (Figure 1). However, recent evidence[45] challenges this view. These authors tested the accessibility of the δ-δ disulfide bond to hydrophilic reducing agents upon reconstitution of the AChR in lipid vesicles. They concluded that the disulfide bond, thought to occur near the C-terminus on the penultimate residue (Cys-500) of the δ chain, lies in the same aqueous space as the ACh binding site, thus supporting the even number of crossings type of model.

A more drastic revision of the existing models, and in particular of the occurrence of the hydrophobic M4 helix (409 to 426 in the α chain) within the lipid bilayer, followed on from more recent experiments from the Lindstrom group: the intracellular location of the β 429 to 441[46] and α 330 to 408[47] segments dismissed the possibility that M4 was embedded in the bilayer. The latter experimental results also eliminated the MA amphipatic helix of Fairclough et al.[36] (Figure 1) as a likely transmembrane segment and forced its occurrence at the cytoplasmic compartment. This radically different view postulates a rather voluminous mass of AChR protein at the cytoplasmic face of the membrane, at odds with both the 4-, 5-, and 7-helix type of model and with the estimates obtained by electron microscopy/image reconstruction[48,49] and neutron[50] and X-ray[51] diffraction techniques on the size of the receptor mass protruding from the membrane.

It is clear that all models lead to different predictions concerning the mechanisms of chain folding, subunit assembly, and, in particular, as to which portions of the polypeptide chains form the walls of the ion channel. It is also clear that the emphasis in, and discrepancies among, all models lies in the occurrence of the transbilayer segments and that experimental tests have heavily relied upon immunochemistry or protein modification from the aqueous phase. Given the varying degree of antigenicity of different nonbilayer AChR segments, it is likely that techniques other than immunochemistry will have to be applied to further refine current models and to solve this discrepancy. (One such approach is the combined use of site-directed mutagenesis and expression of the altered AChR in a heterologous cellular system, as analyzed in the Section IV.I below.) It must also be borne in mind that the algorithms currently used to predict the occurrence of hydrophobic and hydrophilic stretches in membrane proteins[52,53] consider each individual polypeptide chain by successively scanning "windows" a few amino acids long; the hydro-phylic/phobic nature of the stretch weighs only the contribution of residues along the chain, but nearest-neighbor interactions of the stretch with adjacent peptides are not taken into account in this type of analysis. Perhaps some of the differences among current models are related to this deficiency. Wallace et al.[54] have indicated the limited effectiveness of the

prediction methods as applied to membrane-spanning polypeptide segments. In the next section, further implications of protein-protein interactions in the membrane core are discussed in relation to the structure of transmembrane polypeptide chains.

C. MUTUAL INTERACTIONS BETWEEN THE AChR PROTEIN AND MEMBRANE LIPIDS

Regardless of the tentative status and the several still-obscure aspects of the models analyzed above, it can be expected that extensive physical contact occurs between membrane lipid and a significant portion of the AChR protein, and hence that mutual interactions between lipids and the AChR are operative within the bilayer. However, little is known about the nature of such interactions and about their likely consequences on receptor structure and function. As a first approximation, we can consider the impact of the lipid bilayer on AChR structure by recalling the influence of solvent polarity on protein conformation. The five polypeptide chains of the receptor protein are simultaneously exposed to both aqueous (the extracellular and cytoplasmic compartments) and nonpolar (the lipid) media, which certainly favor different secondary structures of the protein domains involved. Resonance Raman spectroscopy of *Torpedo marmorata* AChR in reconstituted lipid systems indicates that 25% of the protein is in α-helical configuration (with 14% disordered α-helical ends) and 34% in β-sheets.[55] In detergent solution, circular dichroism studies of *T. nobiliana* AChR showed 34% α-helix, 29% β-structure, and 37% random coil.[56] Using the same technique, Mielke et al.[57] found 20% α-helix, 50% β-sheets, and 30% random coil in *T. californica* AChR. More recently, Fong and McNamee[58] have analyzed the secondary structure of *T. californica* AChR in reconstituted vesicles by Fourier transform infrared spectroscopy. They found that steroids increased the helical structure of the AChR molecule, whereas negatively charged lipids augment from about 20 to 24% the proportion of β-sheet structure.

At the level of individual AChR subunits, 18% of the ligand binding sites present in the purified α-subunit recover affinities one order of magnitude lower than those of the native receptor in the presence of 1% asolectin, suggesting that a significant refolding of the chain occurs in the presence of lipids.[59] Guy[60] carried out secondary structure calculations of AChR subunits on the basis of limited amino-terminal sequence data[6] available at that time. These calculations suggested a considerable proportion of ordered structure in the amino-termini of all subunits, which are now generally assumed to be exposed to the extracellular face of the postsynaptic membrane. On the other hand, we still ignore (see discussion in the preceding section) the actual mass and hence the surface areas of AChR domains in contact with each of the above media. Both the spectroscopic and the theoretical data on this topic should thus be considered tentative values averaged over the entire AChR molecule.

At this point an additional concept needs to be brought into the discussion, that is, the existence of nonnegligible areas of the AChR protein not exposed to either lipid or aqueous media. Such surfaces constitute an additional type of interface resulting from juxtaposition of segments within the core of the receptor protein itself and between masses of transmembrane segments of adjacent AChR monomers. The amino acid sequence of such transmembrane portions is highly conserved.[8] Finally, given the channel-forming property of other transmembrane AChR portions — those involved in the actual lining of the water-filled pore — a fourth type of interface (AChR/channel) needs to be considered, since the properties of the channel contents may not necessarily be identical to those of the extracellular milieu.

Within the scope of this review, it is those portions of the AChR protein in direct contact with the lipid bilayer that will receive closer attention. The study of the influence of the lipid environment on AChR structural and functional properties can be tackled at various levels of complexity and with different aims in mind. However, any initial description should minimally consider lipid compositional and metabolic studies in order to understand the overall characteristics of the lipid milieu and the time domains of its turnover, as well as biophysical studies in

TABLE 1
Phospholipid Composition of Acetylcholine Receptor-Rich Membranes of *Torpedinidae* Electric Tissue[a,b]

		Torpedo marmorata[c]			*Discopyge tschudii*[h]	*Torpedo californica*		*Torpedo oscellata*[h]
	—[b]		—[d]	—[e]		—[f]	—[g]	
PC	41.3 ± 0.2	38.8 ± 0.3	46	40.7	44.2	40.6	38.4	27.9—42.3
EGP[i]	33.2 ± 0.5	38.6± 2.1	31	34.8	32.1	29.1	42.2	33.1—44.0
PE	17.3 ± 0.3							
Plas-E	15.9 ± 0.2							
PS	13.6 ± 1.8		14		12.5	10.7	10.9	10.1—15.1
PI	3.8 ± 0.3	16.9 ± 1.1	2 ± 1	6.9	1.9	1.7	N.F.	1.21—1.88
PIP	0.07 ± 0.05	—	—	—	0.14	—	N.F.	—
PIP_2	0.12 ± 0.03	—	—	—	0.04	—	N.F.	—
SPH	5.5 ± 1.2	1.1	7	8.0	8.0	9.3	2.3	1.41—1.93
PA	0.6 ± 0.2	—	—		0.4	0.9	2.9	—
CL	1.0 ± 0.2	—	—	9.5	0.7	0.9	3.3	1.03—1.93
Phospholipid/ cholesterol ratio (molar)	1.73 ± 0.15	1.1—1.18	3.5	0.91 ± 0.2	1.30	—	1.1 ± 0.27	—
Lipid/protein ratio	0.69 ± 0.08	0.45—0.67	0.43	0.48	0.62	—	0.43 ± 0.12	—

[a] Expressed as percentage of total lipid phosphorus. N.F., expressely stated as not being found.
[b] Data from Reference 66. The specific activity of the membrane fractions, reflecting their enrichment in AChR protein, ranged between 850 and 3900 pmol α-bungarotoxin per milligram of membrane protein (*T. marmorata*, n = 9) and 1000 pmol/mg protein for *D. tschudii* (n = 3).
[c] Data from Reference 63. PS and PI were summed up. Specific activity of the membranes ranged between 3000 and 4500 pmol/mg protein.
[d] Data from Reference 64. PS and PI were summed up. The same applies to PA + CL. Specific activities: 2000 to 4000 pmol/mg.
[e] Data from Reference 238.
[f] Data from Reference 62.
[g] Data from Reference 65. Specific activity was 1500 pmol/mg protein.
[h] Data from Reference 61.
[i] Ethanolamine glycerophospholipids. They were resolved into phosphatidylethanolamine (PE) and plasmenylethanolamine (Plas-E) fractions, as indicated above.

order to define the local microenvironment of the AChR. To further define the essential lipid requirements for its function, it is also necessary to resort to reconstituted model systems in which the contribution of individual lipid classes on AChR (1) ligand binding and (2) channel gating function can be assessed. These topics are covered in the following sections.

III. STRUCTURE AND METABOLISM OF LIPIDS IN AChR MEMBRANES

A. LIPID COMPOSITION OF THE TORPEDINIDAE ELECTROCYTE AND AChR MEMBRANES

Partial phospholipid[61-65] (Table 1) and fatty acid[63,65] compositions of AChR membranes have been reported. A comparative study of the lipid composition of the electrocyte and AChR membranes from three *Torpedinidae* sp. (*T. marmorata*, *T. californica*, and *Discopyge tschudii*) has also been produced.[66] The phospholipid composition of the electric organ and AChR membranes thereof is similar for a given fish species. Variations among species are small. On average, the phospholipids of choline, ethanolamine, and serine represent in all cases 80 to 90%

TABLE 2
Fatty Acids of Glycerophospholipids in AChR-Membranes from *T. marmorata*[a]

	Major phospholipids				Minor phospholipids			
	PC	EP	PE	PS		PI	PA	Sph
14:0	1.6	0.6	0.1	0.1	14:0	0.2	0.6	4.2
16:0	57.5	10.2 } 18.9	15.2	7.6	15:0	0.2	1.0	1.2
16:0 (+16:0 DMA)	8.7	8.7	3.5	4.2	16:0	7.5	16.4	17.0
17:0	0.6	—	1.5	1.2	16:1	1.2	1.0	N.D.
18:0	3.3	6.3	24.5 } 37.2	37.3	17:0	1.3	—	1.1
18:1	15.0	11.2 } 37.9	12.7	13.6	17:0	0.2	—	—
18:1 DMA	—	20.4	—	—	18:0	30.4	23.5	4.7
18:2	0.8	0.7	0.8	1.3	18:1	15.9	15.2	—
20:1	0.8	0.6	0.9	0.7	18:2	0.9	0.9	—
					19:0	—	—	0.5
20:4 n-6	1.5	4.6	6.4	3.4	20:0	—	—	2.0
20:5 n-3	0.8	1.3	0.9	1.0	20:1	1.1	0.8	0.5
22:4 n-6	0.3	0.6	0.8	1.1	20:3 n-9, n-6	0.2	0.6	—
22:5 n-6	0.3	0.5	0.5	1.4	20:4 n-6	24.7	10.4	—
22:5 n-3	0.6 } 9.5	2.9 } 34.1	1.9 } 34.5	3.6 } 28.2	20:5 n-3	1.1	1.5	—
22:6 n-3	8.9	31.2	32.6	24.6	21:0	—	—	0.7
Aldehydes	—	23.5			22:0	—	—	3.8
					22:4 n-6	0.4	—	—
					22:5 n-6	0.4	0.7	—
					22:5 n-3	1.5	1.6	—
					22:6 n-3	12.8	19.1	—
					23:0			2.2
					24:0 + 25:0 + 26:0			4.7

[a] Data are expressed in mol% and represent mean values of at least three samples; the percentages are normalized to 100%. Abbreviations used are PC, phosphatidylcholine; EP, ethanolamine phosphoglycerides; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid; Sph, sphingomyelin; and DMA, dimethylacetals. Data modified from Rotstein, N. P., Arias, H. R., Aveldaño, M. I., and Barrantes, F. J., *J. Neurochem.*, 49, 1341, 1987.

of the total lipid phosphorus. Most of the "choline phospholipids" are made up of PC, the rest being accounted for by sphingomyelin (Sph). Ethanolamine glycerophospholipids (EGP) are unusual in that they are made up of high proportions of plasmalogenic subclasses (50, 30, and 39% plasmenylethanolamines, the rest being accounted for by phosphatidylethanolamine, PE) in *T. marmorata, T. californica,* and *D. tschudii,* respectively. The PE/EGP ratio is relatively constant when comparing whole electric organ and AChR membranes obtained therefrom. There are smaller amounts of polyphosphoinositides in AChR membranes than in whole electric organ[66] (Table 1), but such differences can be explained by the lability of the inositol lipids and the relatively lengthy purification procedures involved. Species-related differences are to be found in the higher PC/EGP and PC/Sph ratios in *T. marmorata* as compared with the two other species.[66]

Monoenoic fatty acids, saturates, and long-chain (20 and 22 carbon) polyenes of the n-3 series are the major acyl chains of glycerophospholipids and neutral lipids in the electrocyte and AChR membranes thereof (Tables 2 and 3).

