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MINI REVIEW

NMR Studies of Drug Interaction with Membranes and Membrane-Associated Proteins

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This review focuses on the recent developments in the study of drug interactions with biological membranes and membrane-associated proteins using nuclear magnetic resonance (NMR) spectroscopy and other spectroscopic techniques. Emphasis is placed on a class of low-affinity neurological agents as exemplified by volatile general anesthetics and structurally related compounds. The technical aspects are reviewed of how to prepare membrane-mimetic systems and of NMR approaches that are either in current use or opening new prospects. A brief literature survey covers studies ranging from drug distribution in simplified lipid matrix to specific drug interaction with neuronal receptors reconstituted in complicated synthetic membrane systems.

KEY WORDS: Model membrane; membrane protein; ion channel; gramicidin; anesthetic; nuclear magnetic resonance; NMR; nuclear overhauser effect.

INTRODUCTION

Biological membranes are central to the very phenomenon of life. They are the sites where different elements of the cellular machinery are brought together. Integrated with but significantly different from bulk water, biological membranes create the environment for many complex enzymatic reactions and bioelectrical and biochemical signaling processes to occur, including the conversion of metabolic energies into osmotic, electrical, and mechanical work, transportation of materials between cellular compartments, and the processing of information. In a broad sense, many cellular activities involve membrane-based ligand-receptor interactions, and are mediated by membrane-associated proteins that are incorporated into the structures of the lipid bilayers. Consequently, biological membranes are the primary targets for many drugs of different therapeutic categories. The knowledge of molecular mechanisms of drug-membrane interaction is not only of theoretical significance,

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but also of potential practical implications in the discovery, design, and screening of novel therapeutic agents.

Biophysical approaches to the studies of membranes aim at the understanding of the most fundamental aspects of the biomembrane functions. Often, the goal is to reveal the underlying physicochemical mechanisms of biological processes with atomic resolution. This, however, can rarely be derived directly from experiments on complex and labile natural membranes and whole cells. Therefore, various membrane models with different levels of complexity have been developed to exploit the power of a wide variety of biophysical techniques.

Among the most commonly used techniques for biomembrane studies are optical, electrical, and magnetic spectroscopy methods, including infrared and Raman spectroscopy, circular dichroism, nuclear and electron magnetic resonance spectroscopy, electron microscopy, X-ray crystallography, and Moessbauer spectroscopy (for introductions to various biophysical techniques, see http://www.cryst.bbk.ac.uk/BBS/Whatis/whatis.html.). Of these, nuclear magnetic resonance (NMR) spectroscopy holds a prominent place for being particularly sensitive to the fine strcuctural and dynamical details of the system in question, allowing for the determination of the three-dimensional structures and the internal motions of the proteins and peptides with atomic resolution. In addition, NMR can also be used for non-invasive measurements of thermodynamic and kinetic parameters of transport processes across the membrane or through the transmembrane ion channels.

The present review focuses on the recent developments in the study of drug interactions with biological membranes and membrane-associated proteins using NMR spectroscopy in combination with other spectroscopic techniques. Emphasis is placed on a class of low-affinity neurological agents as exemplified by volatile general anesthetics and structurally related compounds. The technical aspects of preparing membrane-mimetic systems suitable for NMR investigation are reviewed. A literature survey covers studies ranging from drug distribution in simplified lipid matrix to specific drug binding to neuronal receptors reconstituted in complicated synthetic membrane systems. Methodologically, the discussed approaches can be generalized to other drugs not included in the chosen examples.

MEMBRANE-MIMETIC SYSTEMS FOR NMR

Two general features are required for membrane mimetic media to be suitable for high-resolution NMR investigations. First, they should provide a molecular environment that preserves the correct conformation of the integral proteins under investigation and approximates all interactions as close to those in natural membrane as possible. Second, the dynamic properties of the low molecular weight system should yield NMR spectra of reasonable quality for structural analysis. This usually means that the tumbling of the membrane-mimicking aggregates in solution should be fast enough so that the NMR spectra of the system are well resolved. The principles of choosing the suitable models for specific NMR experimental conditions and the properties and detailed preparation procedures of various model systems have recently been reviewed (Sanders and Oxenoid, 2000).

