Structural and Functional Modulation of Ion Channels by Specific Lipids: from Model Systems to Cell Membranes

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8.1 Introduction.

Biological membranes provide specialized permeability barriers for cells and cell organelles, in which the interplay of lipids and membrane proteins facilitates a wide range of key biochemical processes. These include respiration, photosynthesis, protein and solute transport, signal transduction, and motility. The interactions between membrane proteins and the lipid bilayer have to allow for structural protein rearrangements while keeping the sealed nature of the membrane. This is especially important because many membrane proteins undergo conformational changes that take place in or affect the transmembrane regions which are be essential for their activity. The mobile and flexible lipid molecules are excellent candidates for maintaining this sealing function as they can adhere to the surface of integral membrane proteins and adjust to a changing environment.

A large number of biochemical and biophysical studies have demonstrated the importance of protein–lipid interactions in the assembly, stability, and function of membrane proteins (Lee 2003). Indeed, intrinsic membrane proteins including ion channels, often show an absolute requirement for lipid. In the study of such requirements, different aspects of membrane protein–lipid interactions have to be considered. First, the lipid bilayer provides the matrix in which membrane proteins are partially or fully embedded. However, the bilayer is not a passive homogeneous media, in fact, partitioning of certain proteins is enhanced by specific interactions with lipids. This sometimes leads to the formation of microdomains or "lipid rafts", which are proposed to have a role in signal transduction, membrane transport, and protein sorting, and might serve as mobile platforms for the clustering and organization of bilayer constituents including ion channels (Brown and London 1997; Simons and Toomre 2000; Shogomori and Brown 2003). Second, spin-label EPR experiments show that a first shell of motionally restricted lipids surrounds the transmembrane segments of proteins (Marsh and Horvath 1998). Lipid specificity has been demonstrated as well in numerous biochemical studies, which showed that certain phospholipids are essential for the activity of several membrane proteins (Dowhan 1997; Lee 2004; Tillman and Cascio 2003). Finally, tightly bound lipids have been detected in the X-ray crystal structure of several membrane proteins and have been included in the model and refined with the protein (see refs in Lee 2003). In most cases, these structurally resolved lipids are co-purified with the membrane proteins, and crystallized as protein–lipid complexes. The existence of such high-

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affinity lipid binding sites on the protein provokes discussion and stimulates research to elucidate their possible functions (Pebay-Peyroula and Rosenbusch 2001).

The aim of this chapter is to examine how the nature and properties of the bilayer affect ion channel structure and function. As examples, we have focused on specific K^+ ion channels and on the nicotinic acetylcholine receptor (nAChR), two classes of ion channels representative of voltaged-gated and ligand–gated (LGIC) families respectively and whose structure and function have been documented during the past decade (Doyle et al. 1998; Unwin 2003) The responsiveness of these ion channels to changes in the lipid environment illustrate how ion channels and perhaps many other membrane proteins, may be regulated via cellular control of membrane composition.

8.2 Importance of Lipid-Protein Interactions In Ion Channel Modulation

Ion channels are at the centre of many complex physiological processes such as the control of the beating rate in the heart or the generation of electrical signals in the brain. Ion channels involved in these processes possess two basic characteristics. The first is that they are selective, either for a range of ions (e.g. the nAChR channel, which is cation-selective) or for specific ions (e.g. K^+ channels). Second, the channels have the ability to control movement of these ions, that is, to open or close the ion conduction pathway, a process known as channel gating.

Ion channels accomplish their function of ion permeation after being inserted into the lipid bilayer of the membrane. The process of insertion is poorly understood and most likely rather heterogeneous. Once the ion channel is placed into the lipid membrane, it is believed that the protein assumes an energetic minimum, leading to a stable structure. However, molecular insights into the functioning of ion channels indicate that the processes of ligand-binding (Karlin 2002) and/or voltage sensing (Starace and Bezanilla 2004) are able to modify such structures.

Ion channels are generally multi-subunit complexes, with the ion conduction pathway formed as an aqueous pore at the interface between the subunits. The movement of transmembrane segments is crucial in controlling the diameter of the ion pathway and, as a consequence, determines whether the channel is in either the closed or in the open state. So far three possible alternatives have been entertained to explain movement of the transmembrane helices during channel gating based on the recently determined structures of mechanosensitive, (Chang et al. 1998), ligand-gated (Unwin 2003) and voltage-gated channels (Kuo et al. 2003). All of them move transmembrane segments away from the central ion conduction pathway which results in the displacement of a hydrophobic gate from the ion conduction pathway, allowing ion movement through the channel. The channels would achieve this by moving the transmembrane helices as rigid bodies using three major types of motion: helix tilting as in MscL (Sukharev et al. 2001), rotation as in the nAChR (Miyazawa et al. 2003), or bending as in Kv1.1 (Doyle 2004). In all cases there are

large movements that require a certain degree of flexibility and may cause modifications at the protein lipid interface. Thus, it is possible that changes in the lipid composition may affect channel function by stabilizing distinct functional states.

Reconstitution experiments have established that specific lipids or particular combinations of lipids are often necessary for ion channels to exhibit their native properties (Lee 2004). These preferences may reflect the optimisation of protein structure by the specific lipid environment in which the protein is found, but may also be related to the folding of the nascent protein and its oligomerization, to its targeting to a given membrane microdomain, or to a lipid-mediated regulation of the protein function.

Although several important questions regarding the molecular details of such lipid modulation remain open, emerging data indicate that protein–lipid interactions should be considered as a new explanation for ion channel function modulation that might even result of possible therapeutical interest.

8.3 Hypothetical Nature of Lipid-Protein Interactions

Despite the extensive information obtained on the functional and structural dependence of several ionic channels on its surrounding lipids (Lee 2004; Palsdottir and Hunte 2004), several aspects of the modulation exerted by the different lipid classes on the membrane proteins still remain unclear. Various hypothesis have been implicated in the modulation of ion channels function by lipids: (1) modification of bilayer physical properties, such as fluidity, membrane curvature, and/or lateral pressure (Cantor 1997; de Kruijff 1997; van den Brink-van der Laan 2004), (2) direct effects, exerted through binding to specific sites on transmembrane portions of the protein (Fong and McNamee 1987; Jones and McNamee 1988; Blanton and Wang 1990; Fernández et al. 1993; Fernández-Ballester et al. 1994; Powl 2005), in some cases acting like allosteric modulators and (3) promotion of lateral segregation of specific lipids and formation of lipid domains (Martens et al. 2000; Brown and London 2000). One possibility does not preclude others, the affinity of a protein for specific lipids may either stabilize certain protein conformations, induce domain formation or serve to target the protein to specific membrane domains with different biophysical properties.

