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Structural and Functional Crosstalk Between Acetylcholine Receptor and Its Membrane Environment*

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*Dedicated to the memory of the late E. De Robertis.

Abstract

Nicotinic acetylcholine receptor (AChR) is a transmembrane protein belonging to the superfamily of rapid, ligand-operated channels. Theoretical models based on thermodynamic criteria assign portions of the polypeptide chains to the lipid bilayer region. From an experimental point of view, however, the relationship between the two moieties remains largely unexplored. Current studies from our laboratory are aimed at defining the structural, dynamic, and functional relationship between membrane lipids and AChR. We are particularly interested in establishing the characteristics of and differences between the lipids in each leaflet of the bilayer and the belt or "annular" lipids immediately surrounding AChR and the bulk bilayer lipids. We are also interested in determining the possible implications of lipid modifications on AChR channel properties. Toward these ends, fluorescence and other spectroscopic techniques, together with biochemical analyses and patch-clamp studies, are currently being undertaken. Correlations can be established between structural aspects of phospholipid packing in the immediate perimeter of AChR and other properties of these annular lipids revealed by dynamic spectroscopic and molecular modeling techniques.

Lipid compositional analyses of the clonal muscle cell line BC3H-l and chemical modification studies have been carried out by incubation of intact cells in culture and of membrane patches excised therefrom with liposomes of different lipid composition. These studies have been combined with electrophysiological measurements using the patch-clamp technique, with the aim of determining the possible effects of lipids on the channel properties of muscle-type AChR. A variety of experimental conditions, involving polar head and fatty acyl chain substitution of phospholipids and cholesterol incorporation, are being assayed in the BC3H-1 cells.

Index Entries: Neurotransmitter receptors; ligand-gated channels; protein–lipid interactions; biological membranes; nicotinic cholinergic receptor.

Introduction

Acetylcholine (ACh) is an ubiquitous neurotransmitter. It can be found at the central (CNS) and peripheral nervous systems (PNS). At the PNS, ACh is released from the nerve terminal and diffuses across the so-called synaptic cleft, an intercellular space of about 20 nm, to react with an integral membrane protein located at the postsynaptic membrane, the nicotinic AChR. On binding to this neurotransmitter receptor, the chemical signal coded by ACh is transduced into a local depolarization of the postsynaptic membrane through the transient opening of a cation-selective channel: ACh acts as an agonist.

AChR is still the best characterized cell surface neurotransmitter receptor, owing to inherent properties of AChR 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 (Changeux, 1981; Barrantes, 1983,1988,1989). Sequence comparison has revealed that AChR belongs to a superfamily of ligand-gated, rapid ion channels, whose members share sequence and presumably structural homologies. The main superfamilies, to date, are constituted by multisubunit ligand-gated cation channels (AChR from electric tissues, muscle, and brain; the ryanodine receptor-associated Ca^{2+} channel, the cGMP-gated Ca^{2+} channel of rod outer segments, 5-HT_3 [serotonin] receptor), ligand-gated anion channels ($GABA_A$ receptor from bovine brain), voltage-gated cation channels (for Na^+, K^+, and Ca^{2+}), and the superfamily of single-chain G-protein–coupled proteins (muscarinic, adrenergic, dopamine, $GABA_B$, 5-HT_1, and 5-HT_2 receptors, one type of quisqualate receptor and rhodopsin). The overall structural motifs found in the macromolecular constituents of different receptor superfamilies are depicted in Fig. 1.