Fatty acids of the n-3 series predominate by a factor of three to five over those of the n-6 series in the three principal electrocyte phospholipids (Table 3). In PC, EGP, and phosphatidylserine (PS), respectively, n-3 fatty acids amounted to 81, 80, and 75% for *D. tschudii;* 77, 76, and 75% for *T. californica;* and 80, 85, and 77% for *T. marmorata.* Docosahexaenoate (22:6 n-3) is the most abundant polyene in all major phospholipids in all *Torpedinidae* (Table 3). Qualitative

TABLE 3
Ratios Between Polyenoic Fatty Acids in Major Phospholipids from Electric Organ and AChR-Membranes (mol%)[a]

	D. tschudii			*T. marmorata*			*T. californica*		
	PC	EGP	PS	PC	EGP	PS	PC	EGP	PS
Electrocyte									
20:4 n-6	11.4	8.6	9.8	12.7	13.1	12.6	19.0	20:3	19.0
22:4 n-6	6.5	9.3	12.2	3.6	1.6	6.0	1.8	2.5	3.0
22:5 n-6	1.6	1.9	2.6	3.0	0.8	4.3	2.4	1.2	3.0
20:5 n-3	4.1	1.9	2.4	6.6	2.2	3.9	20.2	8.4	7.3
22:5 n-3	22.8	34.4	33.2	4.2	3.0	7.6	4.2	8.7	9.3
22:6 n-3	53.7	43.9	39.7	69.9	79.3	65.6	52.4	58.9	58.3
n-3/n-6 ratio	4.1	4.1	3.1	4.2	5.5	3.4	3.3	3.2	3.0
AChR membranes									
20:4 n-6	8.4	9.9	6.3	11.6	12.1	9.6	14.7	20.3	15.9
22:4 n-6	9.9	9.1	16.3	2.5	1.0	3.2	6.0	1.7	7.5
22:5 n-6	2.7	2.5	3.4	2.5	0.8	4.1	—	1.0	2.0
20:5 n-3	4.8	3.1	3.5	6.6	3.3	2.9	18.7	7.3	6.9
22:5 n-3	22.0	34.4	32.4	5.0	4.5	10.1	6.0	9.8	12.3
22:6 n-3	52.2	41.0	38.1	71.9	78.4	70.1	54.6	59.9	55.4
n-3/n-6 ratio	3.8	3.7	2.8	5.0	6.2	4.9	3.8	3.3	2.9

[a] Data modified from Rotstein, N. P., Arias, H. R., Aveldano, M. I., and Barrantes, F. J., *J. Neurochem.*, 49, 1341, 1987.

differences can be found in the fatty acid composition of all phospholipid classes, which varies among fish species. Eicosapentaenoate (20:5 n-3) and docosatetraenoate (22:4 n-6) are more abundant in *D. tschudii* lipids, and 20-carbon polyenes such as arachidonate (20:4 n-6) and eicosapentaenoate (20:5 n-3) in *T. californica* lipids, than in the two other species. *T. marmorata* phospholipids have characteristically the highest levels of docosahexaenoate (22:6 n-3). The lower C20:4/22:6 n-6 and C20:5/22:5 n-3 ratios in *D. tschudii* suggest that the enzymes involved in the elongation of polyenes are very active in this species. Conversely, the C22:5 n-3/22:6 n-3 ratio and the C22:4 n-6/22:5 n-6 ratio are higher in *Discopyge*, indicating differences in the activity of the Δ4-desaturases among the three species.

Taking individual phospholipid classes, their fatty acid composition reveals some characteristic features. For instance, the content of polyenoic fatty acids other than arachidonate in phosphatidylinositol of AChR membranes is higher than usual. Diphosphatidylglycerol and triacylglycerols in whole electric organ are rich in oleate, but also contain 20 to 22 carbon polyenes. Sph shows a peculiar fatty acid composition: it displays a high proportion of monoenoic, saturated (especially 14-26 carbon) fatty acids, a large content of 22:1 and very little 18:0 in comparison with mammalian nervous tissue sphingomyelins (Table 2).

Taking all phospholipid classes together, however, one finds compositional trends that merit consideration. For instance, about half the fatty acids in AChR membranes (48 to 58% in the three *Torpedinidae* sp.) are long-chain polyunsaturated fatty acids. Another major feature of the fatty acid composition becomes apparent when examining the composition of two types of phospholipids separately: PC on the one hand and EGP and PS on the other. In contrast with all other phospholipids, PC, the major phospholipid class in AChR-rich membranes (Table 1) is made up of 72% monoenoic and diunsaturated fatty acid species and only 27% of polyunsaturated fatty acids (Tables 2 and 3). In EGP and PS one finds that polyenoic species represent 77

TABLE 4
Molecular Properties of the "Average" Fatty Acid in Major Lipids of AChR Membranes from *Torpedinidae*

Species	Phospholipid (mol%)	mol wt	Unsaturation	Chain length	S/U
Torpedo marmorata	Phosphatidylcholines (41.3 ± 0.2)	269	0.9	17.1	1.7
	Ethanolamine phosphoglycerides (33.2 ± 0.5)	296	3.1	19.4	0.3
	Phosphatidylserine (13.6 ± 1.8)	294	2.1	19.0	0.8
Torpedo californica	Phosphatidylcholines (38.4)	273	1.2	17.4	1.1
	Ethanolamine phosphoglycerides (42.2)	304	3.4	19.9	0.2
	Phosphatidylserine (10.9)	297	2.7	19.9	0.4
Discopyge tschudii	Phosphatidylcholines (44.2)	272	1.1	17.3	1.2
	Ethanolamine phosphoglycerides (32.1)	297	3.2	19.4	0.3
	Phosphatidylserine (12.5)	293	2.4	19.0	0.6

Note: The average values of molecular weight (mol wt), number of double bonds (unsaturation), chain length, and total saturates/total unsaturates ratio (S/U) were calculated from the fatty acid composition of each lipid (aldehydes were excluded in the case of EGP). The data for *T. californica* AChR membranes were calculated from those of Gonzalez-Ros, J. M., Llanillo, M., Paraschos, A., and Martinez-Carrion, M., *Biochemistry*, 21, 3467, 1982. Other data are from Rotstein, N. P., Arias, H. R., Aveldaño, M. I., and Barrantes, F. J., *J. Neurochem.*, 49, 1341, 1987.

and 57%, respectively. The plasmenylethanolamines contain less polyenoic fatty acids than the PEs.[66] Breckenridge and Vincedon[67] had already noted the occurrence of docosahexaenoic acid in the EGP class in *T. marmorata* total electric tissue.

Summarizing the lipid compositional aspects of AChR-rich membranes from *Torpedinidae* fish:

1. Phospholipid class composition is quite similar among the three species.
2. Each of the three major phospholipids in these membranes exhibits characteristic average molecular weight, number of double bonds, and length of its fatty acid acyl chains (Table 4).
3. Lipid compositional differences are not apparent when comparing whole electric tissue and AChR membranes of diverse degrees of purity in terms of receptor content. An important conclusion to be drawn from this point is that the constancy represents a manifestation of the high degree of specialization of the electric organ in subserving the cholinergic system. This is seldom found in other tissues.

Taking all these points together, it is apparent that regardless of the differences in fish habitat (northern and southern Pacific and Atlantic Oceans), there appear to be remarkably conserved features in the lipid compositional patterns, especially those more likely to have an influence on the physical state of the membrane (the opposing trends of PC and EGP + PS; Table 4), suggesting that in analogy with the evolutionary conservation of the AChR primary structure, its lipid microenvironment needs to be kept similarly constant to ensure optimal receptor function.

B. TOPOGRAPHY AND VECTORIAL SIDEDNESS OF PHOSPHOLIPID MOLECULES IN AChR MEMBRANES

A further step in the characterization of the AChR membrane environment involves establishing the location of the lipid components relative to the receptor protein on the one hand and to the two compartments facing each leaflet of the bilayer on the other. In order to study the

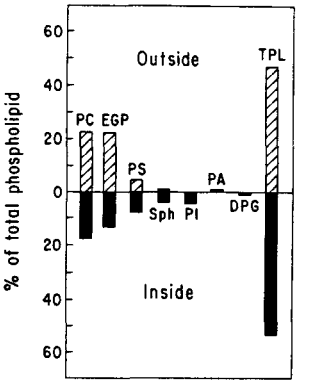


FIGURE 2. Asymmetry of phospholipid molecules in AChR membranes from *T. marmorata*. Phospholipids occurring at the inner and outer leaflets of the bilayer are depicted. See text for further details.[68]

vectorial orientation of phospholipids in AChR membranes from *T. marmorata*, the membrane nonpermeable reagent trinitrobenzene sulfonate was allowed to react with sealed native vesicles. Within 1 h, 90% of the EGP and 40% of the PS had reacted with the probe. Under identical experimental conditions, only 0.5 and 8% of the EGP and PS react in erythrocyte membranes, and 50 and 43%, respectively, in disk photoreceptor membranes.[68] In a second series of experiments the accessibility of phospholipids to phospholipase C digestion was tested in the same membrane preparation. About half the total lipid phosphorus from membrane phospholipids was released within 3 min at 25°C. From these results, the conclusions were reached that (1) about half the total phospholipid faces each side of the membrane; (2) phosphatidylinositol, most of the sphingomyelin (~85%), and 65% of the PS are located on the inner, cytoplasmic-facing leaflet of the membrane; and (3) nearly half the phosphatidate, 57% of the major phospholipid, PC, and 63% of the EGPs face the outer half of the membrane (Figure 2). The phospholipids in the AChR membrane are thus asymmetrically distributed between the two leaflets of the bilayer.

The description of the topology of lipids in AChR-rich membranes involves knowledge of the vectorial sidedness of phospholipids and neutral lipids on the one hand and of their structural relationship with the AChR protein on the other hand. Among the recent attempts to define the lipid "annulus" in the immediate vicinity of the AChR protein, the studies of Giraudat et al.[16] have provided evidence of the contact of PCs and the AChR. Using arylazido PCs carrying their reactive group either at the tip of the aliphatic chain or at the polar head, they demonstrated the contact of all AChR chains with the bilayer. Serial extractions of frozen AChR membranes with solvents of increasing polarity have also been used recently to tackle this question. Hexane, a nondenaturing organic solvent, extracted roughly equal amounts of the two major phospholipid components, i.e., PC and EGP, leaving the AChR protein in the membrane residue. Subsequent extractions with chloroform:methanol mixtures of increasing polarity released an amount of lipid whose composition (percent) had less PE than the lipid removed with hexane, which was richer in PC. The relatively higher concentration of PC than EGP in the AChR-containing phase under those experimental conditions[69] was interpreted to suggest a preferential contact between PC and the AChR (see Barrantes[70]).

C. LIPID METABOLISM IN CHOLINERGIC MEMBRANES

In addition to the structural information obtained on the lipid constituents of the AChR membrane, the study of their metabolism should throw light on the turnover of the membrane framework. Studies on lipid metabolism in electric tissue *in vivo* are rather scarce. Two early

reports on the subject have dealt with the metabolic fate of intraperitoneally injected ^{32}P-phosphate[71] and ^{32}P-glucose-6-phosphate after direct injection into the electric organ.[72] *In vitro*, the incorporation of ^{32}P-phosphate and ^{14}C-glucose has been measured in slices of *Electrophorus electricus* Sach's organ.[73] The incorporation and distribution of ^{3}H- and ^{14}C-labeled glycerol and two fatty acids, ^{3}H-oleate (18:1) and ^{3}H-arachidonate (20:4), into lipids of *D. tschudii* electrocytes has been recently studied after direct injection into the electric organ and incubation of electrocyte stacks with the precursors.[74] *In vivo*, most of the label from glycerol is incorporated early on by phosphatidate and diacylglycerols and later by membrane lipids and triacylglycerols. The glycerol double-labeling experiments have also provided evidence on the synthesis of phosphatidylinositol (PI) via the route phosphatidic acid → CDP-diacylglycerol → PI. Virtually all of the radioactive 20:4 and 18:1 present in the tissue is esterified within 4 and 16 h, respectively. After exhaustion of the free fatty acids, the ratio of labeled phospholipids to labeled neutral lipids is 80/20 for 20:4 and 30/70 for 18:1.