The simplest approach to creating an environment resembling that of the membrane interior or interfacial regions is to use organic solvents with low or intermediate polarity (e.g., octane and carbon tetrachloride for the former, and chloroform and alcohols for the latter), respectively. Although in carefully chosen systems, useful information can be obtained from preparations in organic solvents for small molecules (Borissevitch *et al.*, 1995) or even for protein structures with stable and native folding (Girvin and Filingame, 1994; Rastogi and Girvin, 1999; Taylor *et al.*, 2000), it is generally believed that to use organic solvents to mimic membranes is overly simplistic (Chen and Wallace, 1997; Sanders and Oxenoid, 2000; Tang *et al.*, 2000a). Extreme caution should therefore be exercised in data interpretations.

The simplest true membrane mimetic systems are micelles and reversed micelles formed in water and organic solvents, respectively, by various ionic and non-ionic surfactants. In the absence of added solutes and at reasonably low ionic strength (salt concentrations on the order of 100 nM or lower), micelles are approximately spherical aggregates of about 10–100 kDa. In the presence of large charged molecules, especially integral proteins, the aggregates are far from being classical micelles described by the laws of colloid chemistry. Dozens of surfactants were tested for micelle preparation, with the most traditional being sodium dodecyl sulfate (SDS), cetyltrimethylammonium chloride and bromide (CTAC and CTAB), lysophosphatidylcholine (LPC), and Triton X-100. Dodecylphosphocholine (DPC) and SDS, among other micelles, have been found appropriate as a model membrane system for solution NMR spectroscopy (Arora *et al.*, 2001; Kallick *et al.*, 1995; Opella *et al.*, 1999; Xu *et al.*, 2000c).

In solution, aggregation of surfactants into micelles occurs spontaneously at surfactant concentrations above the critical micelle concentrations, which varies from ~0.01 mM for non-ionic and long-chain zwitterionic surfactants to ~10 nM for SDS and other ionic surfactants with relatively shorter chains in deionized water. Reconstitution of peptides and proteins into micelles, however, is less straightforward and requires special procedures. For short-chain peptides such as functional transmembrane segments of ion channels, the procedure suggested by Tang *et al.*, 1994d, which is a modification of the method by Killian *et al.*, 1994, works in most cases. First, the peptides are dissolved in organic solvents. The use of 2,2,2-trifluoroethanol (TFE) is preferred, although DMSO can sometimes be used. The former promotes helical structures whereas the latter produces mostly random coils. When solubility becomes a problem for highly hydrophobic peptides, pre-dissolving the peptides with volatile acid 2,2,2-trifluoroacetate (TFA) and then drying the acid solution into thin film under a stream of nitrogen gas often facilitate the subsequent dissolution in TFE. Aliquots of the peptide solution in organic solvent are then added to the concentrated aqueous solution of surfactant to reach a peptide-to-surfactant molar ratio of ~1:200. The solvent composition is adjusted to a water-to-TFE volume ratio of 16:1. After vigorous mixing, complete removal of organic solvents is achieved by lyophilization and high vacuum for 24–48 hr. The lyophilized samples are then rehydrated in water, and the pH is adjusted to the desirable values. Thorough removal of organic solvent is especially critical in the studies of protein interaction with low-affinity neurological drugs such as volatile anesthetics and alcohols, since organic solvents themselves are often potent neurological agents.

Despite their structural simplicity, micelles and reversed micelles provide useful insight into the structure and function of more complex bioaggregates (Fendler, 1982). The use of detergent micelles in NMR studies of peripheral and integral proteins and polypeptides has been reviewed (Henry and Sykes, 1994; Opella *et al.*, 1994). The properties of a number of representative surfactants have been compared in a recent work of *E. coli* diacylglycerol kinase solubilized by surfactants (Vinogradova *et al.*, 1988). It is worth noting that although micellar preparation offers many advantages, some surfactants (including the most popular SDS) can potentially denature proteins under unfavorable conditions (Vinogradova *et al.*, 1988). Membrane protein functionality in micelles may be improved by adding a small amount (5–20 mol.%) of lipids to form mixed micelles (Sanders and Oxenoid, 2000; Vinogradova *et al.*, 1998).

Reversed micelles have been used successfully to solubilize large organic and inorganic molecules (Valdez *et al.*, 2001; Yushmanov, 1999) and water-soluble proteins (Flynn *et al.*, 2001) for NMR studies. The packing and dynamics of surfactant molecules in aqueous micelles are more sensitive to the bulky solutes than in reversed micelles, seemingly because of the more restricted motion of surfactant chains in the aqueous micelles (Yushmanov, 1999). The use of reversed micelles to encapsulate large soluble proteins in low-viscosity fluid to increase protein spectral resolution (Wand *et al.*, 1998) has yielded fruitful results. Whether membrane proteins can be solubilized by reversed micelles remains to be determined.