In the case of modulation of ion channels structure or function by changes in bilayer physical properties, it is known that the effect of packing different lipid species with each other and with proteins results in mechanical pressures in the biological membrane, the redistribution of pressures being related to changes in bilayer thickness, curvature stress or hydrophobic matching. Such biophysical phenomena have been suggested as a possible mechanism to explain the influence of bilayer properties on ion channel structure and function. The lateral pressure profile may influence protein conformation directly, by mechanical pressure at the lipid-protein interface or indirectly by changes in other membrane parameters. An

example on ion channel unambiguously influenced by this mechanism is the mechanosensitive channels (Perozo et al. 2002).

On the other hand, biological membranes contain a wide variety of lipids species with different fatty acyl chains so that the lateral diffusion of lipid molecules within the plane of the membrane will result in local fluctuation of membrane thickness. The hydrophobic thickness of the lipid bilayer is expected to match well the region of any protein embedded in the bilayer, because of the high cost of exposing either fatty acyl chains or hydrophobic amino acids to water. Any mismatch between the hydrophobic thicknesses of the lipid bilayer and the protein would be expected to lead to distortion of the lipid bilayer, or the protein, or both, to minimize the mismatch. The activity of a number of membrane proteins is sensitive to the thickness of the lipid bilayer, with the optimal thickness usually corresponding to that of the bilayer of dioleoylphosphatidylcholine (Lee 1998).

A second alternative mechanism to explain modulation ion channel structure and function by lipids may be through a direct interaction. To these respect a large variety of biochemical and biophysical studies have demonstrated the existence of a direct lipid-protein interaction. These studies include fluorescence spectroscopy, electron paramagnetic resonance spectroscopy, photoaffinity labelling, X-ray crystallography and electron microscopy (EM).

The first clue about how lipid molecules might interact with an intrinsic membrane protein came from electron spin resonance (ESR) studies using phospholipid molecules with nitroxide spin labels attached to selected positions in the fatty acyl chains. These studies show the presence of a subpopulation of highly immobilised spin labels, not found in protein-free membranes (Marsh and Horvath 1998; Marsh 2004). The ESR approach can be used to estimate the number of lipid molecules bound to the surface of a membrane protein. In a series of studies, Marsh and Horvath (1998) showed that the number of bound lipid molecules fits reasonably well to the expected circumference of the trans-membrane region of the protein. The close relationship between the number of lipid molecules estimated to surround a membrane protein and the diameter of the protein supports the presence of a distinct annular shell of lipid molecules around that protein.

Fluorescence quenching studies show binding of specific phospholipids with different affinities to some ion channels. These lipids, often bound between transmembrane α-helices either within a protein or at protein–protein interfaces in multisubunit proteins, have been referred to as “non-annular lipids” (Marsh et. al 1982). For example, the KcsA channel requires the presence of some anionic lipids for its function, and fluorescence quenching studies show the presence of two classes of lipid binding sites on KcsA. At one of them (non annular sites) anionic phospholipids bind more strongly than phosphatidylcholine (PC), whereas at the other (annular sites) PC and anionic phospholipids bind with equal affinity. (Williamson et al. 2002).

The high-resolution structure of membrane proteins obtained by X-ray or EM sometimes show tightly bound lipids, thus providing a new insight into protein–lipid interactions. However only a small number of lipid molecules have been resolved from the structures of membrane proteins by high-resolution X-ray or EM. Since

only highly ordered lipid molecules are seen in these structures, such lipid molecules should not correspond to the bulk of lipid molecules surrounding the protein. Even more, annular lipids will normally be too disordered to appear in high-resolution structures. Therefore, most of the lipid molecules resolved in high-resolution crystal structures of membrane proteins are likely to be non annular lipids Their strong binding to the protein leads to immobilisation of, at least, part of the lipid molecule so that they appear in the high-resolution structure. In both X-ray diffraction and EM studies, the lipid headgroups are disordered but many fatty acyl chains are well resolved, mostly bound in distinct grooves on the surface of the protein. Phosphatidylglycerol (PG) is a typical non-annular lipid molecule bound between transmembrane α-helices at monomer–monomer interfaces in the homotetrameric potassium channel KcsA. Again the headgroups of the lipid molecules are not resolved, and the lipids have therefore been modelled as diacylglycerol (Zhou et al. 2001). KcsA requires the presence of PG or some other anionic phospholipid to function and it has been suggested that the presence of the anionic lipid "cofactor" could be important in the gating process (Valiyaveetil et al. 2002; Heginbotham et al. 1998).

The third hypothetical mechanism by which lipids can modulate the function and/or structure of ion channels is the formation of lipids domains. These dynamic structures, generally termed lipid rafts, are rich in tightly packed sphingolipids and cholesterol (Harder et al. 1997) and are believed to exist in a different phase state to that of the surrounding phospholipids (Brown and London 1997). The first report of ion channels localized in lipid microdomains described the targeting of Shaker-like K^+ channels to lipid rafts (Martens et al. 2000). Biochemical isolation of rafts shows that the $K_V2.1$ channel is associated with rafts in transfected fibroblasts and rat brain. Depletion of membrane cholesterol using cyclodextrin, an agent known to perturb raft organization (Brown and London 2000), causes a dramatic hyperpolarising shift in the steady-state inactivation properties of the $K_V2.1$ channel, whereas other K_V channels in the same cell type, such as $K_V4.2$ channels, are unaffected. Therefore, at least for some channel proteins, there is a functional consequence of their association to lipid rafts. Additional reports suggest that other types of ion channels, including both voltage- and LGIC, are also associated with lipid rafts. For example, Ca^{2+}-activated K^+ channels are sorted to lipid rafts on the apical membrane of Madin-Darby canine kidney cells (Bravo-Zehnder et al. 2000). The neural α7 nNAcChR, a protein with a well-known to have preference for certain lipids, was recently shown to target to lipid rafts in the somatic spines of ciliary neurons (Bruses et al. 2001). The muscle like nNAcChR had been shown to produces lateral phase separation of the monoanionic phosphoryl form of the phosphatidic (PA) acid probe, causing the formation of specific PA-rich lipid domains that become segregated from the bulk lipids (Poveda et al. 2002). Although it appears that several ion channels might be localized to these rafts domains, several questions regarding the mechanism and function of ion channel–raft association need to be addressed.

It is clear that while the role that membrane lipids play in membrane structure and function is beginning to be understood, there is still a gap in our knowledge of the

complexity of the specific interactions. Since a detailed understanding of the lipid-protein interactions in the membrane requires the knowledge of the dynamic phenomena involved, it appears that complementary methods, including static, like crystallographic analyses and dynamic like functional assays of reconstituted proteins should be considered to achieve a better comprehension of this interaction.