The incorporation of fatty acids in electrocyte lipids *in vitro* is highly efficient and sustained, especially when compared with the incorporation of ^{14}C-glycerol. When the proportion of polyphosphoinositides among electrocyte lipids is considered, the turnover of arachidonate and oleate is highest in these phospholipids. This and other metabolic studies using ^{3}H-18:1, ^{3}H-20:4, and ^{32}P-phosphate[75,76] indicate that the electrocyte is a cell capable of actively synthesizing its lipids *de novo* and that these lipids undergo a rapid turnover of their polar and hydrophobic moieties. Enzymes catalyzing deacylation-reacylation reactions are important among the enzymes controlling the turnover of electrocyte lipids. These enzymes carry out replacement, exchange, and rearrangement of membrane lipid acyl chains, processes that have been proposed to be involved in the control of membrane properties (e.g. fluidity) that depend on the quality of fatty acyl groups,[77] and could play a key role in the modulation of the physical properties of electrocyte membranes, which depend strongly on the length and unsaturation of lipid acyl moieties. In particular, acylCoA-lysophospholipid acyl transferases appear to be very active in the electrocyte.[74,75] The *in situ* "retailoring" of lipid molecular species mediated by these processes should therefore allow for rapid adjustment of the membrane to abrupt changes in environmental conditions, whereas *long-term* adaptations are likely to be controlled by modifications of the *de novo* lipid synthetic processes.[78] The former, more agile mechanism appears particularly suited to a membrane like the one containing the AChR, since the physiologically relevant activities occur within a short time scale. Thus, the rapid exchange and redistribution of acyl chains could give rise (locally) to new molecular species without gross changes in the overall fatty acid composition of the membrane. The fact that polyphosphoinositides exhibit a high turnover[74-76] and that this minor class of lipids is known to generate second messengers is worth further analysis.

Finally, other chemical reactions involving lipid components may play a role in the structure or function of the AChR protein. Covalent modification of the receptor protein by attachment of palmitate has been reported to occur on the α and β-subunits of the AChR in BC3H-1 clonal cells.[79] It has been proposed that this posttranslational modification plays a role in the assembly and/or membrane insertion of the AChR.

IV. THE STUDY OF AChR FUNCTION IN RECONSTITUTED LIPID SYSTEMS

Some of the inherent limitations in the use of the native membrane vesicles for studying AChR functions such as ion translocation, influence of the lipid environment, etc. are eliminated when working with reconstituted systems. As a trade-off, some difficulties still remain, and new ones are encountered. On the whole, however, the specific field of AChR reconstitution has abandoned the state of semiempiricism that characterized it until a few years ago and has become an established analytical tool in AChR research. It is now possible to study the behavior of the

AChR-controlled ion channel in its minimal molecular form, to assess the contribution of each subunit — either normal or mutationally altered — to the gating activity, and to insert the AChR into supramolecular assemblies of more strictly controlled chemical and physical properties than the native environment. Basically, the AChR can be solubilized from its native membrane in different oligomeric states (see below), separated from other membrane proteins, purified to homogeneity, recombined with mixtures of lipids of known composition, and physically inserted into a synthetic membrane with preservation of activity. This insertion is done in monolayers, planar, or vesicular (liposome) bilayer systems exposed to ionic media that can be easily adjusted. In this manner, gating behavior can be studied independently of the multiple (and often uncontrollable) influences of the cytoplasmic and extracellular environments. The artificial media bathing the two faces of a reconstituted bilayer, and the bilayer itself, can also be manipulated in terms of temperature, imposed voltage, host lipid composition, lipid:protein ratio, presence of chemicals at defined concentrations, ionic gradients, size of the assembly, etc. Such degree of control is certainly not affordable with the native cell. To date, nicotinic AChRs (two peripheral and one central nervous system) from three different sources (*T. marmorata*, *T. californica*, and *Locust migratoria*) have been reconstituted into planar bilayers with apparent preservation of functionality (see below).

In addition to the now classic reconstitution approach using lipid bilayers, AChR and other membrane proteins can be reinserted into lipid monolayers and heterologous cellular systems, as reviewed below. Successful attempts to "reconstitute" AChR in cells devoid of this protein have been reported. Ho and Huang[80] used Sendai virus envelope proteins as fusogen to transfer *Torpedo* AChR into mouse L-929 cells. So far, the technique has not found further application. Probably the most successful heterologous cellular system for testing AChR function has been the amphibian oocyte. The importance of the recent advances in the field emerging from its use are discussed in Section IV.I below.

A. EARLY WORK ON AChR RECONSTITUTED INTO LIPID ASSEMBLIES OF VESICULAR FORM (LIPOSOMES)

Earlier attempts to reincorporate detergent-solubilized protein material into artificial lipid membranes made use of crude or partially purified membrane preparations originating from various biological sources rich in AChR.[81-85] Briley and Changeux[86] have analyzed such early work *in extenso*, and the reader is referred to this review for a critical appraisal. Basically, one of the pitfalls of the earlier attempts lies in the lack of characterization of the AChR preparation itself. Another source of difficulty was the lack of control of the lipid material used or of the conditions of the solubilization process.

In the first attempts to reconstitute the AChR system *in vitro*, agonist-induced permeability changes could be restored when sodium cholate-solubilized AChR membranes were freed of detergent and supplemented with the total lipid extract from *T. marmorata* membranes.[87] Similarly, Schiebler and Hucho[64] used delipidated cholate extracts of *T. californica* membranes, and recombined them with phospholipids, restoring Na^+ efflux. One problem with these attempts was their low reproducibility; eventually, use of purified AChR constituted a step toward understanding the reasons for this lack of reproducibility. Michaelson and Raftery[62] solubilized AChR from *T. californica* in Triton® X-100. Upon reconstitution with phospholipids, such preparations needed a very high agonist concentration to elicit a moderate Na^+ flux, and occasionally they were even found to be unresponsive to agonists. Up to this stage, the attempts to reconstitute the agonist-mediated ion translocation, and in some cases even the mere physical reincorporation of the receptor protein into a lipid assembly, were difficult to control. The credit for establishing a reproducible method for reconstitution of the AChR in liposome systems is to be given to a series of communications from the Racker laboratory that made apparent the importance of lipids in the preservation of AChR ionic translocation. Epstein and Racker[88] included soybean phospholipids in the detergent used for extracting *T. californica* membranes

and subsequently in the reconstitution of purified AChR.[89] Several other laboratories adopted the essential aspects of the Racker approach and contributed to the characterization of the AChR in reconstituted systems;[90-93] since then the use of slow, integrated flux measurements has become an established reproducible technique. Lindstrom et al.[93] have also contributed to the optimization of the reconstitution procedure, emphasizing throughout the need to control the protein composition of the AChR. Criticisms were raised against the use of proteolyzed AChR, specifically as reported by Huganir et al.,[89] whose preparation lacked the α-subunit, and Changeux et al.,[91] in which case the preparation apparently consisted only of α-subunits. Historically, this word of caution is coincident with the prolonged debate on the subunit composition of the AChR protein (see, for example, Conti-Tronconi and Reftery[94]).

B. WHAT IS THE NATURE OF THE PRESERVATION OF AChR FUNCTION AFFORDED BY LIPIDS?

The general concept that AChR function is dependent on the lipid environment has gained acceptance in recent years.[3,95-98] Although no absolute requirement for a unique type of lipid has as yet been identified, as seems to be the case with some membrane proteins and receptors (see, for example, Hasegawa et al.[99]), distinct conditions have to be met to preserve ligand binding and ion channel gating functions of the AChR (see, for example, Fong and McNamee[58,102] and Criado et al.[100,101]). Reconstitution studies aimed at analyzing the nature of the protein-lipid interactions occurring in the membrane, the effect of detergents on AChR structure and function, and more specifically addressed at dissecting the contribution of individual lipid classes to AChR properties, should all contribute toward solving this question.

The early experiments of Epstein and Racker[88] employed a crude mixture of soybean phospholipids ("asolectin"). This is a lipid mixture containing most common phospholipids, variable amounts of lysophospholipids (5 to 20%) and steroids, its exact chemical composition varying with source and batch. The asolectin was supplemented with sodium cholate in a 1:10 (w/w) ratio. Extraction of the neutral lipids, or replacement of asolectin by synthetic phospholipids resulted in the deterioration of the bulk ion flux properties explored. Addition of α-tocopherol, vitamin K, or the coenzyme Q_{10} partly restored the flux values to those obtained with asolectin.[103] The latter authors suggested that these protective effects were mediated by modification of the phospholipid acyl chain packing and not by the antioxidative action of the steroids. Asolectin contains, on average, longer fatty acid chains with a higher degree of unsaturation than those of egg PC or *Torpedo* membrane lipids.[63] The general notion that emerged from this and other reconstitution studies was that lipids exerted a "stabilizing" action on AChR-mediated ion fluxes[88-91] (see also Lindstrom et al.,[104] Anholt et al.,[105] Popot et al.[106]).

Extraction of proteins from *Torpedo* AChR membranes by sodium cholate is maximal at about 2% detergent (half-maximum at 0.8%),[105] attaining about 70% of the original AChR content in the extract. In a more recent study, Jones et al.[107] explored this topic in great detail and found that solubilization depends strongly on the lipid concentration of the membrane; half-maximal solubilization with sodium cholate was attained at 0.6, 0.8, and 7.5% for AChR in dioleoyl-PC, alkaline-extracted native membranes from *T. californica*, and asolectin, respectively. Raising the cholate concentration to 3% already produces irreversible denaturation of the $^{22}Na^+$ flux, even when the lipid:detergent ratio is maintained.[105] Detergent inactivation was found to be dependent on the type of membrane preparation.[107] In this latter study it was found that not all the "endogenous" lipid could be displaced, even at high detergent:lipid ratios; a minimal number of lipid molecules (about 20) always remained associated with the AChR.

The chemical nature of the detergent has been explored in a few cases; replacement of cholate by β-octylglucoside has been reported to result in AChR denaturation.[105] Gonzalez-Ros et al.,[108] however, reported successful reconstitution of *T. californica* AChR into electroplax total lipid vesicles using this detergent, which apparently enhances the proportion of the 13-S dimeric AChR species. In the case of the neuronal AChR from *Locust migratoria*, solubilization was

accomplished using sodium deoxycholate.[109] In the reconstitution studies of Heidmann et al.,[110] special attention was paid to the preservation of agonist-induced state transitions in the absence and presence of lipids.

Studies with various phospholipases of the A_2 type have pointed out the importance of membrane lipid integrity for the preservation of AChR function and to the possible deleterious effects of lipid degradation products. Thus, whereas *C. adamanteous* phospholipase A_2 was relatively ineffective in blocking $^{22}Na^+$ fluxes, the enzyme from *Naja naja siamensis* exerted strong inhibitory action and converted the AChR into the desensitized state.[10,111,112] Hanley[9] had also shown that various phospholipases A_2, including that from crotoxin, inhibited ion transport in lipid vesicles containing *Torpedo* AChR, and that the hydrolysis products (free fatty acids) were also inhibitors. This can be correlated with the earlier work of Brisson et al.,[113] who showed that fatty acids and detergents could exert noncompetitive inhibitory effects on AChR properties. Bon and Changeux[114] had suggested that a toxin from *Bungarus caeruleus* was a channel blocker, but phospholipase A_2 activity was subsequently discovered in the toxin fraction.[115] The lysophospholipids appear to be ineffective in promoting inhibition of AChR-mediated ion fluxes,[9,10] but produce leakiness of the membranes in a detergent-like fashion.[116] More recently, Villar et al.[117] have reported that treatment of AChR-rich membranes with phospholipase A_2 or with free fatty acids, in particular linolenic acid (18:3(n-3)), causes impairment of the other AChR functional property (ligand recognition), as measured by the destabilization of the α-toxin binding ability of the AChR protein. These latter results disagree with earlier reports[112] demonstrating impairment of ion translocation, but not of ligand binding properties of the AChR by fatty acids. In addition to local anesthetics,[113] general anesthetics can block the AChR-mediated permeability response elicited by agonists.[118,119]

In an attempt to study in more detail the influence of lipid chemical structure on AChR function, systematic reconstitution studies were carried out using Torpedo receptor liposomes of pure synthetic lipids.[100,101] With increasing chain length of the saturated PCs, a marked augmentation in carbamoylcholine dissociation constants was observed. The increase affected both the apparent equilibrium dissociation constants K_R and K_{eq}, but, more importantly, the difference between the two apparent dissociation constants (which should be indicative of the AChR capacity to undergo agonist-induced affinity transitions) was maximal for chain lengths of 16 carbon atoms. This is in agreement with the observed composition of the PCs naturally occurring in AChR membranes: palmitic acid constitutes more than 57% of the total (Table 2). Substitution by other dimyristoyl phospholipids for dimyristoyl PC had the same, although quantitatively less pronounced, effects. Introduction of unsaturation in the acyl chains dramatically reversed the effect of increasing chain length. Again, this result concurs with the observed fatty acid composition of the PCs in the native AChR membrane (Tables 2 and 3). Similar experiments have been analyzed in terms of a model considering co-existence of both high- and low-affinity states of the AChR; no effects of lipid composition on agonist affinities were found.[102]