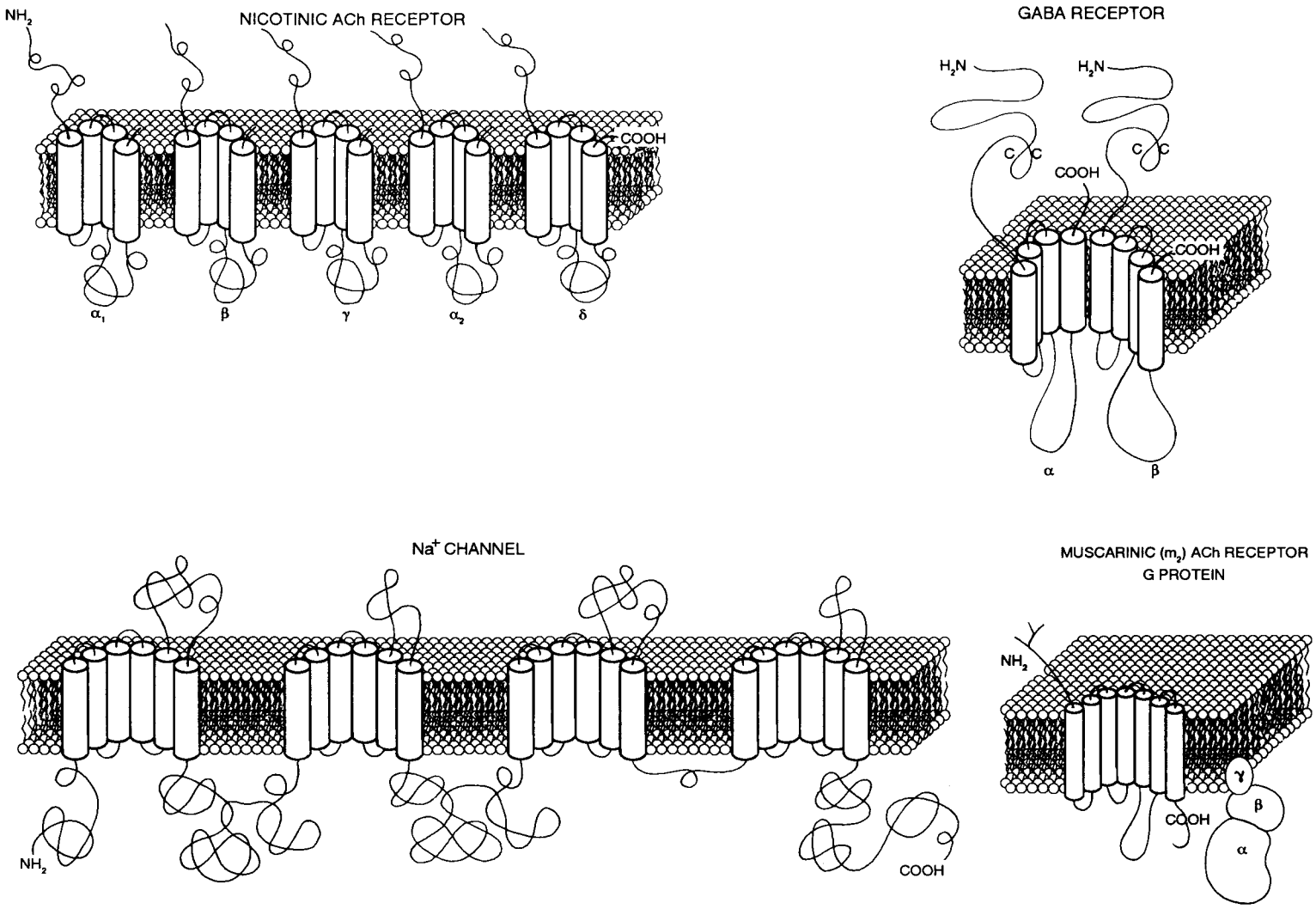

Fig. 1. Comparative schematic representation of the transmembrane arrangements for prototype proteins from different receptor-channel superfamilies. The hydrophobicity profile of the ligand gated cation channel, AChR, indicates the existence of four transmembrane segments, represented by each cylinder, with a similar membrane disposition for all five subunits (top left). The model for the topology of the GABA receptor shows four membrane-spanning helices in each subunit, only some of which will form the inner wall of a central ion channel (top right). The α-subunit of the Na^+ channel, a member of the superfamily of voltage-activated channels, consists of four homologous domains with six transmembrane α-helices (bottom left). The superfamily of the G-protein–coupled receptors, here illustrated with the muscarinic AChR, shares structural features: seven clusters of hydrophobic amino acids with significant amino acid sequence similarity, which may represent membrane-spanning domains. Cytoplasmic regions of the proteins are involved in the interaction with guanine nucleotide regulatory proteins (bottom right).

Nicotinic AChR is a prototype for many membrane-bound proteins that function in the nervous system by transiently altering the electrical properties of the cell membrane in response to the binding of a chemical transmitter. Molecular characterization of this receptor is proving useful for understanding the interactions of small molecules with membrane-bound proteins and the transport of ions across a biological membrane in response to these interactions.

What Makes the Study of AChR Lipid Microenvironment Relevant?

AChR is a phosphoglycoprotein of more than a quarter of a million mol wt composed of two quasiidentical subunits (α) and three additional subunits (β, γ, and δ) in a stoichiometry of α_2, β, γ, and δ (*see reviews* in Changeux, 1981; Barrantes, 1983,1988; Stroud et al., 1990). The functional characteristics of AChR, involving the very fast opening and closing of its cation-selective channel, probably determine the permanent occurrence of five transmembrane polypeptide segments, one contributed by each subunit, in the channel-lining region of the protein. Moreover, the rapidity of the ion transport process probably does not permit this region to be associated with large, energetically costly and time-consuming conformational changes.

This "permanent" structural feature of the channel-forming segments of AChR is likely to determine, in turn, that other portions of the protein are obliged to be topologically related to the adjacent lipid bilayer regions. Interactions between AChR and its surrounding lipids, and between these lipids and the bulk bilayer lipids, are complex in nature and certainly cover a wide time span. Little is known about the nature of such interactions and still less about their consequences for receptor function. Only indirect evidence is available on the alterations of AChR function on disruption of the bilayer by phospholipase A_2 action on the peripheral and neuronal AChR (Barrantes, 1989 and refs. therein). The details of the lipid bilayer structure and the constraints it exerts on the dynamics of AChR in the membrane are also of importance for understanding the function of many integral membrane proteins.

Evidence for Topological Relationship Between AChR and the Membrane Bilayer

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 (Tarrab-Hazdai et al., 1980). The photolabeled portion of the α-chain, a M_r 13,000 hydrophobic polypeptide, was subsequently isolated (Tarrab-Hazdai and Goldfarb, 1982).

Crosslinking other photoreactive probes with the β and γ subunits (Sator et al., 1979) or all four subunits (Middlemas and Raftery, 1983) also has been reported. Arylazido photoreactive phosphatidylcholine (PC) analogs also result in the labeling of all subunits (Giraudat et al., 1985) 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 on desensitizing AChR with high concentrations of carbamoylcholine (Gonzalez Ros et al., 1983), suggesting that exposure of the probe—and by inference of the tagged polypeptide segment in AChR—to the lipid phase varies with conformational transitions of the protein. The same conclusions were reached in previous work from our laboratory in which the intrinsic fluorescence of AChR in its native membrane was quenched with nitroxide spin labels of different degrees of penetration in the bilayer in the presence and absence of the potent agonist suberyldicholine (Barrantes, 1978).

The Transmembrane Nature of AChR Subunits

The abundant evidence on this topic stems from a variety of biochemical studies, including controlled proteolysis (Strader and Raftery, 1980; Wennogle and Changeux, 1980), immunocytochemistry (Tarrab-Hazdai et al., 1978; Strader et al., 1979; Klymkowsky and Stroud, 1979), and lactoperoxidase labeling of portions of AChR from the membrane interior (Hartig and Raftery, 1977). In more recent years, application of cDNA recombinant techniques and the resulting knowledge of complete amino acid sequences, 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: On 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 AChR chains led Noda et al. (1982,1983a,b), Claudio et al. (1983), and Devillers-Thiery et al. (1983) 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 models predicted that both the amino- and carboxyl-termini of the subunits face the extracellular millieu. This type of model—the so-called four-helix model—places about 30% of AChR protein on the cytoplasmic face of the membrane. An alternative type of model (the five-helix model) predicted a much smaller (ca. 20%) portion of AChR on the cytoplasmic compartment; in addition to the four transmembrane helices per chain, it postulated a fifth, amphipathic transmembrane helix (M5, MA, or A) (Finer-Moore and Stroud, 1984; Guy, 1984).

A basic difference between the two models becomes immediately apparent: The five-helix model places the C-terminus on the cytoplasmic compartment. 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 was postulated to provide the lining of the internal walls of AChR ionic channel. Current studies using point mutation and patch-clamp data on the one hand, and immunochemical evidence on the other have made the hypothesis that the MA amphipathic segment is a transmembrane helix less tenable, if not totally indefensible.

Challenging Current Models of AChR Topography

Theoretical models have raised new questions concerning the topography of AChR with respect to the membrane. A drastic revision of the existing models, and in particular of the occurrence of the hydrophobic M4 helix (409–426 in the α-chain) within the lipid bilayer, followed immuno-chemical experiments from Lindstrom's group (Lindstrom et al., 1982): The intracellular location of the β-429–441 (Ratnam et al., 1986a) and α-330–408 (Ratnam et al., 1986b) segments argued against the possibility that M4 was embedded in the bilayer. The latter experimental results also eliminated the MA amphipathic helix of Fairclough et al. (1983) as a likely transmembrane segment and forced its occurrence at the cytoplasmic compartment. This radically different view postulated a rather voluminous mass of AChR protein at the cytoplasmic compartment, at odds with both the 4-, 5-, and 7-helix types of model and with the estimates obtained by electron microscopy/image reconstruction (Zingsheim, et al., 1982a,b; Brisson and Unwin, 1985) and neutron (Wise et al., 1981) and X-ray (Ross et al., 1977) 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 and subunit assembly and in particular,

which portions of the polypeptide chains form the walls of the ion channel or contact other chains and the lipid bilayer. It is also clear that the emphasis and major discrepancies among all models lie in the occurrence of the transbilayer segments and that experimental tests have heavily relied on 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. It must also be borne in mind that the algorithms currently used to predict the occurrence of hydrophobic and hydrophilic stretches in membrane proteins (Kyte and Doolittle, 1982; Hopp and Woods, 1981) consider each individual polypeptide chain by successively scanning "windows" a few amino acids long; the hydrophylic/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. (1986) have indicated the limited effectiveness of the prediction methods as applied to membrane-spanning polypeptide segments. We are currently using molecular modeling and molecular dynamic techniques to explore lipid–AChR interactions by computational methods (Cockroft et al., 1990,1991).