8.4
Influence of Lipids on nAcChR Function

LGICs are membrane proteins that transiently open a pore through the lipid membrane in response to neurotransmitter binding. The nAChR is one of the best understood members of this family, principally due to two factors that have aided in its characterization: (1) the rich source of nAChR present in the electric organ of some fishes (*T.marmorata, T. californica and E. electricus*) and (2) the presence of neurotoxins in snake venoms that bind specifically to the nAChR providing the means for assaying receptor binding and for affinity purification.

nAChRs are heteropentamers comprised of four different but highly homologous subunits designated as α, β, γ and δ (Fig. 8.1; for reviews see refs in Karlin 2002). Each subunit contains an extracellular N-terminal domain (which include the ACh binding sites), four hydrophobic transmembrane (TM) domains (M1-M4), and a small extracellular C-terminal domain. Several studies have provided convincing evidence that the TM2 domain segments from each subunit cluster around a central axis to form the ion channel pore, whereas TM1 TM3 and TM4 domains are in close proximity or exposed to the lipid interface (Miyazawa et al. 2003; Barrantes 2003).

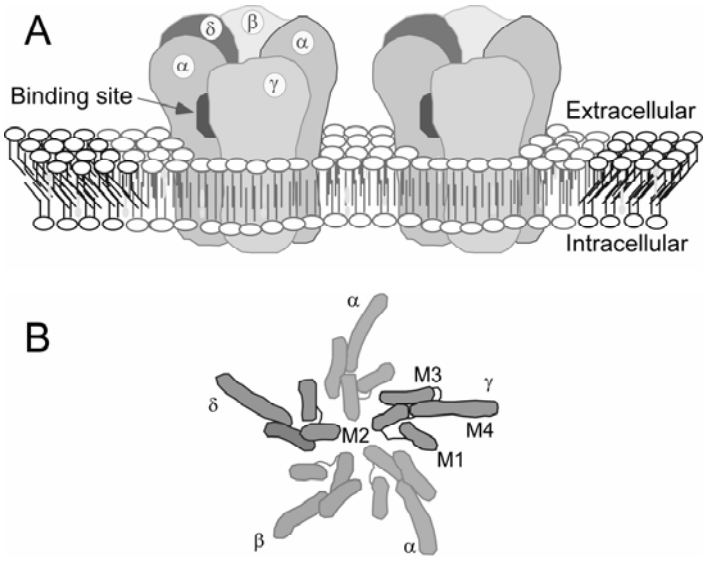

Fig.8.1 (a) Schematic representation of the quaternary structure showing the arrangement of the NAcChR subunits in reconstituted vesicles. (b) Cross-sectional slab through the pentamer at the middle of the membrane showing the four transmembrane segments (M1-M4) of each subunit, based on Unwin (2003)

These receptors are implicated in the propagation of electrical signals between the cells at the neural and neuromuscular synapse. Upon activation by agonist, nAChRs transiently open a cationic channel responsible for the initiation of postsynaptic membrane depolarization. In the continued presence of agonist, the nAChR become refractory to the stimulus and the ionic current declines. This process, called desensitisation, occurs because the fully liganded receptor eventually adopt a stable, high-affinity conformation that is not permeable to ions.

Extensive biochemical studies have demonstrated that the ability of the nAChR to support ion channel function requires the presence of specific lipids. In 1978 Epstein and Racker opened the way for more detailed studies of the influence of the lipid environment on the nAChR by measuring in a reproducible manner integrated flux responses specifically induced by cholinergic agonist in reconstituted systems. Since then many experiments reconstituting nAChRs into artificial liposomes of defined composition have shown that the presence of certain lipids in the reconstituted samples, namely cholesterol and acidic phospholipids, are important in preserving the ability of this protein to exhibit an optimal cation channel activity (Gonzalez-Ros et al. 1980; Criado et al. 1984; Fong and McNamee 1986; Jones et al. 1988; Sunshine and McNamee 1992; Fernández et al. 1993). Such lipid effects on nAChR function are also known to be fully reversible. For instance, McNamee's group used an approach of "re-reconstitution" (reconstituting the protein twice, first in a lipid matrix that does not allow nAChR function, then in whole asolectin lipids) to demonstrate that an apparently "inactive" nAChR, regains its function upon a second reconstitution into an appropriate lipid matrix (Jones et al. 1988). Rapid-kinetics stopped-flow studies have demonstrated that the presence of PA in the reconstituted membranes maintains an optimal nAChR cation channel activity. On the other hand, reconstitution into cholesterol/zwitterionic phospholipids, in the absence of anionic phospholipids, causes a lost in nAChR function (authors submitted manuscript, Fig. 8.2). The lack of ion channel activity in samples containing PC as the only phospholipid present has been reported previously, using several different chemical species of synthetic PCs (Fong and McNamee 1986; Ochoa et al. 1989; Sunshine and McNamee 1992) or egg yolk PC (Fernández et al. 1993). It seems that this lipid

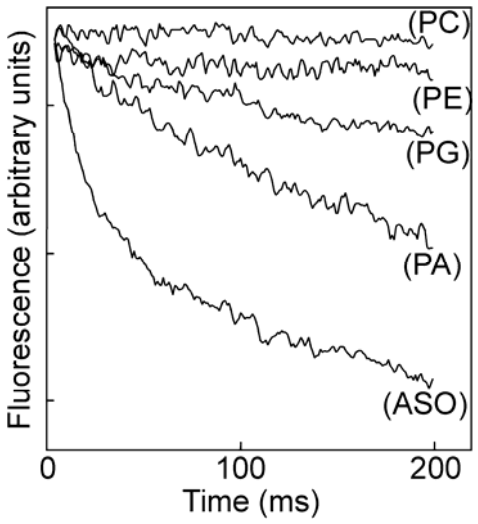

Fig.8.2. Representative stopped-flow traces corresponding to the rapid collisional quenching of the fluorescence on 1,3,6,8-pyrene tetrasulfonate entrapped into reconstituted nAcChR vesicles by externally added Tl^+. The figure shows Tl^+ influx responses to 500 μM carbamilcoline exhibited by reconstituted nAcChR vesicles made from different lipid mixtures

stabilizes the nAChR in a nonresponsive, desensitized state. Also, the need of cholesterol and negatively charged phospholipids, particularly PA, to retain nAChR function upon reconstitution has been widely documented (Criado et al. 1984; Fong and McNamee 1986; Jones et al. 1988; Sunshine and McNamee 1992; Fernández et al. 1993). Similarly, preliminary data using the approach of "transplanting" the nAChR from reconstituted vesicles to the plasma membrane of live *Xenopus* oocytes (Morales et al. 1995 see below), show that microinjecting samples reconstituted in whole asolectin lipids (fully "active" samples) or in just egg phosphatitylcholine ("inactive" samples) produce comparable agonist-induced nAChR ion currents upon incorporation of the protein into the host cell membrane (manuscript in preparation).

These effects of specific lipids in nAChR function may be exerted through binding to specific sites of the protein or by modification of bilayer physical properties. Previous results have demonstrated that membrane lipids interact differentially with nAChR. For example, sterol, PA and fatty acid spin labels have a relative high affinity for nAChR compared with other spin labelled phospholipids (Ellena et al. 1983).