The effect of lipid classes on the channel-gating properties of the AChR has been examined in most cases using radioactive tracer flux measurements. Unsaturated PEs in combination with 28 to 35 mol% of cholesteryl hemisuccinate was the best lipid mixture for reconstitution of the receptor-gating function in artificial lipid systems.[101] Thus, zwitterionic phospholipids such as PE[120] may be important in AChR channel function. An acidic phospholipid, phosphatidate, has also been found to display favorable effects on channel-gating properties.[102] In our hands, when PE was systematically replaced totally or partially by other phospholipids with the same or different acyl chain composition, a marked decrease of ion transport was apparent, even when similar vesicle size, degree of receptor incorporation, and agonist-induced affinity transitions were obtained.[101] One of the conclusions drawn from these experiments is that the maintenance of the affinity state transitions of the reconstituted receptor is a necessary but not sufficient condition for the manifestation of the ion-gating receptor activity. A second conlusion is that the

more unsaturated the acyl chains of PE are, the higher the response that is observed, suggesting that these lipids are important for the ion translocation function of the receptor. Thus, the trends observed in our flux studies with synthetic lipids[101] show concurrence with more recent analyses of the fatty acid molecular species in native membranes[66] showing more than 75% polyenes in PE (of which the majority are hexaenoic molecular species; Tables 2 and 3). There are still no comparable studies in the literature in which the influence of lipids on AChR gating properties has been systematically surveyed at the level of single-channel behavior (see below). In only one special case was a synthetic PC used, but its proportion with respect to residual endogenous lipids was not assessed.[121]

C. THE MONOLAYER AS AN INTERMEDIATE EXPERIMENTAL STAGE IN AChR RECONSTITUTION

AChR-lipid interactions have also been studied using lipid monolayer techniques. The fact that nearly all pure lipids form monolayers (see Gaines[122]), even though not all of them yield stable vesicles (e.g., unsaturated PE and cholesterol), probably constitutes the most powerful asset of monolayer film techniques. Furthermore, relatively small amounts of lipid are required in this type of technique. On the other hand, an important drawback of the monolayer method lies in its inability to provide membrane-spanning proteins with the normal thickness of the lipid bilayer in which these proteins occur. Second, since monolayers are sprayed at the air-water interface, the technique fails to offer the two aqueous phases to which integral membrane proteins are normally exposed.

Monolayer film techniques can be used for the study of lipid-protein interactions in two different ways: (1) as the final assembly in which the properties of channel-forming proteins are analyzed, or (2) as an assembly temporarily serving as host of the protein to be subsequently incorporated in bilayer systems by various fusion techniques (see Hanke[123] for review). In one of the earlier monolayer studies, the penetration of purified AChR from *Torpedo* or *Electrophorus* into films of pure lipids was used as an indicator of the degree of interaction.[63] AChR/monolayer interactions were found to be dominated by hydrophobic interactions; the nature of the polar moiety of the phospholipids had little influence on the rate or extent of AChR penetration. Incorporation into the monolayer was enhanced upon increasing the length of the fatty acyl chains of synthetic PCs.[63] Purified AChR also was found to exhibit a preference for cholesterol films, as compared with monolayers prepared with any phospholipid or with several other types of sterols.[63] Small differences in structure (such as those between cholesterol and ergosterol) have also been reported to influence AChR incorporation, indicating that the observed specificities are not merely the consequence of different bulk physical properties of the films.

D. THE IMPORTANCE OF CHOLESTEROL

As mentioned above, measurement of agonist-stimulated Na^+ flux in vesicles of varying lipid composition suggested the participation of neutral lipids in AChR-mediated ion translocation.[103] Thus, AChR reconstituted with neutral lipid-depleted asolectin or with mixtures of pure phospholipids exhibited diminished fluxes in comparison to those observed with complete asolectin. Inclusion of several neutral lipids (α-tocopherol and some quinones) had the reverse effect, provided PS was present.[103] Dalziel et al.[124] and McNamee et al.[125] subsequently reported an increased receptor-mediated ion influx in PE/PS vesicles when their cholesterol content was raised. These results can be correlated with the preferential affinity for cholesterol exhibited by purified AChR in monolayer[63,126] and bilayer[127] systems, and the importance of this sterol in maintaining the appropriate cohesive pressure for the AChR to function in a bilayer.[126] It has been found that not only is cholesterol necessary for the preservation of the ion flux after reconstitution, but it is also essential for the preservation of the agonist-induced affinity transitions.[100] Zabrecky and Raftery[128] have also reached similar conclusions. Furthermore, we

found different cholesterol requirements for optimal detergent solubilization and subsequent reinsertion of the AChR in a bilayer, respectively. The amount of AChR reinserted in the membrane increased as a function of cholesterol concentration up to a maximum of about 46 mol% cholesteryl hemisuccinate, but decreased at higher concentrations.[100] In other membrane systems, cholesterol has been found to facilitate insertion of integral membrane proteins into preformed lipid bilayers, apparently by lowering the energy barrier for this process.[129] Omission of these considerations can give rise to reconstituted systems with irreversibly altered (>95%) receptor (e.g., Lüdi et al.[130]).

Since the amount of endogenous cholesterol in AChR-rich membranes is relatively high (Table 1), reconstituted AChR may still exhibit conductance responses in the absence of exogenous steroid[131] without necessarily preserving optimal functional properties. The presence of residual endogenous cholesterol in the reconstituted system may in fact hamper the proper assessment of the influence of this lipid on AChR function and account for the apparent discrepancies in the nature and magnitude of the cholesterol effects. Man-tailored lowering of the endogenous cholesterol levels in native *Torpedo* membranes increases agonist affinities for channel activation.[128] Kilian et al.,[103] Dalziel et al.,[124] Criado et al.,[100,101] and Fong and McNamee[102] have also reported on the positive influence of cholesterol on AChR-mediated ion flux in reconstituted AChR, but the effects on ligand binding or agonist-mediated state transitions are still open to controversy.

Optimal conditions for preservation of both ligand binding and permeability responses upon acidic phospholipid and cholesterol concentrations have been reported.[101,102] Concentrations of 30 to 40% cholesteryl hemisuccinate were found to be necessary in the reconstituted system (liposomes made of this cholesteryl derivative and dimyristoyl PC) to mimic the kinetics of agonist-induced state transitions observed in native membranes. Modification of the cholesterol content in *Xenopus* muscle cells results in alteration of AChR channel kinetics but not of channel conductance in the presence of the general anaesthetic halothane.[132]

In spite of the biochemical evidence accumulated over the past years on the importance of steroids in AChR function, there are still discrepancies on the topographical location and even the occurrence of these neutral lipids in the postsynaptic membrane. Cholesterol is characteristically a plasma membrane lipid, the molar ratio to other lipids being 0.7 to 0.8, while in intracellular membranes this ratio may be as low as 0.1 to 0.2.[133] Free cholesterol makes up more than 99% (mol%) of the "total cholesterol" pool in whole electric organ, triacylglycerols and cholesteryl esters representing only 0.7 to 1% (Rotstein et al.[66]). The latter two neutral lipids are practically absent from AChR-rich membranes. The spontaneous exchange of cholesterol between different membrane compartments is reported to be rather slow ($t_{1/2} > 2$ h[134]). In the case of *T. californica* AChR membranes, the existence of two pools of cholesterol has been recently reported.[135] The cholesterol pool available for exchange, about 50% per mol, is depleted within 3 h.

The distribution of cholesterol in the frog cutaneous pectoris and sartorius neuromuscular junction has been studied by Nakajima and Bridgman[136] using the antibiotic filipin, which forms large intramembranous filipin-sterol aggregates.[137] These can be readily detected by electron microscopy (e.g., Orci et al.[138]). The aggregates could be observed in the extrasynaptic regions of the sarcolemma, but were absent in receptor-rich areas, leading the authors to conclude that the AChR environment was devoid of cholesterol. A similar inference has been drawn from observations of *Torpedo* electrocytes after filipin treatment.[139] This conclusion is not necessarily valid in the special case of the postsynaptic membrane, in which filipin-induced deformation of the bilayer may be severely hampered by the dense packing of the AChR protein. In contrast to the latter studies, the presence of cholesterol in *Torpedo* receptor-rich membranes has been indirectly demonstrated by St. John et al.[140] using saponin, a reagent known to interact with cholesterol-rich membrane regions. Upon saponin treatment, reagent molecules as large as ferritin could penetrate the otherwise sealed membrane vesicles. AChR-rich membrane areas

have also been reported to react extensively with filipin in rat but not in chick myotubes.[141] The contradicting reports summarized above point to the difficulties involved in this type of study. The lack of more specific probes contributes to the problem.

Additional pieces of evidence point to the occurrence of cholesterol in the AChR-rich areas of the postsynaptic membrane, and probably in the immediate vicinity of the AChR protein: (1) about 50% of a cholesterol analog, an androstane spin label, is preferentially immobilized in the lipid annular region as judged by electron spin resonance techniques,[142,143] (2) cholesterol diazoacetate, a photoaffinity cholesterol analogue, labels all four subunits of the AChR in the native membrane,[144] and (3) one of the two pools of cholesterol detected in native AChR membranes[135] appears to be tightly associated with the receptor protein.

E. WHAT IS THE MINIMAL AChR STRUCTURE RETAINING GATING PROPERTIES UPON RECONSTITUTION?

Finding the minimal entity that exhibits channel-forming properties is a core issue in any attempt to characterize the macromolecule in question. In the case of the AChR, several studies have been concerned with the actual minimal structure capable of retaining agonist-dependent fluxes upon reconstitution. The early observation that AChR proteolytically degraded to the extent that only the α-subunit was apparent[92] and that it maintained agonist-induced transitions,[110,145] local anesthetic binding,[91,146] and $^{22}Na^+$ uptake[91,145] was certainly disquieting. Furthermore, the fact that when the proteolysis was deliberately extended to the stage where no obvious subunits were left intact (see Lindstrom et al.[104]) the AChR still retained antigenic determinants corresponding to each subunit, its characteristic (low-resolution) morphology, its sedimentation properties, and its agonist-activated $^{22}Na^+$-efflux raised doubts as to the sensitivity of total, integrated flux measurement with low time resolution in establishing preservation of functional attributes of the receptor. Such functional assay is still used, under more stringent experimental conditions, to this day (e.g., Fong and McNamee[102]) and has become an established technique in assessing AChR permeability properties.

The study of this issue has entered a new era with the impact of cDNA recombinant techniques, the derived knowledge of the primary structure of AChR subunits, and the possibility of introducing selective alterations in primary structure in order to define the contribution of individual chains and portions thereof on AChR function. Point mutation and comparative electrophysiological recording of responses mediated by normal and altered receptors now make it feasible to attempt correlation of AChR molecular (and submolecular) structure with function at an unprecedented level of resolution.[70,147]

F. IS THE GATING FUNCTION DEPENDENT ON AChR OLIGOMERIC STATE?

Reconstituted preparations of monomers, dimers, or higher oligomers of the AChR respond equally well in their ability to translocate ions as measured by integrated Na^+ flux.[93] In another study, no differences were found between monomeric and dimeric receptor obtained from AChR membranes by the cholate dialysis technique[148] using either integrated Na^+ efflux or Na^+ uptake measurements. Wu and Raftery,[149] using stopped-flow measurements of Tl^{3+} influx into AChR-asolectin vesicles, compared monomeric and dimeric preparations. No differences could be detected. Boheim et al.[121] tested both monomeric and dimeric AChR purified by affinity chromatography using single-channel measurements. Again, no differences were observed in the gating behavior of the two species. From these studies it was concluded that the M_r 250,000 monomer was the minimal functional entity capable of responding to agonist in a reconstituted system, as judged by both flux and single-channel measurements. The $^{22}Na^+$ uptake assay of normal and glutaraldehyde-crosslinked AChR reconstituted into soybean lipid vesicles could be interpreted as meaning that the monomer does not reaggregate noncovalently into dimers.[148] However, the authors did not discard the possibility that the latter could occur in a time scale inaccessible to the total flux measurements. More recently, Schindler and co-workers[150] have

conducted experiments to test these various possibilities. The AChR dimer was found to exhibit conductances twice as large as the monomer.