Magnetically oriented discotic phospholipid micelles, often referred to as bicelles, are becoming increasingly popular in NMR studies, especially in structural determination of proteins (Glover *et al.*, 2001; Howard and Opella, 1996; Luchette *et al.*, 2001; Sanders and Prosser, 1998). Bicelle structure intermediates that of micelles and of unilamellar vesicles. Bicelles are typically composed of a long-chain phospholipid, such as dimiristoylphosphatidycholine (DMPC), forming a bilayer, and a short-chain one, preferably dihexanoylphosphatidylcholine (DHPC) (Struppe and Vold, 1998) or dicaproylphosphatidylcholine (DCPC) (Raffard *et al.*, 2000), coating the hydrophobic edges of the bilayer. At a total lipid concentration above 2–5% (w/w) and DMPC-to-DHPC (or DCPC) molar ratio of 2–6 bicelles align spontaneously in the magnetic field with the average bilayer normal perpendicular to the applied magnetic field (Struppe and Vold, 1998). In the presence of less than 1 mol.% of the trivalent lanthanide ions (EU^{3+}, Er^{3+}, Tm^{3+}, Yb^{3+} or their chelates, the bicelle alignment is flipped by 90°, with the average bicelle normal being parallel to the magnetic field (Prosser *et al.*, 1999; Prosser *et al.*, 1998). Incorporation of membrane peptides into bicelles may be accomplished using the procedure similar to that described above for micelles. Bicelles are usually too large to allow for solution NMR experiments on large integral proteins. Therefore, the search for smaller fast-tumbling aggregates of lipid bilayers is in order (Glover *et al.*, 2001; Luchette *et al.*, 2000). Optimization of bicelle preparation often involves varying the lipid mole fraction, the temperature, and the water content (Raffard *et al.*, 2000).

Bilayer and multilayer vesicles formed by synthetic phospholipids are popular membrane models of next level of complexity. They are mostly used in biophysical studies designed to address questions pertinent to the physical properties of membrane components that are relevant to interactions with drugs. Phospholipid vesicles

may exist in unilamellar or multilamellar forms. The size of the vesicles varies depending on the preparation procedures. To prepare small vesicles, the solution of lipids in organic solvents (typically chloroform) is dried into a thin film under nitrogen gas and then vacuumed for the complete removal of the solvent. The film is then re-hydrated with water or D_2O and ultrasonicated into vesicles (Tang *et al.*, 1997; Xu and Tang, 1997a). The diameter of the vesicles can be controlled by adjusting the power of sonication. To make large unilamellar vesicles with a uniform size distribution, extrusion method is preferred (de Arcuri *et al.*, 1999; Duzgunes *et al.*, 1983; Walde and Ichikawa, 2001). Because sonication at high power has the tendency to damage the proteins, incorporation of transmembrane proteins and peptides into the vesicles is often achieved by adding aliquots of the protein solution in organic solvents (e.g., TFE) to preformed vesicle suspensions (Tang *et al.*, 1999a). Incubation at slightly elevated temperature (e.g., 50° C) for several hours (e.g., 3–24 hr) is often required for the incorporation to complete. Removal of organic solvents is achieved by several stages of dialysis (Tang *et al.*, 1999a). Because of their relatively large size (typically on the order of MDa), lipid vesicles are usually not suitable for structural studies of integral proteins based on the NMR resonance of the proteins. They are, however, suitable for monitoring the drug-protein interactions by using the NMR peaks of the drugs (Xu *et al.*, 2000a). An example of this is the analysis of drug binding to nicotinic acetylcholine receptors (nAChR) reconstituted in 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) vesicles (Xu *et al.*, 2000a). Multilamellar bilayers are used in the solid-state NMR studies of membrane proteins (Opella *et al.*, 1994; Opella *et al.*, 2001). Preferential orientation of the bilayer normal parallel to the direction of the magnetic field may be achieved by sandwiching multilamellar vesicles between glass plates, or by centrifugal forces (Opella *et al.*, 1994; Sanders and Oxenoid, 2000).