Additionally, several lines of evidence indicate a separate binding site for neutral lipids, namely non annular sites. McNamee´s group used the ability of brominated lipids to partially quench the intrinsic or modified fluorescence of the nAChR to monitor contacts with the surrounding lipid in reconstituted membranes. They found that quenching of PC was independent of and additive with that due to brominated cholesterol derivatives (Jones and McNamee 1988). These results argue strongly for independent binding sites for cholesterol and phospholipids.

Although cholesterol may affect the nAChR directly, it definitely has profound effects on the structure of the membrane environment, most notably in changes of membrane order or fluidity. In earlier studies both the agonist affinity and ion flux seemed to required an optimal fluidity (Fong and McNamee 1986). However subsequent studies showed that while the ion flux activity of the nAChR was strongly influenced by lipid composition (Fernández-Ballester et al. 1994), there was no correlation with membrane fluidity as measured by steady state anisotropy of membrane probes (Shunshine and McNamee 1994). Measurements of membrane fluidity showed that cholesterol further ordered membranes containing PC and PA, but another sterols, like androstanol, did not, however, both neutral lipids supported similar ion fluxes. Thus neutral lipids do not exert their effect on the nAChR by changing bulk membrane order. Nevertheless, effects on bulk membrane order are sometimes different from those at the protein-lipid interface and it is possible that protein promotes the lateral segregation of specific lipids that allows an optimal packing density (daCostaet al. 2002; Poveda et al.2002).

Careful delipidation experiments showed that a lipid/protein mole ratio approximately below 45 caused irreversible inactivation of the nAChR, consistent with the requirement of an annular shell of lipids around the periphery of the hydrophobic region (Jones et al. 1998). This requirement for a lipid annulus of 40-50 lipids molecules is supported by a variety of spectroscopic techniques establishing the presence of a lipid phase associated with the protein that differs from the bulk bilayer lipids in terms of molecular motion (Antollini et al. 1996). It is

also in good agreement with theoretical predictions, which suggests the presence of a inner shell annulus of approximately 42-51 lipid molecules (Barrantes 1993). The nature of the molecular species making up this dynamic annulus has not been wholly ascertained although it seems obvious that both neutral and negatively charged lipids must play a role. It becomes clear that the nAChR annular lipids are important for its correct functional activity, but the precise mechanism by which these annular lipids affect the nAChR are yet unknown.

8.5 nAChR Modulation by other Lipophilic Compounds

As indicated above, some of the lipids surrounding the nAChR play an important role in determining its functional activity. Besides, many other hydrophobic molecules, with astonishingly different molecular structures, modulate the nAChR function, including:

(1) free fatty acids (Andreasen and McNamee 1980, Villar et al. 1988),
(2) steroid hormones, both glucocorticoids (Bouzat and Barrantes, 1996, Nurowska and Ruzzier 1996) and sex hormones (Valera et al. 1992),
(3) local anaesthetics (Katz and Miledi 1975, Gentry and Lukas 2001),
(4) some cholinesterase inhibitors, such as tacrine (Cantí et al. 1998) or BW284c51 (Olivera et al. 2005), and
(4) other lipophilic compounds such as alcohols and general anaesthetics (Liu et al. 1994).

Most of these hydrophobic molecules act as non-competitive blockers (NCB) on the nAChRs. The similar inhibitory effect mediated by such a broad range of lipophilic compounds can be explained assuming that the nAChRs function as an allosteric protein (Changeux 1990; Hogg et al. 2003). This assumption postulates that the protein can exist in different states (closed, open and desensitised, each characterised by its affinity for the agonist, or other ligands, and its conductance) and undergoes spontaneous conformational transitions. At rest, the equilibrium between these conformational states is in favor of the closed state, but when the agonist is bound, the equilibrium shifts towards the active or desensitised states. The binding of molecules at specific regulatory sites (different of the agonist-binding site) would shift the isomerization equilibrium towards one of the possible states (Galzi et al. 1996). Nevertheless, the detailed mechanism by which such heterogeneous group of hydrophobic compounds affects nAChR activity remains largely unknown.

Two main different locations in the nAChR have been proposed for the interaction with NCBs: (1) a high-affinity site, located at, or close to, the lumen of the ion channel and (2) several (up to 30) low affinity sites located at the annular interface receptor-lipid bilayer. The high-affinity site is thought to be at or close to the ion channel pore since photoaffinity labelling experiments using either [^{3}H]CPZ

(Revah et al. 1990) or [^{125}I]TID (White and Cohen 1992) label several residues within the M2 sequence of each subunit, which corresponds to the lining wall of the ion pore. Different experimental approaches further reinforced this view:

(1) Site-directed mutagenesis of the M2 residues in neuronal α7 nAChR affected the apparent affinity of the nAChR for NCB, although also modified the affinity for agonists and competitive antagonists and the desensitisation rate (Revah et al. 1991), and
(2) membrane current recordings of nAChR activity, either in the presence of local anaesthetics (Neher and Steinbach 1978) or TID (Forman 1999), indicate that these compounds cause an open channel block, likely due to their affinity for a site within the open ion channel.

The low affinity sites located at the receptor-lipid interface bind a heterogeneous group of hydrophobic molecules, such as fatty acids, sterols, steroids, alcohols and general anaesthetics. The binding sites for these compounds would be located in the middle (M1, M3) and/or outer (M4) transmembrane domains of the nAChR (Barrantes 2003). Although these segments are not forming part of the ion conduction pathway, in contrast to the M2, they are lipid-exposed and can modulate the receptor function (Arias 1998; Barrantes 2003). As a rule, all NCB acting on these sites cause similar functional effects: they reduce the channel mean open time (τ_{on}), but without changing the maximal agonist binding. Furthermore, some of them enhance desensitisation. Remarkably, comparable changes in gating kinetics are observed when lipid-exposed residues in the M4 domain of the nAChR are mutated (Bouzat et al. 1998).

As for $GABA_A$ and glycine receptors, members of the same superfamily of LGIC, nAChRs seem to have specific binding sites for alcohols and anaesthetics, located in water-filled cavities between the inner (M2) and the outer (M4) set of helices (Miyazawa et al. 2003; Chiara et al. 2003). In the case of steroids, it is known that all of them affect nAChR channel kinetics in a similar way, but the magnitude of their effects are inversely related to their lipophilicity (Garbus et al. 2001). This suggests that their effect is not exerted through a simple perturbation of the lipid bilayer properties but through the binding to a site located at superficial regions of the nAChR-lipid interface, i.e., close to the phospholipid polar head region. In addition, it should be mentioned that when progesterone (Kel and Lukas 1996) or corticosterone (Nurowska and Ruzzier 1996) are bound to bovine serum albumin, forming a cell-impermeant complex, they retain their modulating actions on the nAChR. Therefore, these steroids should be acting on an extracellularly accessible site of the nAChR. Although almost all steroids tested to date on nAChR have an inhibitory action (negative allosteric effect) on nAChR, it has been recently reported potentiating effects of 17β-estradiol on rat (Paradiso et al. 2000) and human (Paradiso et al. 2001, Curtis et al. 2002) neuronal α4β2 nAChRs. The potentiating effect is mediated at a site in the C-terminal tail of the α4 subunit (Paradiso et al. 2001; Curtis et al. 2002), being therefore markedly different to that involved in the steroid inhibiting action. Finally, it should be mentioned that there is no competition

between the inhibitory actions of steroids (hydrocortisone) and local anaesthetics (QX-222), as it would be expected from their different acting sites on the nAChR (Bouzat and Barrantes 1996).