G. AChR-MEDIATED ION TRANSLOCATION AT THE SINGLE-CHANNEL LEVEL

During the last few years, successful attempts to reconstitute AChR into lipid bilayers have prepared the way for application of powerful biophysical techniques for monitoring electrical activity at the level of individual molecules. The experiments of Schindler and Quast[151] made use of native AChR vesicles from *T. californica* originally dispersed in an aqueous phase (hypophase) together with lipid vesicles made from endogenous *Torpedo* lipids and various ratios of soybean lipid+cholesterol. The ratio of AChR to lipid was, in turn, varied. The vesicles were allowed to spread at the air-water interface to form monolayers, whose composition was primarily determined by that of the vesicles in the hypophase. The monolayer formation is a self-assembly process, originating in the disintegration of the vesicular structure at the interface to generate a surface-adsorbed monolayer in equilibrium with the parental structures. A clear demonstration of this equilibrium and its control by the cohesive energy ("surface pressure") at the monolayer, vesicle size, and lipid concentration has appeared.[152] The study was concerned with the quantitative treatment of the problem for the case of a homogeneous lipid system, i.e., dioleoyl PC.[152] Once the equilibrium was established, the surface pressure became independent of vesicle concentration. Analogous treatments of the more complex cases of a protein-containing lipid vesicle, or of heterogeneous mixtures of pure lipid and protein-lipid vesicles are not as yet available. Regardless of the exact mechanism of vesicle-monolayer assembly, two monolayers formed in two separate compartments can be made to fuse at the orifice of a Teflon® septum separating the compartments into a bilayer, as in the Montal and Mueller[153] technique (see Montal et al.[96] for review).

In the Schindler and Quast[151] study, carbamoylcholine and succinylcholine were used to elicit conductance changes and d-tubocurarine and α-bungarotoxin to inhibit them. The agonist-induced responses showed the concentration and time dependence exhibited *in vitro* by native AChR membranes in the desensitized state.[18,154] At low concentrations of AChR-containing vesicles in the hypophase, single-channel fluctuations were observed; their amplitude was ionic strength-dependent. Channel open times were in the range 1 to 2 ms. Cation selectivity was determined; Na^+ or K^+: Cl^- ratios were about 7. Asymmetric bilayers with respect to the AChR recognition site could be made, but the conductances were insensitive to voltage reversal (no rectification was observed).

Another approach to the study of the AChR gating behavior at the single-channel level is that of Nelson et al.[131] Technically, the procedure differs from that of Schindler and Quast[151] in a few details. The AChR was purified, by any of the five methods reported by Lindstrom et al.,[93] and reincorporated into soybean lipids as stated in the preceding sections. The AChR-containing vesicles were then supplemented with soybean lipid liposomes and spread by applying drops of this mixture at the air-water interface. The formation of bilayers followed from this intermediate monolayer stage. The current fluctuations obtained within a limited range of carbamoylcholine concentrations (0.25 to 2 μ*M*) were analyzed by means of autocorrelation function. A single exponential curve fitted the function, from which the channel mean open time was estimated to be 32.5 ms. Single-channel recordings illustrated in this work had amplitudes of 60 pS in 0.5 *M* NaCl (–10 mV) and lifetimes of about 50 ms. Asymmetry of the response could also be established.

The study of Boheim et al.[121] focused on the single-channel behavior of the AChR in planar bilayers. The technique differed from the preceding ones in that the lipid bilayer formed prior to the addition of liposomes to the hypophase. Second, in that study[121] a synthetic lecithin (1-stearoyl-3-myrystoylglycero-2-phosphocholine) was introduced in an organic solvent mixture instead of soybean lipids. The pure lipid has a sharp transition temperature. The AChR-

containing vesicles fuse with the preformed lipid bilayer a few degrees below the transition temperature. Native membranes, alkaline-treated membranes (i.e., depleted of nonreceptor peptides), purified AChR in different oligomeric states, and finally purified AChR monomers were studied. Various modalities of channel behavior were described (among them channel flickering) and compared with those observed in intact rat or frog neuromuscular junctions with the patch-clamp technique. Boheim et al.[121] extended their comparison to the pharmacological specificity of the AChR channel properties, as assessed by the progressive lengthening of the mean open channel lifetime in the sequence carbamoylcholine:acetylcholine:suberyldicholine, which as expected was concomitant with an agonist-invariant mean conductance. Channel blockage by the local anesthetic QX-222, whose action on single-channel behavior is well documented,[155] was also demonstrated. A current-voltage relationship, reversal potentials, and permeability ratios for Na^+, K^+ and Cl^- could be furnished.

Subsequent studies by Labarca et al.[156,157] established the existence of two kinetically distinct open states in *Torpedo* AChR reconstituted into planar lipid bilayers. The two states had similar current amplitudes (~45 pS) but differed in their lifetime. A short-lived channel had mean open times of about 0.5 ms; the long-lived channel exhibited lifetimes of about 4 ms with ACh.[158] Short-duration channels remained open for about 1 ms and long-open time channels had average durations of ~11 ms in the presence of 100 n*M* suberyldicholine. Colquhoun and Sakmann[159] had also observed two distinct open-channel lifetimes in skeletal muscle AChR channels.

H. RESTITUTIO AD INTEGRUM?

The studies analyzed above constitute attempts to describe the mean open time and average conductance of the *Torpedo* AChR channel. The merit of these preliminary phenomenological descriptions is to be judged in light of the unavailability of such information in the living electrocyte at the time the reconstitution studies were conducted. They showed the *Torpedo* AChR channel to be voltage insensitive and that its response to cholinergic agonists was similar in many respects to that of the neuromuscular junction. The same apparent ionic selectivity sequence observed *in vivo* for the junctional AChR was also found in the reconstituted AChR *in vitro:* $NH_4^+ > Cs^+ > Rb^+ > Na^+ >> Cl^-$, Fl^-, SO_4^{2-}.[157,160] The agonist concentrations eliciting responses were, however, extremely low, and probably different experimental conditions will have to be met in order to fully characterize the dose-response curve. Furthermore, detailed kinetic analysis of the AChR gating has disclosed rather complicated pathways through which closed and open states of the channel communicate, and the existence of at least two open states.[161] These intricacies should likewise foster additional experimentation.

The strategy of Schindler and co-workers[150-152,162,163] offers the possibility of correlating channel behavior with physical properties amenable to characterization in the intermediate, monolayer state such as cohesive energy of the planar bilayer (surface pressure). In this manner, the physical state of the final bilayer can be adjusted to that of the monolayer and ultimately to that of the parental AChR vesicle, thus matching the thermodynamic state of the host membrane. In the strategy of Montal and co-workers[131,156-158,161] the possibility of combining flux measurements in reconstituted vesicles and electrical properties in the bilayer is suggested. This complementation might aleviate some limitations of the integrated flux measurements, especially in relation to the time resolution problems of the latter.

The work of Boheim et al.[121] constituted the first successful attempt to study the AChR gating behavior in a chemically defined synthetic lipid environment. The different strategies analyzed above point to possible developments that might prove fruitful in the future for investigating the contribution of the AChR polypeptide components, nonreceptor peptides, lipid classes, and environmental factors on AChR channel properties in reconstituted systems. Given the remarkable technical progress accomplished with the use of patch-clamp recording techniques, now making possible the excision of small membrane fragments from the intact cell surface, the control of the sidedness of the membrane patch,[164] and the ability to rapidly modify the

environment of the membrane by perfusion of either of its two faces,[164,165] the major contributions of bilayer measurements are to be sought in their successful combination with chemical dissection of AChR building blocks and other individual constituents of the postsynaptic membrane. It could well be that in some instances planar bilayers prove more apt than intact cells or excised patches for performing experiments aimed at defining molecular properties of the AChR, for example, the basis of cooperative phenomena in cholinergic responses. Given the greater ability of the experimentalist to control a complex set of multivariate parameters *in vitro*, the access to problems requiring a detailed knowledge of geometrical constraints, diffusion barriers, exact composition of reaction partners, and state of aggregation of the AChR, to mention a few, will probably be found in the planar bilayers rather than in the living synapse. In attempting to resolve these problems, the art of reconstitution may achieve the *restitutio ad integrum* of the AChR system. In summary, incorporation of biochemically characterized AChR preparations into lipid bilayers entered the category of a reproducible technique within the arsenal of receptor methodologies due basically to two factors. First, some of the parameters contributing to the failure or success of the reconstitution phenomenon began to be understood (without necessarily implying that these parameters have been completely fathomed). This was partly a costly lesson from a decade of flux experiments in membrane vesicles. Second, the electrophysiological characterization of the AChR gating function in terms of its unitary behavior, as defined by patch-clamp measurements *in vivo*, left little ambiguity as to what to look for in the reconstituted systems.

I. MOLECULAR ANATOMY OF THE AChR CHANNEL

The introduction of molecular genetic techniques in the field of the AChR has paved the way for structural-functional correlation studies of unprecedented depth. Expression of site-mutated messages in heterologous cellular systems had been carried out mostly in the amphibian *(Xenopus)* oocyte, given its enormous biosynthetic capacity, and, most recently, the availability of a technique for stripping away the viteline membrane and thus enabling the application of single-channel recording at the naked plasmalemma.[166] Other heterologous cellular systems have recently been introduced: yeast[167] and different types of fibroblasts.[168-170] The former is a promising avenue, given the wealth of information available on yeast genetics. The second approach is also worthwhile, since it enables the obtention of large quantities of stable, normal, or mutated AChR (or chains thereof) in homogeneous cell populations.

Mishina et al.[8,171] and White et al.[172] carried out site-directed mutagenesis studies testing different combinations of normal and modified cloned AChR subunit DNAs in oocytes. Deletions and replacements of critical amino acids in the α-subunit led to abnormal electrophysiological responses. White et al.[172] synthesized mRNAs coding for *Torpedo* α, β, and γ chains and combined these with mouse δ chain. The hybrid produced larger responses than the normal message, i.e., higher ACh binding, larger single-channel conductance, smaller mean channel-closed times, and slower rates of desensitization were observed with the hybrid than with the native species. These two studies employed conventional voltage-clamp electrophysiological techniques.

Sakmann et al.[173] used patch-clamp recording to further define this problem. When injected in oocytes, normal *Torpedo* and calf AChR displayed currents of about 23 and 1600 nA, respectively. Single-channel measurements revealed that the basic difference between AChR channels of the two species resided in the average duration of the open channel state. *Torpedo* receptors opened much shorter (~0.6 ms) channels than those of calf (~8 ms), conductance of both channels being about equal (~40 pS). In spite of these differences, the two native receptors were similar in ion selectivity and transport properties. Tentatively, the δ-subunit was postulated to govern the rate of channel closing, the time for which an AChR channel remains open apparently being determined by inherent characteristics in both the α- and δ-subunits.

As analyzed in preceeding sections, hydrophobicity profiles of the amino acid sequence of

FIGURE 3. The synthetic 23-mer peptide mimicking the sequence of the *Torpedo* M2 δ segment elicits membrane currents upon reconstitution into artificial PC bilayers (A). The M1 δ 23-mer peptide (B) does not. Upward deflections correspond to channel openings; the single-channel conductance was about 40 pS at an applied potential of 100 mV. (From Oiki, S., Danho, W., Madison, V., and Montal, M., *Proc. Natl. Acad. Sci. U.S.A.*, in press. With permission.)

AChR subunits suggest the presence of membrane-transversing helices. Depending on the model in question (Figure 1), amphiphilic helices with different proportions of polar side chains have been chosen as candidates for lining the actual walls of the AChR ionic channel. Such ideal polypeptides should also meet the appropriate length to span the bilayer and, in forming aggregates, should expose more polar moieties toward the channel lumen, while apolar side chains should preferentially face the hydrocarbon portion of the lipid bilayer for a favorable partition in the membrane. In line with these hypotheses, model peptides have been recently synthesized, reconstituted into planar lipid bilayers, and tested for their ion-conducting properties. One strategy in designing such peptides has relied on the known sequences of AChR chains postulated to be channel-forming segments. Oiki et al.[174] first synthesized a 22-mer peptide mimicking the structure of a putative transmembrane fragment of the voltage-sensitive sodium channel. More recently, they have applied this same concept to the case of the AChR. They synthesized a 23-mer peptide facsimile of the M2 helix of the *Torpedo* δ subunit (EKMSTAISVLLAQAVFLLLTSQR).[175] This model peptide exhibited single-channel conductance, discrimination of cations over anions, and channel mean open- and closed-state lifetimes in phosphatidylcholine bilayers similar to those observed with the authentic AChR. On the other hand, a synthetic peptide mimicking the sequence of the M1 helix did not display such characteristics[175] (Figure 3).