The characteristics of the traditional model membrane systems discussed above are summarized in Table 1. New alternatives recently described in the literature include amphipathic polymers (Nagy *et al.*, 2001; Prata *et al.*, 2000) and perfluorinated surfactants (Barthelemy *et al.*, 1999; Raffard *et al.*, 2000), which offer some new and exotic features.

DRUG INTERACTION WITH MODEL MEMBRANES

In the studies of drug effects on biological tissues at the molecular level, much attention has been paid to the interactions of small molecules with membranes. These interactions determine and modulate other interactions and drug metabolism in organisms. To obtain specific information about the localization of drugs in model membranes and the involvement of different functional groups in the interactions, a variety of spectroscopic methods have been developed and refined.

To localize drugs in micelles and vesicles, the first approximation is to differentiate the hydrophilic (polar) and hydrophobic (non-polar) environments and to quantify the ordering parameters of the hydrophobic chains. High-resolution multinuclear NMR (Tang *et al.*, 1997; Xu and Tang, 1997a), solid-state ^{2}H NMR quadrupole splittings (Saint-Laurent *et al.*, 1998a), wide-line ^{31}P NMR (Forrest and Mattai, 1985), spin-probe electron spin resonance (ESR), and fluorescence quenching (Louro

Table 1 Model Membrane Systems Commonly Employed in NMR Studies of Proteins

Membrane model	Molecules	Main NMR uses	Useful references[a]
Micelles	Wide variety of ionic and non-ionic amphiphilic molecules	Solution-state NMR of drugs and proteins in membrane-mimetic environment	(Killian *et al.*, 1994) (Henry and Sykes, 1994) (Vinogradova *et al.* 1998)
Bicelles	Usually, DMPC with admixture of a short-chain lipid or detergent	Solution-state NMR of water-soluble proteins; Solid-state NMR of membrane proteins	(Sanders *et al.*, 1994) (Sanders and Prosser, 1998)
Liposomes	Amphiphilic lipids (usually phospholipids), sometimes with admixture of cholesterol	Solution-state studies of drug-membrane interaction; Solid-state structural studies of membrane proteins	(Huang and Thompson, 1974) (Silvus, 1992)
Oriented bilayers	Phospholipids	Solid-state structural studies of membrane proteins	(Opella *et al.*, 1994) (Cross, 1997) (Opella *et al.*, 2001)

[a]References covering practical aspects of the use of corresponding models.

et al., 1994a,b) are among the techniques commonly used. For example, the locations of a series of nitrogen-containing aromatic and heterocyclic local anesthetics (e.g., procaine, tetracaine, dibucaine) and structurally related drugs have been extensively studied. The position of aromatic rings of the drugs was found to be rather shallow in phospholipid bilayers (de-Paula and Schreier, 1996; Okamura *et al.*, 2000), in differently charged micelles (e.g., in cationic CTAC, anionic SDS, and zwitterionic LPC and *N*-hexadecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate (Louro *et al.*, 1994a; Perussi *et al.*, 1995; Yushmanov *et al.*, 1995; Yushamanov *et al.*, 1994a,b), and in sarcoplasmic reticulum vesicles (Louro *et al.*, 1994b). Similar localization has later been proposed for other aromatic amphiphilic drugs in model membranes, with polar groups of the drug interacting with water and charged lipid head groups, and aromatic groups situated in the neighboring hydrophobic regions. Examples include the studies of dipyridamole and its derivatives in micelles and reversed micelles of CTAC (Borissevitch *et al.*, 1996; Borissevitch *et al.*, 1995; Yushmanov and Tabak, 1997) using fluorescence spectroscopy. ^{1}H NMR, and ESR, of salicylic acid and a number of antifungal drugs in the DPPC membranes using ^{1}H NMR and differential scanning calorimetry (Deniz *et al.*, 1996), and of loperamide (an antidiarreal agent) using ^{31}P NMR, ^{13}C NMR, and ESR (Coutinho *et al.*, 1999).