In summary, besides the nAChR dependence on specific lipids, such as PA or cholesterol, for its correct function, there are many other lipophilic compounds, including endogenous molecules, acting on specific loci of this protein. This allows a multifactorial and extremely complex modulation of the nAChR function.

8.6 Influence of Lipids on nAChR Structure

Structural knowledge of nAChR and other LGIC has been greatly hindered by the absence of a crystal structure. The most informative structure is from electron micrographs of the tightly packed arrays of nAChR in tubular membranes isolated from the electric organ of *Torpedo* electric rays (Toyoshima and Unwin 1988, Unwin 1995, Miyazawa et al. 2003). These studies indicate that the transmembrane segments (M1-M4) are basically α helices, although M1 seems to have a distorted helical structure, probably due to the presence of a proline residue. These data have been confirmed through NMR and other spectroscopic studies of the peptides corresponding to the different transmembrane segments of the protein or with biochemical approaches such as photo-labelling, protein modification and site directed mutagenesis of the entire protein (Karlin et al. 1986; Akabas et al. 1994; Blanton and Cohen 1994; Blanton et al. 1998; Corbin et al. 1998; Lugovskoy et al. 1998; Opella et al. 1999; Pashkov et al. 1999; Williamson et al. 2004; Barrantes et al. 2000; Tamamizu et al. 2000; Cruz-Martin et al. 2001; Guzman et al. 2003; Ortiz-Acevedo et al. 2004; Santiago et al. 2004). Finally, site-directed mutagenesis and NMR experiments, propose that M3 contain a mixture of alpha helix and 3_{10}-helix (Lugovskoy et al. 1998; Guzman et al. 2003). The secondary structure of the entire protein has also been studied through spectroscopic techniques such as Raman, FT-IR or CD. Calculated α-helix content ranges from 20 to 43%, β sheet content from 29 to 48%, and non-ordered structure from 20 to 28% (Moore et al. 1974, Mielke and Wallace 1988, Yager et al. 1984, Fong and McNamee 1987, Butler and McNamee 1993, Methot et al. 1994, Castresana et al. 1992). On the other hand, theoretical predictions estimate 44% α-helix and 27% β sheet (Finer-Moore and Stroud 1984). The lack of concordance of these data is probably due to the low sensitivity of CD to detect beta structures, the diversity of the FT-IR quantification methods, and finally to the different conditions used to reconstitute the protein.

As stated in the previous section, lipid surrounding nAChR modulates protein function, possibly through changes in membrane general properties or by direct binding to the protein. This modulation should be caused by some effect on the protein conformation, probably acting on one or several of the transmembrane domains that are in direct contact with lipids (M1, M3 and M4). In fact, site directed mutagenesis studies report many examples of residues located at the lipid-protein interface whose mutation to tryptophan dramatically affects protein function

(Tamamizu et al. 2000; Guzman et al. 2003; Ortiz-Acevedo et al. 2004; Santiago et al. 2004). In order to detect the possible structural changes associated to lipid nAChR modulation, different spectroscopic studies have been done, mainly through analysis of the FT-IR amide I' band. Lipid membranes where nAChR is fully functional, typically those containing PA and cholesterol, are those where the protein presents a higher α-helical content relative to non-ordered structure, whereas nAChR in lipid membranes where it is less active, such as those with only zwitterionic phospholipids, shows a larger non-ordered structure concomitant with a decrease in α-helical one. Meanwhile, β sheet content remains basically unchanged (Fig.8.3).

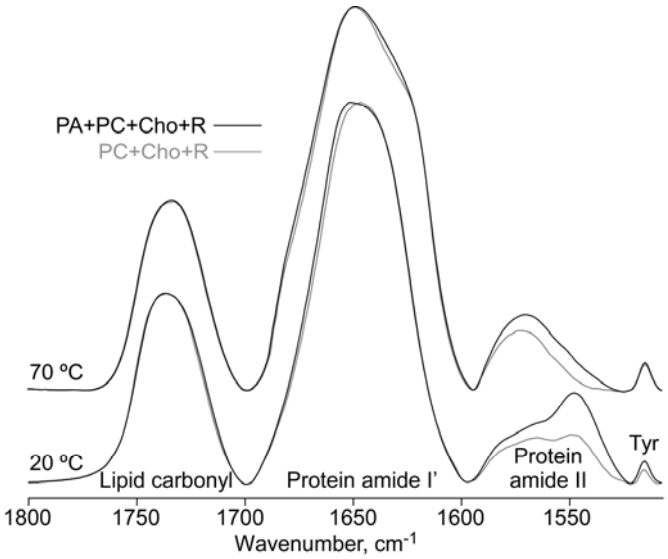

Fig.8.3. Infrared spectra (1800-1505 cm^{-1}) of nNAcChR reconstituted in different lipid vesicles at 20 and 70 ºC. Spectra shown lipid carbonyl, amide I', amide II and tyrosine bands.

All these experiment where done after submitting the samples to a D_2O for H_2O exchange. This process can be followed through the FT-IR amide II band, which diminishes with the H-D exchange, and depends on the accessibility of the different aminoacids to the solvent, so it reports information about the tertiary protein structure (Hvidt and Nielsen 1966; Pershina and Hvidt 1974). Once reached the equilibrium for the H-D exchange, the remaining amide II band was quantified, resulting that those samples with a larger remaining amide II, that is, with the lower H to D exchange, are precisely those with less non-ordered structure. Furthermore, if those samples are constantly heated this amide II band disappears showing a sigmoidal behaviour. From these curves, it is possible to calculate a temperature (Tm) for the collapse of the tertiary structure. Again it is observed that samples with a higher content of ordered secondary structure are those with a higher Tm. All these experiments support nAChR structure data calculations from amide I' analysis, since those samples with more ordered secondary structure, typically alpha-helical, are those more resistant to H-D exchange (author's submitted manuscript). The way through which anionic lipids, especially PA, stabilizes α helix structure of nAChR is not clear, although some authors have pointed to an interaction between the dipole from the α-helical of nAChR and the PA phosphates (Hol et al. 1978; Sali et al. 1988). In addition, there are some works with model peptides that also detect

stabilization of α-helical structure by anionic phospholipids (Liu and Deber 1997). However, other authors have reported an increment in β sheet content upon addition of anionic phospholipids (Butler and McNamee 1993; Fong and McNamee 1987), although these studies were done in a region of the infrared spectrum where bands are rather weak and its assignation to secondary structure is not clear. It has also been reported that cholesterol favours an increment in α-helical structure (Fernandez-Ballester et al. 1994; Fong and McNamee 1987; Butler and McNamee 1993) and even β sheet (Fernandez-Ballester et al. 1994). To explain this result it has been postulated that the rigid sterol ring, oriented parallel to the receptor axis, could localize in between helices at the lipid/protein interface, causing their stabilization (Fong and McNamee 1987).