Lear et al.[176] have taken a different approach: their synthetic peptide was devised choosing leucine as the amino acid for the apolar face of the channel because of its hydrophobicity and helix-forming tendency, serine was selected for the polar face on account of its polar but uncharged nature, and a linear repeat of seven residues (either LSSLLSL or LSLLLSL) was picked to provide registry of polar and apolar surfaces in linked heptamers. From each heptamer, 21-mer peptides were finally synthesized; their CD spectra in methanol were characteristic of α-helices. The $(LSSLLSL)_3$ peptide incorporated into diphytanoyl phosphatidylcholine bilayers produced channels with one predominant conductance state of about 70 pS in 0.5 *M* KCl, with a mean open time between 3 and 8 ms and cation selectivity. Lear et al.[176] undertook energy minimization calculations to produce molecular models of the peptides congruent with the conductivities observed in planar bilayer. Their conclusion was that the 21-mer of LSSLLSL is most likely in the form of hexameric or higher-order aggregates. In contrast, the LSLLLSL 21-mer, which exhibits proton selectivity, would either form trimeric or tetrameric aggregates in the membrane. The cation-selective LSSLLSL 21-mer thus appears to be a closer counterfeit of the AChR channel. A 14-mer version did not suffice to span the bilayer and failed to form stable channels. These two bioengineering-based studies offer interesting possibilities for the comprehension of channel structure-function relationships and, as an offspring, for exploring the design of man-tailored selective pores.

J. THE LIPID-AChR PROTEIN INTERFACE AS THE SITE OF ACTION OF NONCOMPETITIVE CHOLINERGIC ANTAGONISTS

Some noncompetitive antagonists can bind to the AChR agonist recognition site with high (micromolar) affinities. Thus, Heidmann et al.[177] reported a single high-affinity site per AChR monomer for phencyclidine, meproadifen, and Triton® X-100 (K_d 5 μM). Chlorpromazine and trimethisoquin binding, on the other hand, exhibited 10 to 30 low-affinity sites per AChR monomer. Since the stoichiometry of this class of sites depended linearly on the lipid-protein ratio in reconstituted AChR membranes, it was suggested that the low-affinity sites occurred at the lipid-protein interface, distant from the channel itself.[177] These noncompetitive antagonists displayed affinities related to their partition coefficient in lipids and their charge. Two sites have been detected on the AChR with ESR techniques.[178] A third "nonsaturable" binding site in the bulk lipid bilayer has also been proposed.[177] This proposal was based on the high lipid solubility of several of the noncompetitive blockers. Even if there was one saturable high-affinity binding site per AChR monomer responsible for blocking agonist-stimulated ion translocation, the lipid membrane provided the receptor with a locally high concentration of drug that may have its own effects on AChR function through specific effects on the lipid-protein interface, or by affecting the local charge environment, or by a combination of both.[179] This appears to be the case with spin-labeled local anaesthetics that sense strongly immobilized spectra in the presence of agonist, suggesting agonist-induced conformational changes of the AChR protein.[180] The possibility that the electron spin resonance (ESR) signal arises partly from protein-lipid interactions has been considered by the cited authors.

V. DYNAMICS OF AChR AND LIPIDS IN THE MEMBRANE

The AChR molecule is one of the few membrane receptors for which a great variety of techniques has provided a detailed picture of its dynamic aspects. One of the salient features of this picture is the enormous temporal range covered by the phenomena considered.

Some of these phenomena are related to metabolic processes in which the membrane itself is unlikely to play any relevant role, and are therefore beyond the scope of this review. Others, for example, lateral and rotational motions of the AChR protein and lipid molecules in the membrane, are of particular interest here and will be considered in more detail.

A. MOTIONALLY RESTRICTED LIPIDS IN AChR MEMBRANES

Several natural membranes studied to date exhibit immobilization of the lipid surrounding integral membrane proteins. The immobilized lipid has been termed "boundary lipid"[181] or lipid "annulus".[182] It should be stressed that such immobilization is relative to the bulk bilayer lipid, and that it refers to the time window (10^{-9} to 10^{-7} s) covered by the technique presently employed to study these dynamic properties, i.e., ESR. Densities of more than 10,000 AChR particles of 8 to 9-nm diameter per square micrometer are observed in the neuromuscular junction and even 50,000 units per square micrometer in the *Electrophorus* electromotor synapses (see review in Barrantes[183]). AChR-rich membranes purified from *Torpedinidae* are also densely covered by receptor particles and exhibit a relatively high protein-lipid ratio (Table 1). In fact, the AChR membrane is far from conforming to the "fluid mosaic" concept (see Bartholdi et al.[184]), its lipids filling the interstices left by closely packed integral membrane proteins rather than the other way around. It is therefore not surprising to find clear indications of motionally restricted lipid in the ESR spectra of lipid spin labels incorporated into such membranes. This is particularly evident when the probe senses motion close to the end of the fatty acyl chain, given the large degree of averaged spectral anisotropy available there to detect any immobilization induced by the protein.

ESR spin label studies make use of the rotational diffusion constant, the order parameter S, and the maximum line-splitting, T, to describe rotational motions of lipid analogue spin labels

in the membrane (see reviews by Marsh[185,186]). Early studies failed to detect immobilized components in the spectra of fatty acid derivatives incorporated into native membranes, leading to the postulation of a fluid lipid environment in the immediate vicinity of the AChR.[187] Stearic acid (18:0) constitutes a major component of the EGP's (25% in PE) and the principal fatty acid in PS (37%) in *T. marmorata* membranes (Table 2). A nitroxide spin label derivative of this fatty acid (16-SASL) was chosen in ESR studies of native membranes and aqueous dispersions of the extracted membrane lipids. By using differential electron spin resonance spectroscopy, two components could be observed, one of which corresponded to motionally restricted lipid in the membrane.[142] Similar immobilized components were observed with phospholipid[143] and steroid[127,142] spin labels. The latter authors calculated that the proportion of the protein-perturbed lipid was about 38%. Rousselet et al.[188] had observed immobilization with fatty acid probes but not with phospholipid spin labels, from which they inferred specific binding of fatty acids to the AChR protein.

The work on reconstituted membranes by Ellena et al.[127] finally settled this controversial issue. First, they demonstrated some degree of specificity in the association of the fatty acid and steroid labels with the receptor protein. Secondly, from experiments with spin-labeled PC, which showed no preferential selectivity in interaction with the protein as judged by ESR criteria, they clearly demonstrated the immobilized component. Furthermore, the fluid and motionally restricted spin-labeled lipid could be separately quantitated by difference spectroscopy. For purified membrane proteins reconstituted in bilayers, the lipid/protein ratio may vary, and it has been found that a fixed stoichiometry is maintained for the motionally restricted lipids, independent of the total lipid/protein ratio. For two lipid species (L^1, L^2) competing for sites on the surface of the protein, an exchange equilibrium can be assumed between the lipid free in the bulk bilayer, L_b, and that associated with the protein, L_p:

$$PL^1 + L^2 \overset{K_r}{\rightleftharpoons} PL^2 + L^1 \tag{1}$$

The apparent association constant for lipid L^2 with respect to L^1 is given by:

$$K_r = [L^2]_p \cdot [L^1]_b / [L^1]_p \cdot [L^2]_b \tag{2}$$

The total number of association sites on the protein can be determined from ESR spectra.[185] Ellena et al.[127] demonstrated that there is a preferential interaction of the negatively charged phosphatidic acid (PA) with AChR characterized by a threefold greater K_r with respect to PC, a result recently confirmed by fluorescence studies[189] (see also Table 6). Approximately 40 lipid molecules (40 ± 7) are estimated to be motionally restricted by direct interaction with the hydrophobic surface of the protein (Table 5), in good agreement with estimates of the number of phospholipid molecules that may be accomodated around the intramembranous perimeter of the AChR, based on structural data available to date (Figure 3). No information is available as yet on whether cholesterol molecules are in direct contact with the AChR protein. A recent study using [3H]cholesteryl diazoacetate demonstrated covalent labeling of this probe to all receptor subunits,[144] thus confirming our ESR studies suggesting direct interaction of an androstanol spin probe with the AChR.[142] Cholesterol does not appear, however, to effectively compete for the phospholipid annular sites presumed to surround the immediate perimeter of the AChR,[189] although there is a positive specificity ($K_r \sim 4.3$) in the interaction of androstanol with the AChR.[127] The recent results of Jones and McNamee[189] are consistent with the existence of additional, nonannular sites for cholesterol on the AChR that are not accessible to phospholipids. Their number is 5 to 10 per AChR monomer. Further studies will be necessary to establish the exact stoichiometry of the total (i.e., phospholipid+cholesterol) lipid annulus of the AChR. In the case of the AChR, the projected surface of the intramembranous portion has been determined

TABLE 5
Stoichiometries of the Motionally Restricted Lipid Component in Reconstituted AChR Membranes and Other Lipid-Protein Systems

Protein/host lipid membrane	M.W. $\times 10^{-3}$	N_l^{exp} (mol/mol)	$N_l^{exp}/\sqrt{\text{mol wt}}$	N_l^{calc} (mol/mol)	Ref.
Acetylcholine receptor/DOPC	250	43 ± 4	0.080 ± 0.014	43 (51)	127
Na^+,K^+-ATPase/DOPC	314	63 ± 3	0.112 ± 0.005	(~57—72)	239
Na^+,K^+-ADTase shark rectal gland	265	58 ± 4	0.112 ± 0.008	(~57—72)	240
Cytochrome oxidase/DMPC	165	45 ± 4	0.110 ± 0.011	40—45	241
Ca^{2+}-ATPase/egg PC	115	22 ± 2	0.065 ± 0.006	(~23—27)[a]	242
Ca^{2+}-ATPase/sarcoplasmic reticulum	115	24	0.071	(23—27)[a]	243
Rhodopsin/bovine rod outer segment disk	39	25 ± 3	0.125 ± 0.016	24 (±2)	244
Rhodopsin/frog rod outer segment disk	39	23 ± 2	0.114 ± 0.010	(24)	240

Note: Modified from data compiled in Reference 185.

[a] Calculated assuming a dimer, with monomer radius 2.0 nm. N_l^{exp} is the effective number of motionally restricted lipids per protein deduced from ESR experiments. N_l^{calc} is the calculated number of lipid molecules that can theoretically be accommodated around the intramembranous perimeter of the integral protein. Abbreviations used: DOPC, dioleoylphosphatidylcholine; and DMPC, dimyristoyl-phosphatidylcholine.

by electron microscopy at a resolution of about 2 nm.[49] The corresponding radius of the membrane-embedded domain is about 4 nm, which correlates well with estimates obtained from our previous data on noncrystalline specimens[48,190] and those of other groups.[25,191,192] The projected area of an average phospholipid molecule is about 0.6 nm^2 (McLaughlin[193]) (radius 0.49 ± 0.03 nm[194]). Assuming that phospholipid molecules are arranged as cylinders around the AChR molecule, one shell of lipids occupies a perimeter of 2π (4 ± 0.49 nm) = 25.1 nm (the perimeter is projected to intersect the center of each lipid molecule). Ellena et al.[127] had previously calculated a perimeter of about 21.3 nm. The number of lipid molecules in the annulus would thus be given by (25.1 nm/2 × 0.49 nm) × 2 (i.e., two leaflets) = 51. It thus follows that the AChR monomer could accomodate the motionally restricted lipid detected by ESR spectroscopy within the first shell around its intramembranous perimeter (Figure 4). The stoichiometry of motionally restricted lipid molecules scales approximately with the square root of the protein molecular weight for this and other membrane proteins (Table 5).

It is now generally accepted that the strong immobilization of the AChR in the postsynaptic membrane arises from protein-protein rather than protein-lipid interactions[195] (reviewed by Barrantes[2]). The immobilization of the protein obviously has consequences on lipid mobility: the rotational correlation times of the motionally restricted lipid in native AChR membranes are about one tenth that of the fluid lipids.[142] Both lipid components, however, are relatively more mobile than an average integral membrane protein (~20 μs). For a correlation time of about 50 ns (Table 6), the lipid-lipid exchange rate in a fluid bilayer is estimated to be ~2.10^7 s^{-1}. The rotational correlation time compounds motional contributions of (1) the lipid acyl chains interacting directly with the protein and (2) the exchange of lipid on and off the protein surface. The effective lifetime of the lipids on the protein boundary has a lower limit of about 50 ns, shorter than the lifetime of average lipid-lipid exchange in fluid lipid bilayers resulting from lateral diffusion. Thus, lipids in the immediate vicinity of the AChR exhibit motion, restricted in comparison to that of the fluid phase and independent of the exchange by diffusion (see below). It has been demonstrated in ^{2}H-NMR studies that the first layer of lipids around integral membrane proteins is more disordered than, and in fast exchange with, the bulk fluid lipid.[196] Slower motion and greater disorder of the motionally restricted lipids may thus constitute the ideal interface between protein and the fluid bilayer, allowing rapid exchange of lipids between the two lipid "phases" (Figure 5).