Ecological Niche of AChR

The lipid composition of the *Torpedinidae* electrocyte and AChR membranes has been the subject of study in our laboratory. Phospholipid and fatty acid compositions of electrocyte and AChR membranes from three *Torpedinidae* species (*Torpedo marmorata, Torpedo californica*, and *Discopyge tschudii*) have been analyzed and compared (Rotstein et al., 1987a). 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 80–90% of the total lipid phosphorus. Most of the "choline phospholipids" are made up of PC, the rest being accounted for by sphingomyelin (Sph). One interesting observation stemming from this series of studies was the hypothesis 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 + phosphatidylserine[PS]), suggesting that in analogy with evolutionary conservation of AChR primary structure, its lipid microenvironment needs to be kept similarly constant to ensure optimal receptor function *(see review* in Barrantes, 1989).

Time Domains of Lipid Turnover in AChR Membranes

Metabolic studies using [^{3}H]-18:1,[^{3}H]-20:4, and [^{32}P]-phosphate (Arias and Barrantes, 1987a,b; Bonini de Romanelli et al., 1987; Rotstein et al., 1987b, summarized schematically in Fig. 2) 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 and could play a key role in the modulation of the physical properties of electrocyte membranes. These latter depend strongly on the length and unsaturation of lipid acyl moieties. In particular,

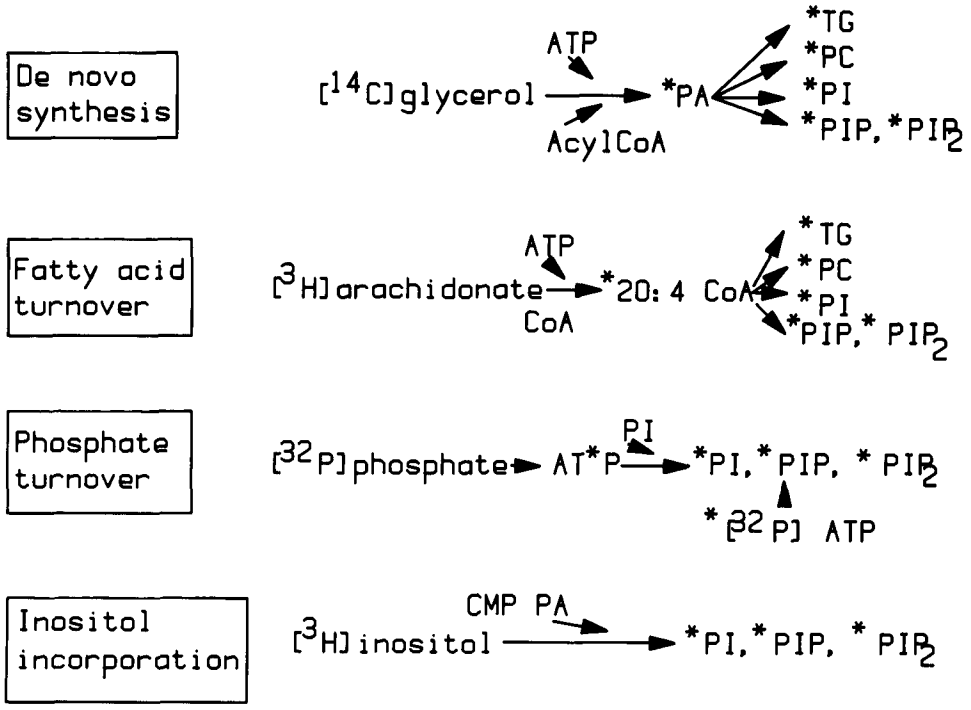

Fig. 2. Diagrammatic summary of the metabolic studies carried out in *Torpedo* AChR-rich cells: the electrocytes (from Arias and Barrantes, 1987a,b; Rotstein et al., 1987b; Bonini de Romanelli, Roccamo de Fernandez and Barrantes, 1987).

acylCoA-lysophospholipid acyl transferases appear to be very active in the electrocyte (Arias and Barrantes, 1987a). 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. The former, more agile mechanism appears particularly suited to a membrane like the one containing 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 (Rotstein et al., 1987b; Arias and Barrantes, 1987a,b) and that this minor class of lipids is known to generate second messengers is worth further analysis.

Mutual Interactions Between AChR Protein and Membrane Lipids

Regardless of the tentative status and the several still obscure aspects of the models analyzed above, extensive physical contact can be assumed to occur between membrane lipid and a significant portion of AChR protein, hence yielding a certain degree of reciprocity in the interactions between lipids and AChR. Little is known, however, about the nature of such interactions and 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 considering 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 *T. 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 (Aslanian et al., 1983). In detergent solution, circular dichroism studies of *T. nobiliana* AChR showed 34% α-helix, 29% β-structure, and 37% random coil (Moore et al., 1974) . Using the same technique, Mielke et al. (1984) found 20% α-helix, 50% β-sheets, and 30% random coil in *T. californica* AChR. More recently, the secondary structure of *T. californica* AChR was studied in reconstituted vesicles by Fourier transform infrared spectroscopy; steroids were found to increase the helical structure of AChR molecule, whereas negatively charged lipids augment from 20 to 24% the proportion of β-sheet structure (Fong and McNamee, 1987).

The effect of protein-AChR-on lipid is even less known. One could extrapolate from other membrane systems and hypothesize about disordering effects of integral membrane proteins on surrounding lipids or on modifications of thermotropic lipid phase transitions resulting from the presence of protein. The available information is far too scarce, however, to be able to elaborate on these topics in the case of the AChR system. A recent calorimetry study (Bhushan and McNamee, 1991) indicates that AChR reduces the enthalpy change associated with the lipid phase transition in reconstituted C18:1, trans 9,10-PC membranes. The lipid phase transition temperature and the cooperativity were found to be reduced. Modeling the behavior indicated a stoichiometry of about 95 lipid molecules per AChR, i.e., higher than had been previously observed with electron spin resonance (ESR) techniques (Ellena et al., 1983, reviewed in Barrantes, 1989). Whereas ESR identifies motionally restricted first-layer lipid surrounding AChR (the "annulus"), differential scanning calorimetry reports on first- and second-shell AChR-*perturbed* lipid. Using infrared spectroscopy (IR), Bhushan and McNamee (1991) found that the interactions between C18:1-PC and AChR were short-range and weak.