Among the different regions that conforms nAChR, the M1 transmembrane segment could be a good candidate to be modulated by lipids (Williamson et al. 2004; dePlanque et al. 2004). NMR studies of this transmembrane segment reconstituted in lipids show that some portions adopt an α-helical conformation but that the presence of a proline located in the middle of the segment significantly disrupts the α-helical structure. In fact, a proline and about four surrounding residues typically form a kink in the transmembrane stretch with an angle that can vary between 5º and 60º, and these hinge regions are thought to play a key role in membrane proteins because of their expected inherent flexibility (Cordes et al. 2002; Arshava et al. 2002). Furthermore, conformational studies as a function of the lipid environment suggest that the degree of helicity in this region strongly depends on the lipid environment, and that M1 orders DMPC acyl chains and interact more favourably with cholesterol containing PC bilayers, mimicking several aspects of the effect of the entire nAChR on model membranes (de Planque et al. 2000). This flexibility could be maintained in the entire protein as transmembrane segments in nAChR are loosely packed, and M1, M3 and M4 are largely separated by water filled cavities from the inner ring of M2 helices (Miyazawa et al. 2003). These results, together with the observed M1 labelling from both hydrophobic and hydrophilic probes (Blanton and Cohen 1994; Karlin et al. 1986), and the close proximity between M1 and M2, suggest that the conformational flexibility around the proline in the M1-transmembrane may be important for the modulation of channel gating by the lipid environment and by other molecules which partition into the lipid bilayer, such as general anaesthetics.

Opposite to these works are those using ATR spectroscopy (Ryan et al. 1996; Baenziger et al. 2000). These authors find very faint variations in amide I' band for nAChR reconstituted in different lipid vesicles, only detectable after band deconvolution. They propose that these little variations are caused by the different rate of H-D exchange for the different samples, due to subtle variations in the protein dynamics that do not involve changes in the secondary structure. These variations would be those causing the lipid modulation on nAChR function. This could explain the little differences they find in the amide I' band, but not for the large differences above referred, as there are evidences showing that large variations in H-D exchange do not cause significant changes in the quantification of secondary structure motifs from amide I' band (author's submitted manuscript). One reason to

explain why these authors do not detect large variations in amide I' band could be the fact that nAChR-containing samples are submitted to a drying cycle accompanied by a long period of re-hydration (up to three days) before doing the FT-IR experiments. The consequences of this process have not been tested, since no functional experiments are done after these treatments. It is possible then, that nAChR is in a desensitised-like state, independently of the lipids where it is reconstituted. In the same sense, cholesterol has been proposed to modulate nAChR function without varying protein structure. To do so, it would localize in the spaces between different nAChR subunits facilitating the sliding between them, so making possible the conformational changes necessaries for channel function (Corbin et al. 1998).

nAChR like other ligand gated ion channel, after binding of the corresponding agonist, suffers a conformational change in transmembrane segments, probably a rotational movement, destabilizing the hydrophobic girdle that forms the channel gate and thus allowing ions to pass through the pore. The mechanisms and pathway to transform the energy of ligand binding at the extracytoplasmic domain of the protein into the movements of the M2 segments are largely unknown. It has been proposed some electrostatic and hydrophobic interactions between the M2-M3 loop and other loops in the agonist-binding domain as the responsible of this transmission (Kash et al. 2003; Miyazawa et al. 2003). The action of phospholipids to modulate nAChR function should interfere either the transmission between ligand-binding domain towards transmembrane segments or the subsequent transmembrane movement. A possibility to do that is causing the protein to enter in a non-active conformation, through changes in the secondary and/or tertiary nAChR structure. Considering the above results, zwitterionic lipids would stabilize a conformation with less ordered α helical content that would impede some of the steps that allow the protein function. By contrast, anionic lipids and cholesterol would stabilize a more compact conformation able to transmit movements of the binding domain towards the transmembrane domain.

8.7 PA-nAChR Interaction

As stated above, nAChR binds preferentially anionic lipids, which are positive modulators of its function. Among them, PA seems to interact in a special fashion with this protein. In vitro studies with nAChR reconstituted in lipid vesicles of controlled composition show that PA is among those phopholipids that bind the protein with a higher affinity, and it is the most effective lipid in preserving nAChR function (Jones and McNamee 1988; Marsh and Barrantes 1978; Ellena et al. 1983; Esmann and Marsh 1985; Dreger et al. 1997), possibly through an stabilization of the resting versus the desensitised state of the protein (da Costa et al. 2002). On the other hand, as if a bi-directional coupling takes place, nAChR in PA-containing membrane leads to a dramatic increase in both the lateral packing densities and the gel-to liquid crystal phase-transition temperatures of the reconstituted lipid bilayers

(da Costa et al. 2002; Wenz and Barrantes 2005). This strong interaction leads to the segregation of a PA enriched domain from a complex mixture of lipids at determined lipid to protein ratios (Fig.8.4, Poveda et al. 2002; Wenz and Barrantes 2005). However, nAChR has no detectable effect on the lateral distribution of lipids when PA is substituted by other zwitterionic or anionic phospholipids such as PC, PG or phosphatidylserine (PS) (Poveda et al. 2002; da Costa el al. 2004), although it has been recently reported the segregation of a saturated PC from a unsaturated one by the action of nAChR (Wenz and Barrantes 2005). In this case the authors suggest that the maintenance of this domain is predominantly due to lipid-lipid interactions opposite to that with PA, more stable and mainly maintained by protein-lipid interactions. The PA domain has been detected either through fluorescence, or FT-IR and DSC techniques, the later sensitive to macroscopic events, indicating that macrodomains should be form. In addition, from resonance energy transfer experiments it has been shown that these domains are located next to the protein (Poveda et al. 2002).