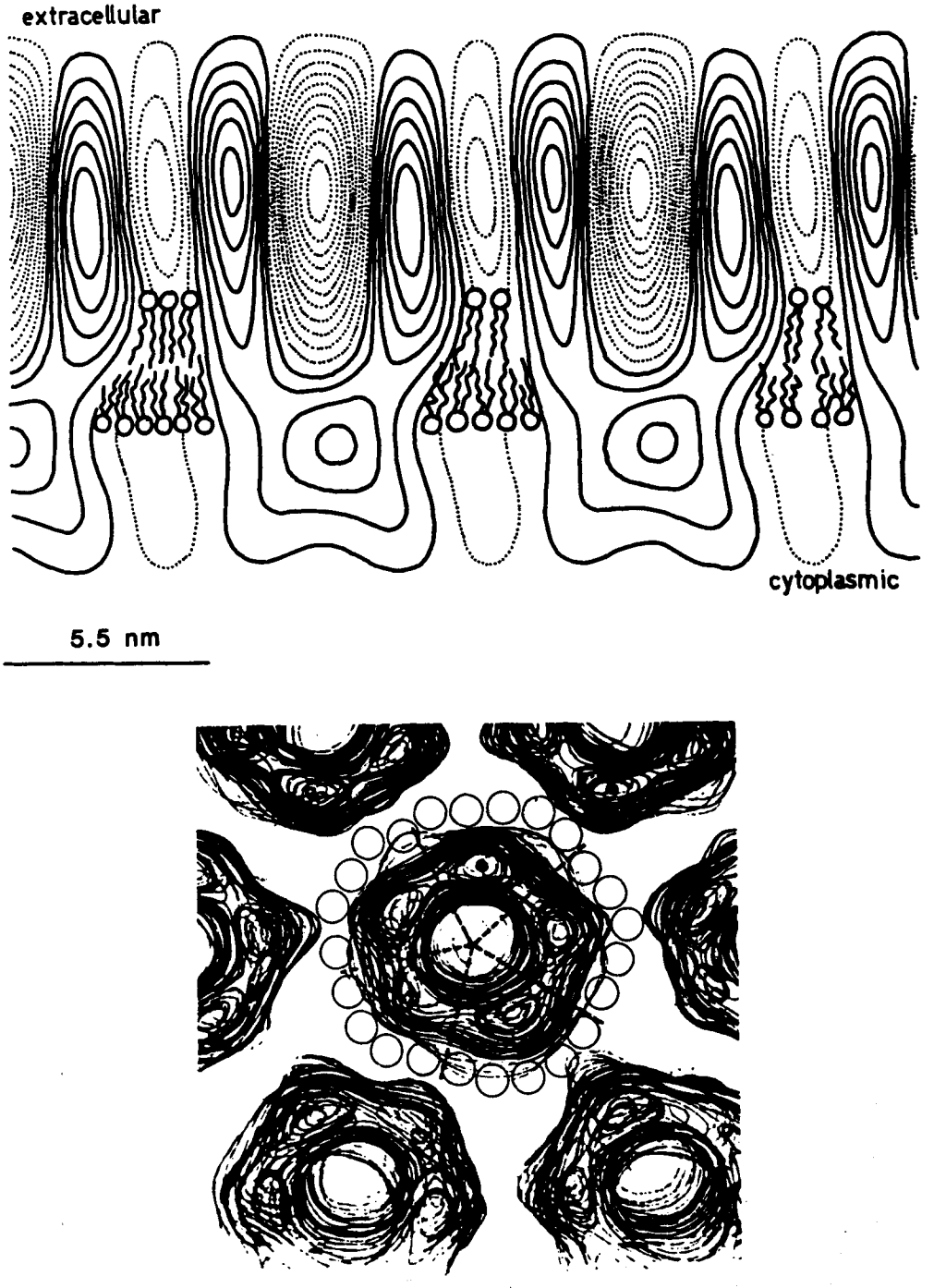


FIGURE 4. Phospholipid annulus around AChR molecules. The AChR particles and the bilayer thickness have been drawn to scale according to the electron microscope data of Brisson and Unwin.[49] (A) Lateral view. (B) Top view. The 24 phospholipid molecules drawn in the immediate perimeter of an AChR particle constitute the average number of lipid molecules per hemiannulus. The cytoplasmic half of the bilayer could accomodate more phospholipid molecules, provided bilayer curvature is not too pronounced and there are similar volumes for the phospholipids in the two hemilayers. The average total number of phospholipids (48) in the annulus is in agreement with the figures calculated from ESR experiments.[127]

TABLE 6
Molecular Mobility Parameters of Lipid Spin Labels in the Bulk Bilayer and the Motionally Restricted Lipid Regions of *Torpedinidae* AChR-Rich Membranes

Membrane and spin label	Temperature (°C)	$^{r}R(ns)^{a}$	$^{r}R(ns)^{b}$	$^{r}R(ns)^{c}$	Ref.
T. marmorata					
12-Doxyl-stearic acid	34	50	46	12	142
16-Doxyl-stearic acid	–4	34	41	45	
Doxyl androstanol	14	45	73	47	143
Protein perturbed component:		42%			
Exchange rate (immobilized-fluid lipid)		$<2 \times 10^{7}\ s^{-1}$			
T. californica					
16-Doxyl-phospholipids	0	16	Protein-perturbed component, 38%		127
	0	1	Not strongly perturbed, 62%		
Exchange rate (immobilized-fluid lipid)		$<5 \times 10^{7}\ s^{-1}$			
Relative affinities for AChR (K_{AV})[d]			Doxyl-androstanol-SL 4.3		
			Stearic acid-SL 4.1		
			Palmitic acid-SL 2.7		
			Phosphatidylethanol-amine-SL 1.1		
			Phosphatidylcholine-SL 1.0		
			Phosphatidylserine-SL 0.7		

[a] Deduced from the low-field line-width, ΔH_l.
[b] Deduced from the high-field line-width, ΔH_h.
[c] Deduced from the outer splitting, A'_{zz}.
[d] SL = spin label. All phospholipid analogs contain 16-doxyl stearic acid in the *sn*-2 position.

B. LATERAL DIFFUSION OF AChR AND LIPIDS IN NATURAL AND RECONSTITUTED SYSTEMS

Lipid lateral diffusion, that is, the two-dimensional translation of lipid molecules parallel to the plane of the membrane, appears to be an important physical property because of its functional consequences. Lipid motion in mono- and bilayers appears to follow in general terms the predictions of the so-called free area theories, leaving aside interactions of the diffusant phospholipid head group with the aqueous phase and of the fatty acyl chains with the opposing monolayer. The aforementioned theories basically envisage diffusion of a lipid molecule as the resultant of two combined processes: (1) creation of a free volume (a sort of hole) adjacent to the lipid molecule in question, and (2) subsequent filling of the free volume created by the diffusant molecule jumping into the hole. The hole left by a molecule on leaving can be used by another molecule or the initial one can jump back. Free area theories have been used to explain the lateral diffusion of fluorescent lipid analogs in reconstituted AChR liposomes and in corresponding bilayers devoid of the receptor protein. NBD-dimyristoyl-PE exhibited values of D, the lateral diffusion coefficient, of $8 \times 10^{-8}\ cm^2\ s^{-1}$ (Criado et al.[197]). The Stokes-Einstein equation provides estimates of the diffusion coefficient for translation and rotation of diffusant molecules:

$$D = kT/f \qquad (3)$$

where k is the Boltzman constant and f the frictional coefficient. In the "stick" limit for rotational motion, when the molecule is immersed in a solvent of viscosity n, the frictional coefficient

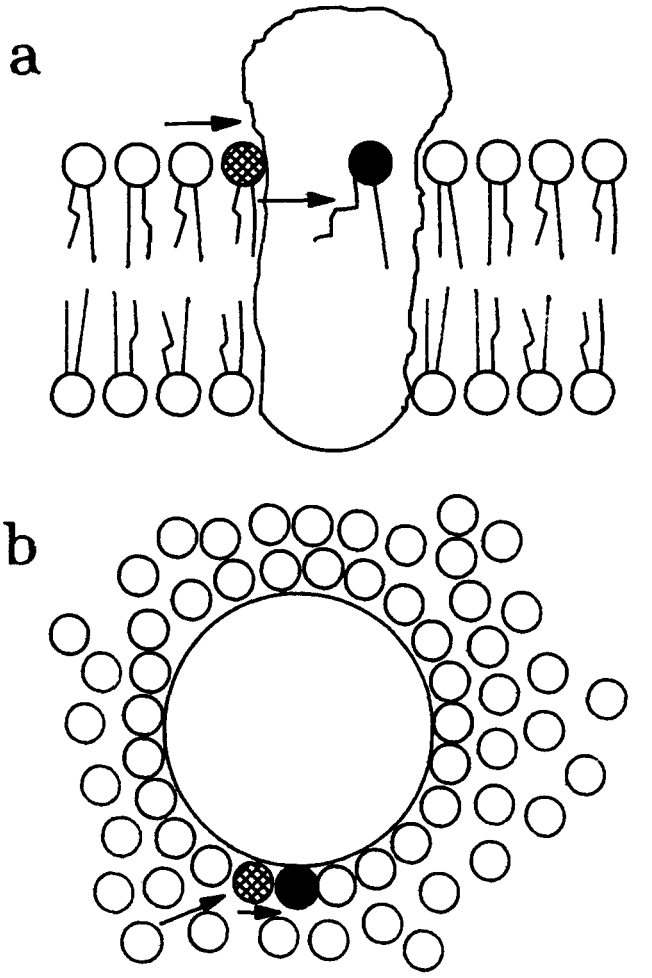


FIGURE 5. Two lipid pools, the fluid bilayer lipid and the less mobile annular lipids associated with the AChR (see Figure 3) exhibit exchange rates of $2\text{-}5 \times 10^7$ s^{-1}.[127,142] When a phospholipid molecule in the fluid lipid region (cross-hatched polar head region) gains access (arrow) to the annular region (filled polar head region), its average motion is reduced to about one tenth that in the bulk bilayer (see also Table 6).

becomes $f_{rot} = 8\pi\eta S r^3$, where S is a shape factor that modifies the volume and is dependent on molecular shape. For *translational* diffusion, $f_{trans} = 6\pi\eta R$. In water at 300 K, a spherical protein of radius $R = 1$ nm would exhibit a diffusion coefficient $D_{rot} = 1.65 \times 10^6$ s^{-1}, but as D_{trans} is approximately equal to R^2_{Drot}, the translational diffusion coefficient $D_{trans} = 1.6 \times 10^{-10}$ cm^2 s^{-1}. Translational diffusion coefficients can be measured either by excited-state Stern-Volmer quenching experiments or by spatial techniques, for example, fluorescence recovery after photobleaching (FRAP), fluorescence microphotolysis, or transient grating generation. The latter type of technique is based on the measurement of the distance diffused by the molecule per unit time. As in nanosecond fluorescence depolarization or polarized phase fluorometry, FRAP techniques rely on photoselection: the probability of light absorption by a chromophore is proportional to the product of the incident light intensity and the cosine squared of the angle subtended by the incident polarization and the absorption dipole moment. Experimentally, laser-produced photobleaching pulses, shorter than the time regime of interest, are used to annihilate

the fluorescence in only a relatively small area of the assembly containing a uniformly distributed chromophore. The recovery of the fluorescence is then followed as a function of time, monitored with an attenuated light source.

In the case of large integral membrane proteins such as the AChR, the study of their dynamics is simplified by tagging with appropriate extrinsic chromophores and subsequently reconstituting them into artificial lipid bilayers at relatively low protein/lipid ratios. Under such conditions, the lateral diffusion of the protein can be estimated by the Saffman and Delbrück[198] formula. This is based on the Stokes-Einstein treatment of motion in a solvent medium, in which lateral motion can be considered as originating in random collisions of the macromolecule with the solvent molecules, and is opposed by frictional forces of the solvent. The diffusion coefficient D, follows the same dependence on the frictional coefficient f and the Boltzman constant as in solution (see above). In the case of a spherical molecule of radius r diffusing in a medium of viscosity, $D = kT/6\,\pi\,\eta\,r$ (the Stokes-Einstein equation). Saffman and Delbrück applied this formula to the case of a cylinder of radius r oriented perpendicular to and diffusing in a thin viscous sheet of viscosity η and thickness h (equal to the height of the embedded cylinder), a treatment that is more relevant to a protein transversing a membrane, and the AChR in particular. The diffusion coefficient becomes:

$$D = (kT/4\pi\eta_m h)\,\ln(\eta_m h/\eta_w r - 0.5772) \quad (4)$$

where η_m is the viscosity of the viscous sheet ("the membrane") and η_w ($\sim 10^{-2}$ poise) is the viscosity of the aqueous medium in which the sheet is immersed. The sheet is treated as a continuum of solvent molecules. The formula predicts a *weak* dependence of D on protein radius, a prediction that appears to be followed roughly by the few integral membrane proteins studied to date, including the AChR.[197]

It should be noted that the theory finds validity only in the case of very diluted proteins (as with proteins reconstituted in lipid bilayers). When AChR monomers and dimers were reconstituted into soybean lipids and dimyristoyl-PC bilayers, we found that the lateral diffusion coefficient in both cases lay in the range of 1 to 3×10^{-8} cm^2 s^{-1} (between 14 and 37°C).[197] The presence of cholesterol did not appear to modify D. In the fluid phase, cholesterol has been reported to decrease the lateral diffusion constant of membrane lipids as studied by FRAP.[199,200] Similarly, using pulse NMR techniques, Kuo and Wade[201] have observed that small amounts of cholesterol (<10%) produce a large increase in lipid lateral diffusion and a gradual decrease as cholesterol levels are increased. More recently, Yin et al.[202] have employed electron-electron double resonance and saturation-recovery spectroscopic techniques to dissect the effect of cholesterol on lipid lateral diffusion. They found that (1) cholesterol significantly orders the part of the bilayer that it occupies and disorders the interior region of the bilayer; (2) alkyl chain unsaturation of the host lipids moderates the effects of cholesterol; and (3) the effects of cholesterol are different in the 0 to 30 mol% and 30 to 50 mol%, respectively, suggesting inhomogeneous distribution of the steroid in the membrane. In our studies, AChR was reconstituted in multilayers of dimyristoyl-PC containing 45 mol% cholesteryl hemisuccinate. D changed monotonically from 6×10^{-9} cm^2 s^{-1} at 14° to 2×10^{-8} cm^2 s^{-1} at 36° (Criado et al.[197]). No phase transition was observed, as previously reported with this cholesteryl derivative,[100] and the values of D in liquid-crystalline multibilayers of DMPC were similar to those in cholesteryl-hemisuccinate-containing DMPC mutilayers at comparable temperatures. Further studies will be necessary using other cholesterol probes and exploring a wider range of cholesterol concentrations in order to fully characterize the effect of steroids on AChR and lipid mobility.