Elucidating Structure–Function AChR–Lipid Motifs

The most firm candidate among the transmembrane portions of AChR to be in contact with the lipid is M4 (residues 409–426 in the *Torpedo* α-chain) segment: It is the least conserved among the putative transmembrane segments of nicotinic AChR, an argument that has been invoked by Donnelly et al. (1989) in the case of muscarinic AChR to postulate lipid contacts for analogs portions of this protein. M4 is also the most hydrophobic; it even has a face virtually devoid of H-bonding groups. Giraudat et al. (1985) showed that arylazido photoreactive PC analogs result in the labeling of all subunits, predominantly α, from the lipid phase.

Only recently have experiments been conducted on single-site mutations of residues presumably located at AChR/lipid interface (Pradier et al., 1989; Li et al., 1990) and voltage-clamp recordings conducted on these mutants to test eventual effects on ion permeation. No patch-clamp recordings are as yet available. Pradier et al. (1989) mutated *Cys*-416 and *Cys*-420 of AChR γ subunit in order to test the possibility that the amphipathic MA segment, purported to be a transmembrane domain, constituted the lining of the ionic channel. Mutations of *Cys*- to *Phe*-reduced ionic conductance by 30%; mutations of *Cys*-to *Ser*-had no effect, and the authors concluded that MA had only an indirect relationship with the channel.

Li et al. (1990) conducted site-directed mutagenesis on *Cys*-451 of the *Torpedo* γ-subunit, which lies in the M4 segment. Tobimatsu et al. (1987) had previously shown that deletion of two to four amino acids in M4 of the *Torpedo* α-subunit or total replacement by foreign transmembrane sequences resulted in no loss of AChR channel activity. In some cases, increases of up to 20% ion conductance were achieved, and shortening of M4 by eight residues completely eliminated ion flux. The recent results of Li et al. (1990) appear to indicate that M4 is indeed involved in chan-

nel-gating and/or folding/assembly of AChR: Mutation of *T. californica* γ *Cys*-451 to *Ser*- or *Trp*- would abolish possible fatty acid acylation via an ester linkage.

The α-subunit also possesses amino acid side chains in the M4 segment that are amenable to derivatization. Thus, arylazido photoreactive PC analogs, having their photoreactive group either at the tip of the aliphatic chain or at the phospholipid polar head region, have been successfully used to label all subunits, predominantly the α, from the lipid phase (Giraudat et al., 1985). Interestingly, since α-*Cys*-424 in *T. californica* is uniquely substituted by *Ser*-424 in *T. marmorata*, and the fivefold higher labeling of the α-chain occurred only in the latter species, the difference was attributed to the existence of the reactive *Cys*-424 in the M4 segment of the α subunit in *T. marmorata* AChR. Blanton and Wang (1990) recently have undertaken a similar study using a photoreactive arylazido PS analog. As in the Giraudat et al. (1985) study, all subunits were labeled but α twice as much. Given the position of their probe relative to the bilayer and the positive charge in PS, the authors postulated that *His*-408 and *Arg*-429, located at about the cytoplasmic and extracellular-facing interfaces of α-M4 with the bilayer, respectively, were the likely candidates for the arylazido PS covalent labeling. White and Cohen (1988) also have shown the labeling of α-M4 with a hydrophobic photolabel, trifluoromethyl iodophenyl-diazirine, in an agonist-independent manner, whereas all four subunits—*Torpedo* γ- subunit about four times as much as each of the others—incorporated the probe in an agonist-dependent fashion into a large fragment that contained M1, M2, and M3 segments. Marquez et al. (1989) could identify *Cys*-222 as the residue that was labeled in the M1 transmembrane segment of the α-chain with the fluorescent hydrophobic probe *N*-pyrenemaleimide. In the case of cholesterol analogs, Middlemas and Raftery (1983) were able to label all four subunits of AChR with the photolabel amantane-diazirine (Middlemas and Raftery, 1983).

Structural Asymmetries of AChR

The Annulus

The very nature of the receptor protein—an asymmetric body vectorially oriented with respect to the plane of the membrane—is certain to impose asymmetry on the lipid components, a feature also observed in other natural membranes. One thus can envisage a first type of asymmetry, i.e., the lateral distribution of lipids in the plane of the membrane, which determines in turn the existence of lateral separation among different lipid pools, receptor-associated and bulk lipid, for instance. The lipids at the intramembranous surface of integral membrane protein can be detected using spin-labeled lipid molecules. This is fortunately a sensitive dynamic biophysical method to evaluate the mobility of the lipids at the protein interface. One finds that lipid mobility at the belt surrounding the protein (the "annulus") is reduced relative to that of the bulk membrane lipid, giving rise to a two-component ESR spectrum from which the number and selectivity of the lipids at the lipid-protein interface may be quantified (*see* e.g., Marsh and Watts, 1982). In this way, we were first able to demonstrate the protein-induced restriction in mobility of spin-labeled fatty acids and spin-labeled androstanol (Marsh and Barrantes, 1978) and of spin-labeled phospholipids (Marsh et al., 1981) in AChR-rich membranes from *T. marmorata* (Fig. 3).

It has subsequently been demonstrated that spin-labeled sterols, phosphatidic acid, and fatty acids associate preferentially with AChR rather than with other kinds of lipid (Ellena et al., 1983). In parallel studies, it was found that cholesterol and negatively charged phospholipids were required to support the ion-gating activity of AChR (Fong and McNamee, 1986) whereas fatty acids block the ion-flux response (Andreasen and McNamee, 1980). The latter was interpreted as the perturbation of the functionally significant interaction between AChR and cholesterol or negatively charged phospholipids.

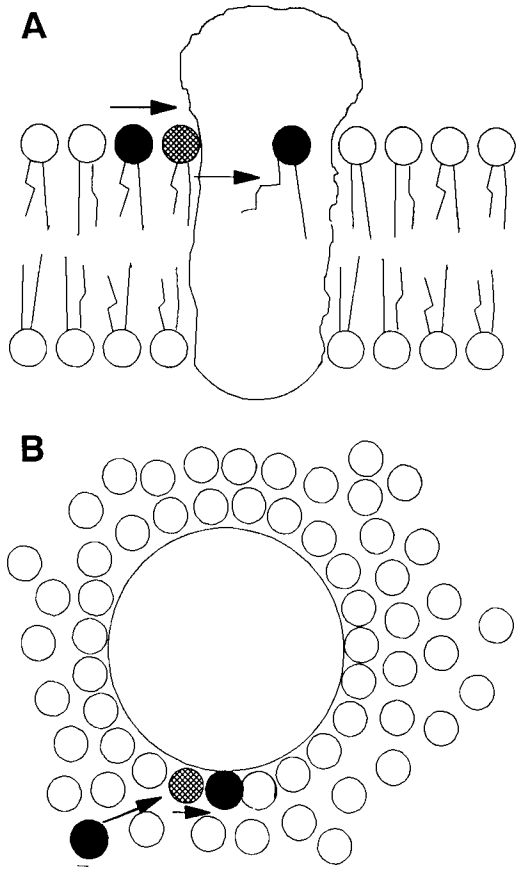

Fig. 3. Fluid bilayer lipid (their polar head region is represented with an empty circle) and less mobile annular lipids (crosshatched circles) associated with AChR exhibit exchange rates of $2–5 \times 10^7$ s^{-1} (Marsh and Barrantes, 1978; Marsh, Watts, and Barrantes, 1981; Ellena, Blazing, and McNamee, 1983). The lateral translation of a nonannular phospholipid (full circle) into the belt, annular region occupied by the less mobile lipids (crosshatched circle) is schematically depicted in a lateral view **(A)** and in an end-on view **(B)**.