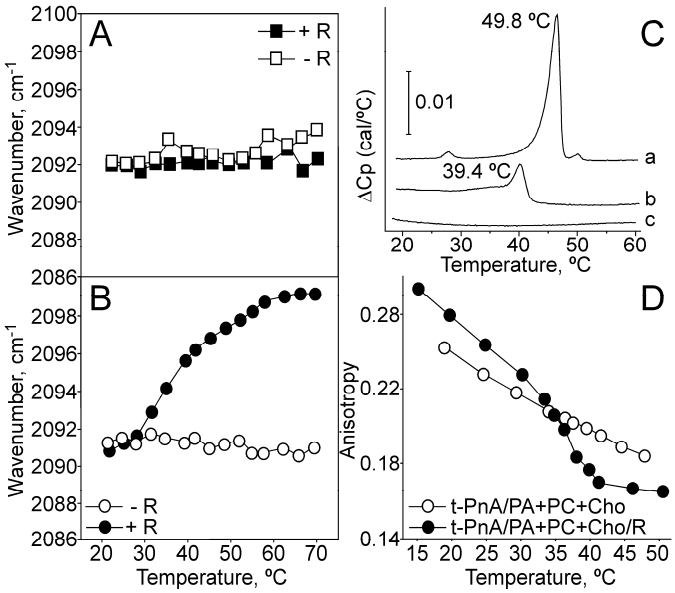

Fig.8.4 (**a, b**) Representative temperature dependence of the infrared CD2 symmetric stretching vibration from perdeuterated phospholipids contained in reconstituted vesicles. Vesicles were prepared by detergent dialysis, in the absence (open symbols) or in the presence (filled symbols) of nAcChR protein, from identical amounts of lipid mixtures containing 25 mol % of cholesterol, 50 mol % of egg PC and 25 mol % of either d-DMPC (panel A) or d-DMPA (panel B). Protein containing samples were prepared at a protein to phospholipid molar ratio of 1:3500. CaF_2 windows were used in the spectrometer cell. (**c**) Differential scanning calorimetry studies on the effect of nAcChR on lipid organization in reconstituted vesicles. The scans correspond to: pure DMPA (a), 25 mol % of cholesterol, 50 mol % of egg PC and 25 mol % of DMPA with (b) or without (c) nAcChR. (**d**) Fluorescence anisotropy of the trans-parinaric acid (t-PnA) probe incorporated into vesicles composed by 25 mol % of cholesterol, 50 mol % of egg PC and 25 mol % of either DMPA in the absence (open symbols) or in the presence (filled symbols) of nAcChR protein.

Phospholipid in membranes, including those interacting with membrane proteins, diffuse very fast, around 10^8 cm^2/s (Ellena et al. 1983; East et al. 1985), so the ability of nAChR to change the lipid lateral distribution segregating PA around it will dramatically enhance its interaction with this phospholipid, hence explaining the strong modulating effect of PA on nAChR.

There are several works describing the segregation of lipid domains by peptides or extrinsic proteins but only one for a membrane protein, rhodopsin (Polozova y Litman 2000). This kind of domains whose organization is directed by a transmembrane protein seems opposite to that of the so-called "rafts" (Simons and Ikonen 1997), where it is postulated that the physical properties of lipids is responsible for the segregation. However, it has been proposed a "clustering" model that could be valid for the nAChR-PA interaction. In it, the action of certain proteins could "cluster" little initial "rafts" dispersed in the membrane (Harder et al. 1998). On the other hand, there are some examples dealing with a special selectivity of certain proteins for PA, as that of the vesicular-stomatitis virus envelope-proteins (Luan el al. 1995). In spite of the low PA concentration in host membranes, these proteins interact specifically with PA giving cause for PA domains, what is an essential event for new viral particles to be formed. Moreover, PA domains have been proposed as scavengers of other essential biological anionic phospholipids such as PIP_2 (Denisov et al. 1998), so the domain formation could configure an important regulation site in the membrane. PS is another anionic phospholipid present at a high level in membranes form the *Torpedo* electric organ (Gonzalez-Ros et al. 1982), so it could be a good candidate to enter the PA domain as in the stomatitis virus occurs where PS is only segregated if PA is present (Luan et al. 1995).

The fact that PA is the main component of the nAChR segregated domain (Poveda et al. 2002), opens a new regulation via for the nAChR activity since PA level at the membrane can be varied through phospholipase D activity, which transforms PC in PA (Exton 1990; Billah and Anthes 1990). In addition, the activity of this enzyme can be regulated through the agonist binding to receptors coupled to G-proteins. In this way, addition of phospholipase D to nAChR enriched vesicles results in an increase in protein function (Bhushan and McNamee 1993). Finally, cells can synthesise PA through the diacylglycerol kinase action on the diacylglycerol formed after breaking phosphatidylinositol through phospholipase C.

Another important question about the segregation of the PA domain is related to the determinants in the protein and in PA responsible of this strong interaction that finally leads to the domain segregation. Calorimetric studies and other experiments using proteases point out to the transmembrane segments as the main structure responsible for the domain segregation (Poveda et al. 2002). It has been proposed that some positive charged aminoacid present at the end of the nAChR transmembrane helices, such as Arg-429 or His-408 at the M4 could be responsible for the stronger binding to anionic phospholipids, although there are no definitive results (Blanton and Wang 1990; Blanton and Wang 1991; Blanton and Cohen 1992). On the other hand, the exact determinants in the PA molecule that could explain its strong interaction with nAChR are also unknown and only general properties of this phospholipid have been pointed out, such as its negative charge, its

very small headgroup or its high capacity to form hydrogen bonds (Baenziger et al. 1999). Evidently, the negative charge is not the only factor, as salt screening or pH titration does not destabilize the domain. Moreover, other anionic lipids are not segregated by nAChR. At this point it is important to stress that PA has a higher pK_a when the protein is present, so its anionic charge is diminished (Poveda et al. 2002). This could facilitate the formation of the PA domains since a lower repulsion and an enhanced attraction through hydrogen bonds between PA molecules would occur (Garidel et al. 1997), decreasing the electrostatic contribution to the free energy of the system (Denisov et al. 1998) as to overcome the entropic effect that favours the homogeneous mixing of lipid components.

8.8 From Model In vitro Systems to Cell Membranes: the Xenopus Oocyte as a Cell Model for the Study of Lipid-Protein Interactions

Most of the studies dealing with the functional and structural dependence of nAChR on its surrounding lipids have been carried out on model membrane systems to avoid the complexity of the cell membrane and to prevent the changes that a single variable can make in the whole system. Though these model systems are useful providing a reductionism approach we must develop novel methods allowing the study of the lipid-protein interaction in native cell membranes in order to confirm the results obtained in artificial systems.