Drug distributions in more complicated unilamellar and multilamellar vesicles have been studied by NMR chemical shift analysis of the drug molecules and by ^{2}H NMR. Many volatile anesthetic agents are fluorinated, thus allowing for the use of ^{19}F NMR with high detection sensitivity at clinically relevant concentrations and without the interference from background signals. The method has been successfully used to distinguish the distributions of volatile general anesthetics and structurally

similar nonanesthetics in model membranes (Tang *et al.*, 1997). Changes in resonance frequencies of the drugs were analyzed using a multiple-site exchange model. It was found that at clinically relevant concentrations, the anesthetics preferentially distributed near the membrane-water interface with constant access to the aqueous phase, whereas nonanesthetics solubilized deeply in the phospholipid core with very limited access to water. The same conclusion was obtained for the same fluorinated compounds and other anesthetic (e.g., α-chloralose) and nonanesthetic (e.g. β-chloralose) compounds using ^{2}H-NMR measurements (Forrest and Mattai, 1985; North and Cafiso, 1997). Different distributions of anesthetics and nonanesthetics in the lipid bilayers may account for their different availability to interact with membrane proteins and receptor channels, and hence their different drug potencies.

To understand the molecular mechanisms of drug action, it is imperative not only to know the drug distribution in the membrane, but also to resolve the drug-membrane interaction with atomic or sub-molecular resolution. Various NMR techniques have been proposed for this purpose. Because the interactions are mostly intermolecular, the non-coherent energy transfer methods are frequently used. The NMR relaxation time measurements and nuclear Overhauser effect (NOE) are particularly sensitive to molecular dynamics and interatomic distances, and truncated and transient NOE and selective spin-lattice relaxation experiments are well suited for studying drug binding in immobilized systems such as biomacromolecules and membranes. The use of the nuclear relaxation measurements in biomedical research has been reviewed recently (Yushmanov, 2000).

Perhaps one of the best examples of using NMR to quantify low-affinity drug interaction with a biological membrane is the intermolecular NOE measurement of xenon in phosphotidycholine lipid vesicles. Noble gas xenon is a clinical anesthetic agent. Because of its chemical inertness and lack of structure, xenon is an unbiased molecular probe for identifying the physicochemical sites in the membrane that are potentially relevant to the mode of action of inhalational anesthetics in general. Using site-selective, truncated driven ^{129}Xe-$\{^{1}$H$\}$ NOE, Xu and Tang (Xu and Tang, 1997b) quantified the interaction strength, as measured by the intermolecular cross relaxation rate constants, between xenon and protons in different regions in the lipid membrane (see Fig. 1). This approach can be generalized to apply to many types of drug-membrane interactions, provided that spectral resolution is high enough to distinguish different sub-molecular sites and the spin diffuse is not too fast to spoil the site-specific selections.

Nuclear (typically ^{1}H and ^{13}C) relaxation caused by the endogenous paramagnetic centers, such as paramagnetic metal ions in metalloenzymes (Sette *et al.*, 1992) or metalloporphyrins (Mazumdar, 1990), can be used to evaluate inter-atomic distances. Paramagnetic centers also provide a convenient measure for analyzing aggregation processes, because when aggregated, many paramagnetic molecules lack paramagnetism due to the antiferromagnetic coupling between metal ions in the aggregates and, therefore, may be readily distinguishable from monomers in high-resolution ^{1}H NMR spectra or by magnetic relaxation measurements (Yushmanov *et al.*, 1996a). The studies with the Fe(III) derivative of *meso*-tetrakis(*p*-sulfonatophenyl)porphyrin ($FTPPS_4$) in micelles (Gandini *et al.*, 2001; Yushmanov, 1999) have shown that aggregation processes in micelles is similar to that observed in