The Two Leaflets of the Bilayer

The second type of asymmetry that may be present in the AChR-rich membranes and by extension in the postsynaptic membrane is that occurring between the two leaflets of the bilayer, facing the extracellular and cytoplasmic compartments, respectively. Given the shape of the AChR macromolecule, the high-density packing of AChR assemblies in the postsynaptic membrane is likely to be facilitated by and coupled with an asymmetric disposition of lipid classes between the two leaflets.

Recently, we have conducted a series of studies in order to establish the phospholipid distribution between the two leaflets of the lipid bilayer in AChR-rich membranes from *T. marmorata* with two complementary techniques: chemical derivatization with the membrane-impermeable reagent trinitrobenzenesulphonate (TNBS) and *B. cereus* phospholipase C hydrolysis. AChR membranes were reacted with TNBS at 0–4 and 37°C, and the accessibility of their aminophospholipids was compared to that of rod outer segment and erythrocyte membranes. The results showed that more of the total ethanolamine glycerophospholipid than of the total phosphatidylserine is located in the outer monolayer, whereas phosphatidylinositol, most of the sphingomyelin, and about 65% of the phosphatidylserine are located on the inner leaflet (Bonini de Romanelli et al., 1990).

Lipid–AChR Interface as the Site of Action of Some Noncompetitive Antagonists

Using spin-labeled cholestane, we have found recently that the probe was incorporated into AChR-rich regions that were to a certain extent enriched preferentially in the steroid (Arias et al., 1990). The ability of certain local anesthetics to modify steroid–AChR interactions was investigated by fluorescence techniques. The effectiveness of the anesthetics in inhibiting cholestane-induced AChR intrinsic fluorescence followed the order: procaine >> benzocaine > tetracaine > QX-222. The data could be interpreted in terms of a model requiring specific association sites for local anesthetics on the hydrophobic surface of the AChR protein that at least partially overlap with those for steroids (Arias et al., 1990). We also have used these two techniques to establish the relative affinities of various local anesthetics for

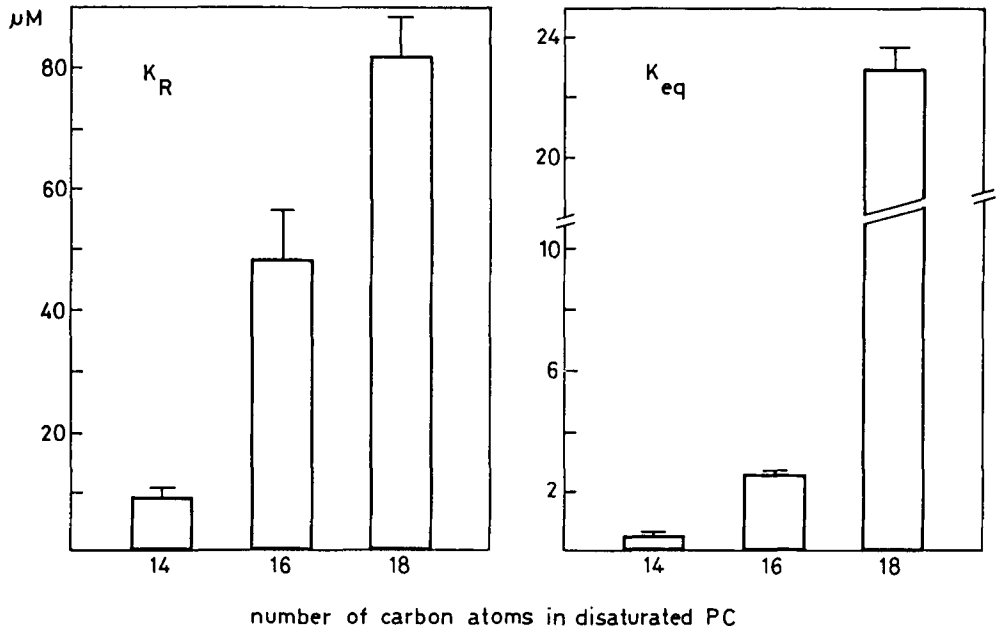

Fig. 4. Increasing fatty acid chain length of phosphatidylcholines in reconstituted AChR diminishes the affinity of AChR for the agonist carbamoylcholine. (Criado, Eibl, and Barrantes, 1984).

AChR by comparison with the relative association constant for spin-labeled phospholipid (K_{ro}). It was possible to differentiate between high ($K_r/K_{ro} > 2$), intermediate (1.6–1.9), and low (< 1.3) specificity and to calculate the fraction of protein-associated local anesthetic in each case (Horvath et al., 1990).

Lipid Composition

Systematic reconstitution studies have been carried out in our laboratory using *Torpedo* AChR liposomes of pure synthetic lipids (Criado et al., 1982,1984). With increasing chain length of the saturated PCs, a marked increase in carbamoylcholine dissociation constants was observed (Fig. 4). The increase affected both of 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 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 (Rotstein et al., 1987a). Substitution by other dimyristoyl phospholipids for dimyristoyl PC had the same, though quantitatively less pronounced, effects (Fig. 5). Introduction of unsaturation in the acyl chains dramatically reversed the effect of increasing chain length (Fig. 6). These results concur with the observed fatty acid composition of PCs in the native AChR membrane (Rotstein et al., 1987a).

In another series of studies from our laboratory, the effect of lipid classes on the channel-gating properties of AChR has been examined using radioactive tracer flux measurements. Unsaturated PEs in combination with 28–35 mol% of cholesteryl hemisuccinate was the best lipid mixture for reconstitution of the receptor-gating function in artificial lipid systems (Criado et al., 1984). From this we concluded that zwitterionic phospholipids such as PE may be important in AChR channel function. An acidic phospholipid, phosphatidate, also has been found to display favorable effects on channel-gating properties (Fong and McNamee, 1986).