When AChR monomers and oligomers (predominantly tetramers, as obtained by the technique of Criado and Barrantes[203,204]) are compared, no differences are observed in the value of the translational diffusion coefficient.[205] Measurements of translational diffusion of AChR molecules in the living cell have also been undertaken. Two populations of AChRs could be

defined in the plasmalemma of developing rat muscle cells on the basis of this type of measurement: a diffusely distributed population having $D = 5 \times 10^{-11}$ cm^2 s^{-1}, and a localized, patched population with $D \sim 10^{-12}$ cm^2 s^{-1} (Axelrod et al.[206]). In blebs of myoblast sarcolemma, Tank et al.[207] found D to be 3×10^{-9} cm^2 s^{-1}, similar to the values of 2.6×10^{-9} cm^2 s^{-1} found by Poo[208] in *Xenopus* embryonic myoblasts. Thus, D in reconstituted membranes is at least tenfold higher than the highest values found in native membranes, that is, the AChR in synthetic lipid membranes undergoes essentially free lateral diffusion. The discrepancy between the D values in reconstituted bilayers and in intact cell membranes could be due to several factors. Reconstituted membranes differ from natural membranes in their lipid composition, concentration of protein, and absence of peripheral proteins. The former factor was not found to influence the lateral diffusion of the AChR at very high lipid:protein ratios. It is likely that the extensive protein-protein interactions occurring both between AChR molecules on the one hand and between AChR molecules and other nonreceptor proteins, perhaps involving cytoskeletal elements, on the other are mainly responsible for the relative immobilization of AChR in the native membrane and particularly in the synaptic region.

A recent pressure-perturbation study is worth discussing here, since it bears on the possible relationship between volume changes, which affect lipid translational diffusion, and AChR function. Single-channel recordings were made under high hydrostatic pressure; information was thus obtained on the volume changes associated with the transition undergone by the AChR from the agonist-free to the open state. Pressurization to 40 MPa (~400 bar) increased both mean open and mean closed times, giving apparent activation volumes of 59 and 139 Å^3, respectively, implying a net volume increase of about 80 Å^3 upon channel activation.[209] For any given AChR-agonist reaction scheme, the pressure dependence of individual rate constants is associated with a true activation volume, which is the volume change needed to overcome the free enthalpy barrier characteristic of the specific reaction step. Due to instrumental limitations, the volume changes could not be ascribed to any given reaction step; both ligand binding and channel opening may involve volume changes arising from compressions or expansions of the AChR protein as a whole or of limited regions thereof. The possibility that part of the volume changes were associated with the lipid bilayer was not considered. In this context it is relevant to mention that lipid translational diffusion is affected by pressure; the volume of activation, V_{act}, depends on lipid chain length.[210]

C. ROTATIONAL MOTION OF THE AChR PROTEIN

Considerations similar to those outlined above for the translation mobility of the AChR are also applicable to the rotational motion of the protein. This has been studied by means of ESR or optical techniques, such as time-resolved phosphorescence anisotropy, fluorescence depletion anisotropy, or the recently applied polarized fluorescence photobleaching recovery (PFPR).[211] The rotational correlation time ø of the monomeric AChR is about 10 to 25 μs as measured by phosphorescence anisotropy,[184,212] in full agreement with the expected value. The slower relaxation times observed are probably related to the existence of higher oligomeric AChR species. In living, nondeoxygenated rat myotubes in primary culture, Velez and Axelrod[211] measured values of rotational diffusion coefficients of about 15 ± 3 s^{-1} for the mobile AChR in nonclustered (diffuse) areas of the cell; clustered AChR exhibits immobility even for sampling times as long as 0.3 s. The mobile fraction of receptors is 0.2 and 0.8 in clustered and nonclustered regions, respectively. Since the translational motion of the AChR in reconstituted lipid bilayers can be practically considered a free, unrestricted lateral diffusion, D can be related to the rotational relaxation time by $D \sim 4\ r^2/ø$.

D. EFFECT OF EXTRINSIC MEMBRANE PROTEINS ON THE PHYSICAL STATE OF MEMBRANE LIPIDS

The influence of extrinsic membrane proteins on the structure of biomembrane is well

recognized. That extrinsic membrane proteins may play an active role in the organization of the membrane, however, is less known and has only recently received attention. Peripheral membrane proteins appear to interact with specific types of lipid. The specificity can be rather wide, involving, for instance, negatively charged lipids, as is the case with myelin basic protein, or absolute, as in the case of the β subunit of cholera toxin and ganglioside GM_1.[213] The fact that particular types of lipid are involved has an important consequence for membrane structure: the protein-lipid interaction affects not only the specific protein and lipid class involved but the membrane in general. Segregation of lipids occurs, i.e., lateral phase separation. This is well documented in the case of vesicular stomatitis viral matrix protein.[214] The membrane regions in contact with the peripheral protein are thus enriched in the particular lipid with a concomitant relative depletion of the latter in the rest of the membrane bilayer. As discussed below, peripheral proteins in AChR membranes may also exhibit interactions with certain membrane lipids.

A case of a peripheral membrane protein affecting lipid acyl chains in a specific manner is clathrin. This is an extrinsic membrane protein (M_r 180,000) associated as a trimer of M_r 650,000 together with three additional proteins of M_r 30,000 to form the triskelion, the basic unit of the membrane coat. Membrane coated pits are specialized membrane domains, precursors of the coated vesicles; both participate in various dynamic processes involved in endocytosis in eukaryotic cells. Coated pits differ from other membrane regions in that they contain a higher density of intramembranous particles and integral membrane receptors. They were initially believed not to contain cholesterol. The lack of cholesterol was interpreted as a reflection of the high degree of fluidity required to facilitate the membrane structural changes involved in coated vesicle formation. More recently, however, cholesterol has been found in coated vesicles and its levels do not appear to be very different from those in other membrane domains. These studies also made apparent that the lack of reactivity of filipin — see section on the importance of cholesterol — was not due to abnormally low cholesterol levels but to the stabilizing influence of the clathrin coat, which inhibits the perturbation induced by filipin on cholesterol-rich areas. Clathrin is postulated to be one of the major factors inducing disorder in fatty acid chains of the lipid bilayer responsible for membrane invagination and coated vesicle formation. This is apparently mediated by the increase in the number of gauche conformers in the acyl chains, resulting in augmented disorder of the lipid bilayer in areas of interaction with clathrin.[215] The relevance of this type of study to the AChR case is made apparent in recent Raman spectroscopy experiments on AChR membranes.[216] These studies indicate the acquisition of a temperature-dependent phase transition in the membranes upon extraction of peripheral proteins, suggesting a similar type of interaction between the latter and AChR membrane lipids.

In addition to the AChR protein, receptor-rich membranes from electric tissue contain other nonreceptor, peripheral membrane proteins. The most outstanding of these are various proteins in the M_r 43,000 region, formerly referred to as 43 kDa protein,[217] ν-peptide[218] ν-doublet,[219] or M_r 43,000 protein.[220] They are all peripheral proteins that appear only to have in common the property of being extracted by procedures known to release such proteins from membranes.[204,221,222] Several effects have been reported to occur upon depletion of these proteins from the membrane, such as alterations of the thermal stability[223] and freedom of motion of the AChR,[184,212,219] but only one specific function has been unambiguously ascribed to a particular species of this group of proteins when it was demonstrated that the pI 6.5 to 6.8 M_r 43,000 polypeptides are an isoenzyme of creatine kinase.[224-228] On the other hand, kinetic and equilibrium binding of cholinergic ligands and channel blockers[221,222] and ion-flux response[221,229] have been reported not to be affected by NaOH treatment of AChR membranes. Moore et al.[229] have correctly pointed out, however, that caution should be exercised in interpreting flux experiments in which more than 95% of the AChR channels remain silent in the assay. In other words, any effect introduced by base extraction on AChR-mediated ion fluxes would have passed inadvertently unless it affected the great majority of the components involved in the regulation of ionic permeability.

Removal of substantial amounts of extrinsic proteins from AChR membranes can be

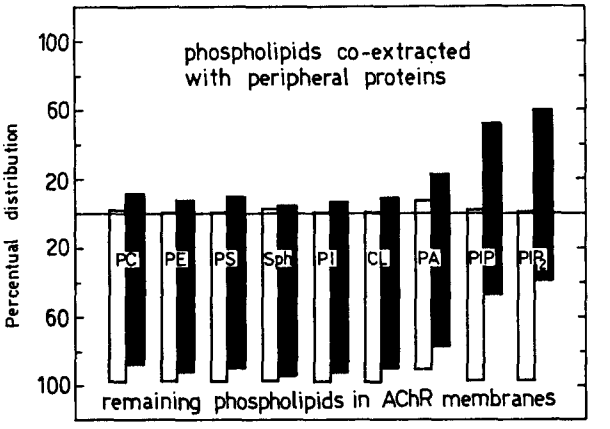


FIGURE 6. Percentual distribution of phospholipid classes co-extracted with nonreceptor, peripheral membrane proteins by alkali stripping of AChR membranes. Empty bars correspond to lipids released from control membranes by hypotonic buffer. Solid bars correspond to the phospholipids extracted by NaOH at pH 11.0. (Drawn from the data of Bonini de Romanelli, I. C., Roccamo de Fernández, A. M., and Barrantes, F. J., *Biochem. J.*, 245, 111, 1987.)

accomplished by various procedures, alkaline treatment being the most commonly employed. This treatment results in changes in the mobility of the AChR (see above) that are sensitive to the lipid environment. Fusion of the alkaline-treated membrane fragments with pure phospholipid vesicles brings about a dispersion of the AChR in the expanded bilayer, while enrichment with cholesterol produces its aggregation and immobilization.[230] The changes brought about by alkaline or diiodosalicylate treatment of AChR membranes have been attributed solely to peripheral protein depletion. We have recently found, however, that minority but metabolically very active acidic phospholipids are also extracted from these membranes together with the peripheral proteins. As shown in Figure 6, only trace amounts of (all) phospholipids are released from AChR membranes by repeated low ionic strength washings. In contrast, extraction of peripheral proteins from these membranes is accompanied by the selective depletion of acidic phospholipids. We have not observed a loss of PE as reported by Martinez-Carrion et al.[231] On a percentage basis, polyphosphoinositides (PIP and PIP_2) constitute by far the most conspicuous phospholipid loss from the AChR membrane.[232] As mentioned above, Aslanian and Négrerie[216] have recently reported that AChR membranes acquire the property of undergoing a thermotropic phase transition (centered around 21°C) upon alkaline treatment, as measured by Raman spectroscopy. The change brought about by NaOH treatment was also attributed solely to peripheral protein depletion, but these results could be reinterpreted in light of our findings on the selective extraction of certain phospholipid classes. No such changes were observed in other studies of AChR membranes before or after NaOH treatment by differential scanning calorimetry[233] and fluorescence anisotropy.[231]

The 43 K protein from *Torpedo* has recently been cloned.[234,235] Froehner[236] has cloned the muscle 43K protein and found in this and its homologous *Torpedo* protein possible sites for fatty acid acylation, as is found with other peripheral membrane proteins (e.g., ligatin[237]). Such sites may provide the anchoring of the 43 K protein to the membrane bilayer and its interaction with certain phospholipids.

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