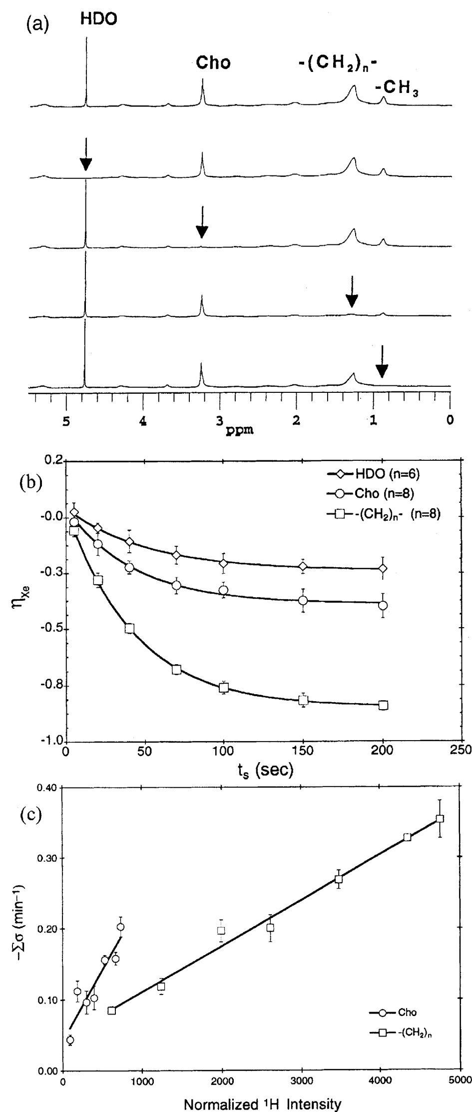


Fig. 1 An example of using noncoherent energy transfer NMR techniques to quantify specific interactions between low-affinity drugs and biological membranes. (a) Selective saturations of particular molecular groups, marked here by vertical arrows for the lipid methylene($-(CH_2)_n-$) and terminal methyl ($-CH_3$) groups, the lipid choline head group (Cho) and the deuterated water (HDO), allow for investigation of the site-specific drug-membrane interaction by measuring the selective $^{129}Xe-\{^1H\}$ NOE buildup. (b) Truncated driven intermolecular NOE from ^1H in $-(CH_2)_n-$, Cho, and HDO groups to ^{129}Xe is measured as a function of ^1H saturation time. The NOE values, η_{xe}, are calculated as relative changes in ^{129}Xe magnetization to its thermal equilibrium value, determined experimentally by placing the saturation frequencies outside the ^1H spectral bandwidth. The solid lines are best fit to the data using the theory described by Xu and Tang, 1997b, yielding the site-specific cross-relaxation rates $\langle\Sigma\sigma_{Xe-H}\rangle$, between ^{129}Xe and the specific groups of protons. (c) The pair-wise interaction strength is quantified by the slopes of the linear relationship between $\langle\Sigma\sigma_{Xe-H}\rangle$ and normalized ^1H intensities when the lipid concentration increases. Xenon interaction with Cho group is at least three times stronger than with $-(CH_2)_n-$. Adapted from Xu and Tang (1997b) with permission.

porphyrin complexes with proteins and DNA (Gandini *et al.*, 1999; Tominaga *et al.*, 1997; Yushmanov *et al.*, 1996b).

In the absence of endogenous centers, the specificity of relaxation data can be substantially improved by introducing paramagnetic probes into the system of interest at known locations, e.g., doxyl stearic acids with definite position of the doxyl fragment in the hydrocarbon chain (Yushmanov and Tabak, 1997). The unpaired electron spin of the probe enhances the relaxation rate of the nuclei of the surrounding surfactant and drug molecules in a distance-dependent fashion (Godici and

Landsberger, 1974). The partitioning and the exact location of paramagnetic membrane probes may be established by ^{1}H NMR relaxation jointly with spectrophotometric and fluorescence quenching measurements (Almeida *et al.*, 1998). This approach was successfully used to study the localization of papaverine in ionic micelles by ^{1}H NMR (Yushmanov *et al.*, 1995) and that of M13 coat protein in SDS micelles by ^{13}C NMR of the SDS chains, and by ^{1}H-^{15}N HSQC and TOCSY (Papavoine *et al.*, 1994). In another application, ^{19}F NMR relaxation of trifluoromethyl-labeled atrazine induced by paramagnetic probes gadolinium ethylenediamine tetraacetic acid and 2,2,6,6-tertramethyl-piperidine-*N*-oxyl has been used to show that atrazine solubilized by humic micelles occupies a hydrophobic domain accessible only to neutral hydrophobic molecules (Chien *et al.*, 1997). The use of paramagnetic spin probes may provide additional information from ESR measurements (Papavoine *et al.*, 1994), e.g., correlation times (τ_c) of molecular motion in different parts of the micellar core (Yushmanov *et al.*, 1995). The paramagnetism approach, however, has its limitation that the unpaired electrons often strongly broaden and displace the NMR peaks, and suppress the intensity of fluorescence.

Studies of interaction drugs with micelles and lipid vesicles can also shed new light on the mechanisms of drug delivery. In this regard, large multilamellar vesicles, also referred to as liposomes, have become the center of focus in different liposome formulations. The measurements of the transverse relaxation times T_2 of polyoxoethylene protons in polyethyleneglycol (PEG) in the PEG-PE conjugates upon their interaction with phospholipid bilayers have demonstrated a destabilizing effect of the short chain PEG-PE (1–3 kDa) and stabilizing effect of cholesterol on liposomal formulations for a long circulating drug delivery system (Bedu-Addo *et al.*, 1996a,b). ^{1}H and ^{31}P NMR were used to elaborate pH gradient-sensitive and tunable pH-sensitive liposomes (Hafez *et al.*, 2000; Maurer *et al.*, 1988) as well as liposome formulations suitable for nucleic acid delivery in gene therapy (Maurer *et al.*, 2001; Semple *et al.*, 2001).