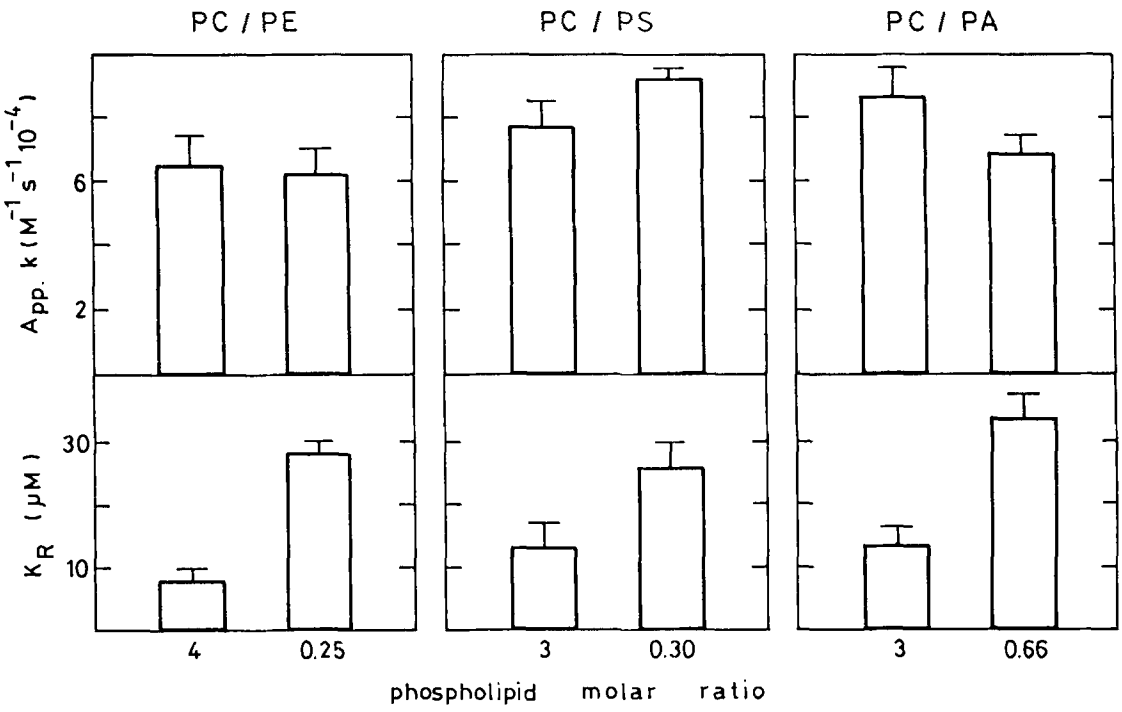

Fig. 5. Apparent toxin association rates (k) are not significantly modified through substitution of dimirystoyl phosphatidylcholine by other glycerophospholipids with different head group. PE, ethanolamine; PS, serine; and PA, phosphatidic acid. On the other hand, the apparent dissociation constant for the agonist carbamoylcholine (K_R) tends to increase with increasing degree of substitution of PC by other phospholipids while maintaining the same fatty acid chain (14:0). (Criado, Eibl, and Barrantes, 1984).

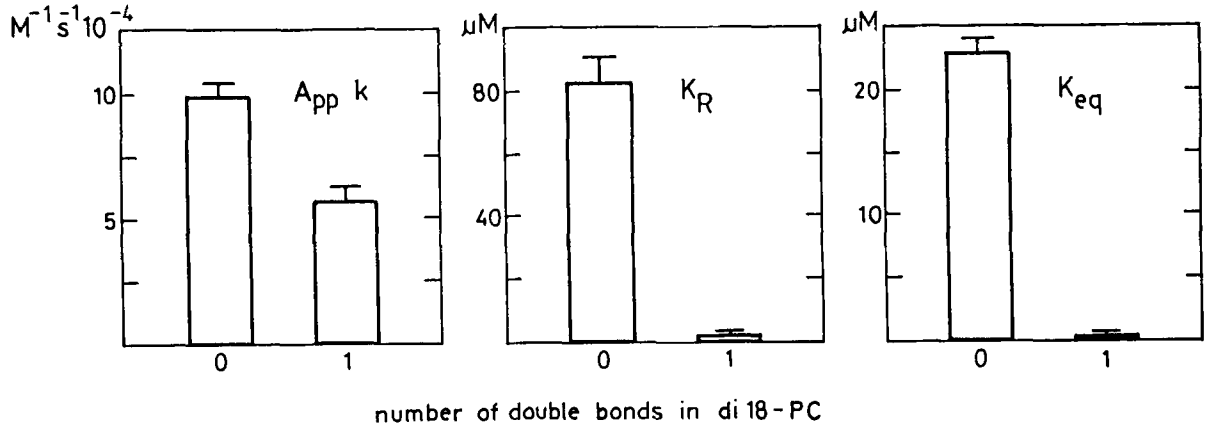

Fig. 6. Introduction of a single double bond in dipalmitoyl PC has a dramatic effect on the apparent dissociation constants of carbamoylcholine (K_R and K_{eq}) The affinity for the agonist is higher in a monosaturated lipid environment. (Criado, Eibl, and Barrantes, 1984).

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 in ion transport was apparent, even when similar vesicle size, degree of receptor incorporation, and agonist-induced affinity transitions were obtained (Criado et al., 1984). One of the conclusions drawn from these experiments is that maintenance of affinity state transitions of reconstituted receptor is a necessary but not sufficient condition for the manifestation of ion-gating receptor activity. A second conclusion 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 (Criado et al., 1984) show concurrence with more recent analyses of the fatty acid molecular species in native membranes (Rotstein et al., 1987a) showing more than 75% polyenes in PE (of which the majority are hexaenoic molecular species). 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 (but *see below).* In only one special case a synthetic PC was used, but its proportion with respect to residual endogenous lipids was not assessed (Boheim et al., 1981).

Lipid Modification

One of the approaches we have adopted in our laboratory is the study of the possible influence of lipid modifications on the function of AChR *in situ.* One of the reasons for undertaking this type of study is that lipids appear to affect the function of some membrane proteins involved in ion transport. Three transporter systems from muscle membranes—the Na^+, K^+-ATPase, and the Na^+–Ca^{2+} exchange system from the sarcolemma and the Ca^{2+}-ATPase from the sarcoplasmic reticulum—have been analyzed in this connection. Cholesterol appears to modulate the activity of the two former, whereas it does not influence that of the latter (Vemuri and Philipson, 1990). The effects exerted by a given type of phospholipid fatty acyl chain are similar for the three transporter systems. Thus, optimal conditions were met for fully saturated PC and unsaturated PS and different chain lengths for fatty acids in these two phospholipids (Vemuri and Philipson, 1990). These authors hypothesize that it is disorder, mainly provided by unsaturated anionic amphiphiles such as PS, that exerts the greatest influence on exchange systems.