One of the putative cell models for these studies is the *Xenopus* oocyte. These cells have been widely used for the biophysical characterization of many ion channels, neurotransmitter receptors and transporters thanks to their ease of use, amenability for electrophysiological recordings and their capability to translate efficiently and faithfully exogenous mRNAs, (Miledi et al. 1989; Soreq and Seidman 1992; Miller and Zhou 2000). Though *Xenopus* oocytes are capable for make a large number of post-translational modifications of the proteins coded by exogenous mRNA (as acetylation, glycosylation or phosphorylation), and to assemble oligomeric receptor/channel complexes, they cannot always match the processing carried out by the cells that natively express them. Almost certainly, this is the reason for the failed or altered function of some foreign proteins expressed in oocytes. So, for instance, *Torpedo* nAChRs expressed in oocytes display an altered pattern of glycosylation (Buller and White 1990) and neuronal nAChRs do not exhibit the properties of native receptors, likely because oocytes fail to assemble correctly their different subunits (Sivilotti et al. 1997). Besides there are specific lipids requeriments of membrane proteins, which might constitutes a handicap for heterologous expression in a functional form (Opekarová and Tanner, 2003). To overcome this handicap, nAChRs, and other membrane proteins, have been functionally transplanted to the *Xenopus* oocyte membrane by intracellular injection of plasma membranes (Marsal et al. 1995; Aleu et al. 1997; Sanna et al. 1998; Miledi et al. 2002; Palma et al. 2003; Miledi et al. 2004) or proteoliposomes bearing the purified protein (Morales et al. 1995; Le Cahérec et al. 1996; Ivorra et al. 2002).

Oocyte injection of proteoliposomes bearing a purified protein, instead of fragments of cellular membranes, has several advantages:

i) it allows to study single molecular entities,
ii) the transplanted protein does not need to be one of the most abundant in the cellular membrane, although the presence of a large amount of protein simplifies its purification, and
iii) it makes possible to study the influence that the lipid composition of the reconstitution matrix has on the functional properties of the transplanted protein.

The later point has a special relevance, since many proteins are, and need to be, surrounded by specific lipids for developing their full functional activity (see above). Therefore, microtransplantation of purified proteins into the *Xenopus* oocyte membrane arises as an excellent way to unravel lipid-protein interactions, since it allows to insert proteins with specific lipids bound to them. Moreover, using this approach it is possible not only to change the ratio of different phospholipids surrounding the protein, to determine their functional relevance, but also the length of the acyl chains, to induce local changes in bilayer thickness and elasticity that might also be important for the protein activity (Martinac and Hamill 2002; Lundbaek et al. 2004).

Besides, an additional advantage of using *Xenopus* oocytes as the cell model for functional and biophysical studies of heterologous proteins is that their membrane lipid composition is well known (Caldironi et al. 1996; Stith et al. 2000) and can be, at least partially, customized. For instance, the cholesterol content in the oocyte membrane can be easily modified, inducing not only changes in bilayer stiffness but also in the functional activity of different proteins, including nAChRs (see above). The normal cholesterol/phospholipid (C/P) molar ratio in the *Xenopus* oocyte membrane (about 0,5) can be almost duplicated by incubating the cells in a solution containing cholesterol-enriched liposomes, whereas a significant decrease in this ratio is obtained by incubating them with methyl-β-cyclodextrin (Santiago et al. 2001). Likewise, the content of other specific lipid molecules can be modify either by oocyte incubation with lipid-defined liposomes or by activating specific pathways of lipid metabolism. It should be noticed that some lipids are charged molecules and hence certain changes in the lipid composition around some proteins, mainly ion channels, might affect their function by an electrostatic mechanism. So, it is well known that the ion channel biophysical properties can be modulated by fixed charges present in the protein itself or by charged molecules in its surrounding, specially phospholipids. This is because a charged surface in the neighbouring of the ion channel influences the concentration of ions at the channel mouth and consequently its conductance (Latorre et al. 1992; Anzai et al. 1994).

Interestingly, the PA modulation of nAChR observed on “in vitro” systems has been corroborated “in vivo” using the *Xenopus* oocyte model (Morales et al. in preparation, Fig. 8.5). In these experiments, purified nAChRs reconstituted in either PA:PC:Chol (25:50:25 molar ratio), PC:Chol (75:25 molar ratio), or soybean lipids

are injected in oocytes, where they are efficiently inserted in the plasma membrane. Then, the functional activity, and properties, of the transplanted nAChRs are assessed using the voltage clamp technique. The amplitude of the acetylcholine (ACh)-elicited currents in the injected oocytes depended on the reconstitution matrix used. The ACh-current was higher when the nAChR was reconstituted in PA than when it was reconstituted either in soybean or PC lipids, which were very similar each other (See fig 5). This effect was not due to a different fusion efficiency of the different proteoliposomes to the oocyte membrane. It is worthy of note that when nAChRs are reconstituted with those lipid mixtures in vitro, the activity is higher for soybean lipids than for the PA-mixture and no activity is found with PC:Chol. The fact that in the cell membrane the nAChR in PC:Chol reversibly recovers its function suggest that the system is sufficiently dynamic as to allow the injected lipid around nAChR be exchanged for the own oocyte membrane lipids. Then, why oocytes injected with nAChR reconstituted in PA displayed larger currents?. Taking into account the above "in vitro" results, one possibility would be that nAChR binds PA tightly, impeding its free exchange with other bulk membrane lipids, and leading to the formation of a PA rich domain segregated around the protein. The permanent interaction with PA, a positive modulator, would result in the enhanced protein activity. An interesting observation that supports this hypothesis is the fact that, as nAChR is purified from *Torpedo* electric organ, the PA content of the lipids which accompanies the protein is progressively increased from 0.5-1.6% up to 2.2-2.9% (Gonzalez-Ros et al. 1982).

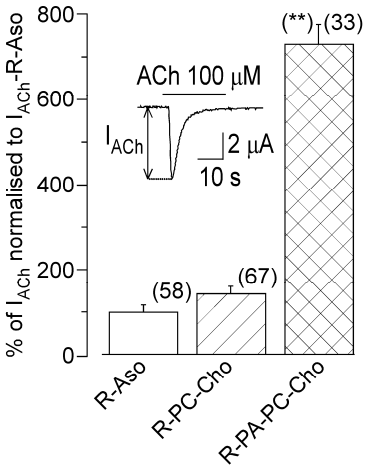

Fig.8.5 Bar diagram showing the amplitude of the peak ACh (100 µM) currents (I_{ACh}) elicited in oocytes previously injected with nAChRs reconstituted in asolectin (R-Aso, open bar), a mixture of PC (75%) and cholesterol (25%, R-PC+Cho, hatched bar) or a mixture of PA (25%), PC (50%), and cholesterol (25%, R-PA+PC+Cho, crossed bar). Values were normalised to the amplitude of the currents obtained in the R-Aso group. The inset shows a representative record of the IACh recorded in the R-Aso group. The arrow indicates de measurement of IAch and the bar the ACh application time. In all experiments the membrane potential was held at −60 mV. The number of observations is given in brackets. Asterisks indicate significant differences with the R-Aso group ($p<0.01$).

Many more studies are needed on in vivo models to fully understand the functional modulation of membrane proteins by their surrounding lipids, but undoubtedly these are the first steps in this way.

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