One of the experimental systems used in our laboratory is the mouse muscle clonal cell line BC3H-1. This cell undergoes developmental changes involving the appearance of functional AChR in its plasmalemma. With the aid of a phospholipid exchange protein, we have managed to introduce exogenous phospholipids by incubation of intact cells in culture and of membrane patches excised therefrom with liposomes of different lipid composition. The mouse muscle AChR from the BC3H-1 cell line has been extensively characterized, and in particular, its physiological and pharmacological properties have been worked out in great detail, thus making these cells a very suitable model for studying subtle changes following environmental modifications. Figures 7 and 8 show examples of one of the variables amenable to study—the AChR channel mean open time-using the patch-clamp technique. The examples illustrate the sensitivity of the AChR to chemical modification of the protein moiety.

With the aim of determining the possible effects of lipids on the channel properties of muscle-type AChR, we are currently undertaking patch-clamp recordings of AChR channels in the normal and lipid-modified BC3H-1 cell line using a variety of experimental conditions involving polar head and fatty acyl chain substitution of phospholipids (different concentrations and application time, sidedness of application, and use of phospholipid exchange protein or not). The PC or PC-PS mixtures having different degrees of fatty acyl chain saturation have been assayed

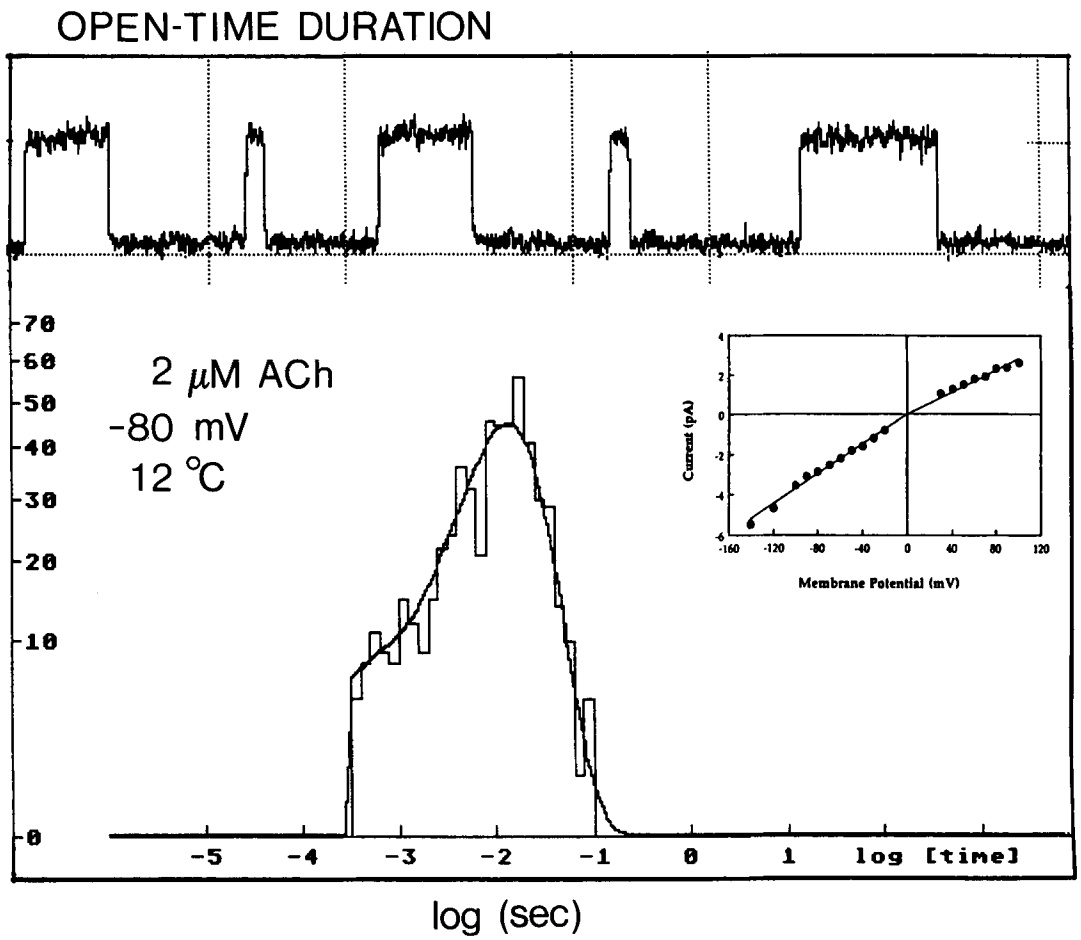

Fig. 7. Open-time duration of single-channel events in a BC3H-1 muscle clonal cell. Raw single-channel traces (top part), open time duration histogram (central part), and current-voltage relationship (insert) from a cell-attached patch obtained from a cell using the low ACh concentration, the membrane potential, and the temperature indicated in the figure. The histogram indicates that there are two types of AChR channels in BC3H-1 cells: a briefly opened channel (mean open time 0.44 ms) and a major longish component (mean open time 14.4 ms) (modified from Bouzat, Barrantes, and Sigworth, 1991, *Eur. J. Physiol.* Pflugers Arch **418**, 51–61).

and shown to be effectively incorporated into BC3H-1 cells for up to 48 h. The fatty acid moiety of the donor phosphatidylcholines is subsequently actively redistributed among most phospholipids in these cells (Table 1).

The imposed changes of the phospholipid composition appear to have little or no effect on the number of AChR molecules at the cell surface (data not shown).

The preliminary single-channel recordings (Table 2) are indicative of very small changes in the kinetic properties of AChR channel; the maintenance of one of the key functional properties of the receptor in the modified lipid millieu probably reflects the homeostatic ability of the cell to cope with fluctuations in its surface membrane environment.

We also have followed the incorporation of [^{3}H]-cholesterol into BC3H-1 cells. The steroid is actively incorporated into preconfluent and differentiated cells, reaching plateau values in a 48-h period. One of the striking effects of choles-

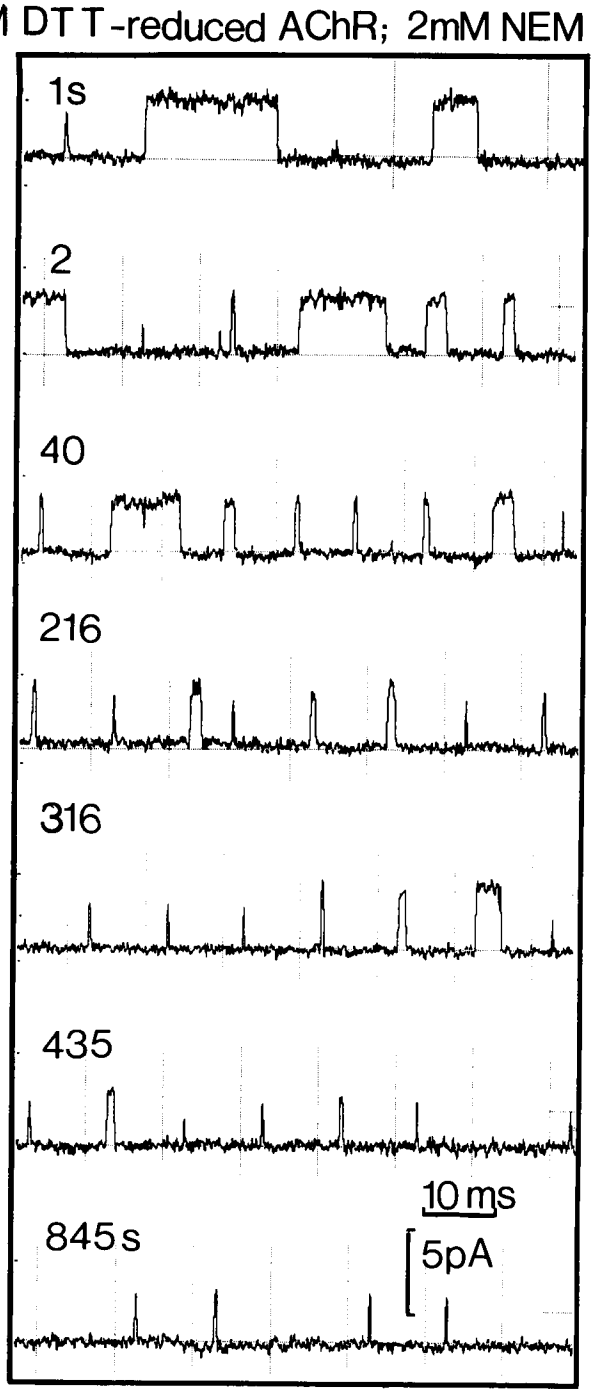

Fig. 8. Example of the sensitivity of AChR channels to chemical modification. A BC3H-1 muscle clonal cell was treated with 2 m*M* dithiotreitol, and then single channels were recorded by the patch-clamp technique in the presence of 2 μ*M* ACh and 2 m*M* *N*-ethylmaleimide in the pipette solution at a membrane potential of –80 mV and at a temperature of 12°C. The traces from top to bottom correspond to successively acquired traces, expressed in seconds (modified from Bouzat, Barrantes, and Sigworth, 1991, *Eur. J. Physiol.* Pflugers Arch, **418**, 51–61).

Table 1
Distribution of Label Different in Lipids of BC3H-1 Cells
After Incubation with 1-Palmitoyl-[^{14}C]Arachidonoyl-Phosphatidyl-Choline[a]

Lipid	% of incorporation	
	24 h	48 h
PS	4.0 ± 2.0	0
PA	1.2 ± 0.8	0
PI		3.3 ± 1.6
PC	31.0 ± 7.0	51.0 ± 12.0
PE	0.6 ± 0.5	9.3 ± 4.0
Free fatty acids	45.8 ± 9.0	24.8 ± 6.0
Neutral lipids	17.4 ± 4.0	11.6 ± 4.0

[a]BC3H-1 cells were incubated with [^{14}C]arachidonyl-PC for 24–48 h in the presence of a nonspecific phospholipid transfer protein. After the incubation periods, the cultures were rinsed with phosphate buffer, scraped with a Teflon spatula, transferred to glass tubes, and processed for lipid determination. The results represent the average of three determinations ± SD (Bouzat et al., unpublished results).

Table 2
Mean Open Time and Conductance of AChR Channels
Recorded from Cells Incubated with Liposomes of Different Lipid Composition[a]

Condition	Mean open time, ms	Conductance, pS
Control	8.51 ± 0.68	35 ± 1.0
Unsaturated PC[a]	9.00 ± 0.30	35 ± 1.2
Saturated PC[a]	7.98 ± 1.23	36 ± 0.9
Saturated PS-PC[b]	7.53 ± 1.87	36 ± 1.1

[a]Postconfluent cells were incubated with medium containing liposomes of dimiristoyl-PC (saturated) or PC isolated from retina (with a high degree of unsaturated fatty acids) during 5 d. The lipid concentration in the medium was 40 μg/mL.

[b]Postconfluent cells were incubated for 1 h at 12°C with medium containing liposomes of 70% dioleoyl-PC-30% dioleoyl-PS. The final lipid concentration was 100 μg/mL medium. Recordings were made at a membrane potential of –80 mV in the presence of 2 μ*M* ACh in the pipette solution. Open time histograms for channels lasting more than 1 ms were fitted with a single-exponential function (Bouzat et al., unpublished results).

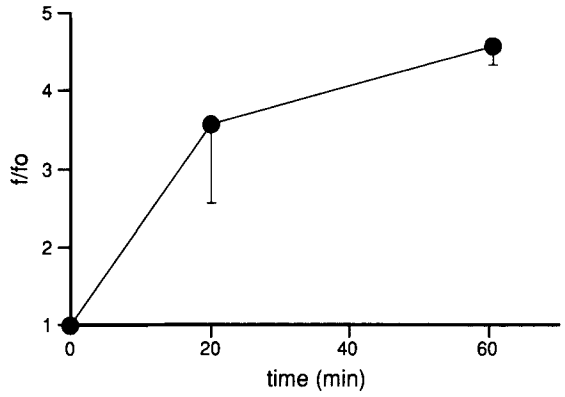

Fig. 9. Cholesterol affects the frequency of single channel events in the intact BC3H-1 cell. The frequency of opening events was determined in cells incubated with 0.5 m*M* cholesterol at room temperature and for periods of 20 and 60 min. Channels were then recorded in the cell-attached configuration in the presence of 100 μ*M* ACh in the pipette and at a membrane potential of –80 mV. The figure shows the relationship between the number of channels/s and the time of incubation with cholesterol. The f_o corresponds to cells incubated in cholesterol-free medium. The number of channels per second is the average over 240-s recording intervals. Each point corresponds to four experiments with the corresponding standard deviation (Bouzat et al., unpublished).

terol is that it produces a 40% increase in the frequency of single-channel events when applied to excised membrane patches, without affecting the conductance or mean open time of AChR channel (Bouzat et al., unpublished and Fig. 9).

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Corrigendum

Axon–Myelin Transfer of Phospholipids and Phospholipid Precursors: *Labeling of Myelin Phosphoinositides Through Axonal Transport*
by Robert W. Ledeen, Francis Golly, and James E. Haley
Molecular Neurobiology
volume 6, numbers 2–3, 1992

Humana regrets that an error was introduced into the final version of Table 2. The corrected Table 2 appears here.

Table 2
Incorporation of [^{3}H]inositol into Myelin Phosphoinositides

	7 d		21 d	
	CL–IL	CL/IL	CL–IL	CL/IL
Optic tract				
PI	2070	14	1090	12
PIP	834	7.6	571	6.9
PIP_2	234	4.1	744	7.8
Superior colliculus				
PI	125	3.1	176	2.9
PIP	122	3.9	296	7.0
PIP_2	243	13	200	4.6

[a]These data were derived from the same experiment depicted in Fig. 1 following isolation of doubly labeled phosphoinositides. ^{3}H counts are expressed as DPM/10 mg myelin, actual counts (CL–IL) in any given sample being approx 35–75% of those shown. Values shown are the average of two independent determinations.