STRUCTURES OF AND DRUG INTERACTION WITH MEMBRANE-ASSOCIATED PROTEINS

Most of the NMR techniques elaborated to examine drug interactions with model membranes can be applied directly to peptides and small proteins integrated in the membranes. Such peptides included virus protein fragments (Hall *et al.*, 1988), synthetic transmembrane and membrane-binding peptides (Belohorcova *et al.*, 2000; Langlais *et al.*, 1999; Montserret *et al.*, 2000; Pare *et al.*, 2001; Prosser *et al.*, 1999; Sharpe and Grant, 2000), and natural venom peptides (Yu *et al.*, 2001). Studies of membrane-associated proteins in membrane-mimetic media are generally more challenging. To date, the largest integral protein, of which the structure has been determined by solution NMR, is a 19 kDa (177m residues) transmembrane domain of the outer membrane protein A of *E. coli* in DPC micelles (Arora *et al.*, 2001), The number and the size of the membrane proteins with structures determined by NMR are expected to rapidly increase. Bacterial expression and site-directed mutagenesis have been used to incorporate 5-^{19}F-tryptophan residues into D-lactate

dehydrogenase of *E. coli* to enable the structural study in LPC micelles by ^{19}F NMR (Peersen *et al.*, 1990). This approach allows one to examine specific sites in the enzyme, their exposure to solvent and lipids, sensitivity to substrates and other intermolecular interactions, and motional properties.

Solution- and solid-state NMR, and the combination of the two, prove to be powerful tools for structural studies of ionic channels. The structures of gramicidin A (Cross, 1997), the putative channel-lining second transmembrane (TM2) segments from nAChR and NMDA receptor (Opella *et al.*, 1999), the TM2 segments from the human glycine receptor α1 subunit (Tang *et al.*, 1999c; Xu *et al.*, 1999), and transmembrane region of the M2 protein from the influenza virus (Song *et al.*, 2000; Wang *et al.*, 2001) have been determined by NMR. Structure-function and dynamics-function relationships of membrane-bound gramicidin A have been extensively investigated by ^{15}N and ^{2}H NMR (Hu and Cross, 1995). It was found that the indole dipole moments are important for cation conductance and that the gating mechanism involves an energy barrier at the bilayer center. Different ionophores have been characterized by direct measurements of sodium transport across unilamellar vesicle membranes, using 1D and 2D ^{23}Na NMR. A comprehensive review of NMR studies of ion-transporting biological channels has been published recently (Hinton, 1999).

Knowledge of the structure and dynamics of membrane-associated proteins in model membranes enables one to address the mode of interactions of drug molecules on membrane proteins. NMR is particularly useful in analyzing drug-protein binding kinetics, especially the low-affinity binding kinetics when the conventional Scatchard type of analysis fails. The conventional biochemical methods depend on the effective separation of the bound from the free drug molecules at equilibrium. This type of analysis is accurate only when the association constants, K_a, of the drug at the possible protein targets are greater than $10^5\,M^{-1}$. Competition measurements between strongly and weakly bound drugs can potentially extend the lower limit of measurable affinity to $10^4\,M^{-1}$. However, many centrally active neuronal agents, such as volatile general anesthetics and alcohols, have an effective concentration (e.g., EC_{50}) in the range of a few hundred μM to a few hundred mM, corresponding to a K_a in the range of 10–10^4 M^{-1}. For hydrophobic drugs in a membrane environment, the on-rate of binding (k_{on} is approximately diffusion-limited (Xu *et al.*, 2000b), i.e., about 10^8 $M^{-1}\,s^{-1}$. Thus, the off-rate ($K_{off} = k_{on}/K^a$) is in the range of 10^4 to $10^4\,s^{-1}$. As a consequence, the mean lifetime of most general anesthetics and other low-affinity drugs in the bound state is in the range of 0.1–100 μs. Although specific bindings are still possible in this time range, rapid exchange among multiple sites often confounds the interpretation of results in the conventional binding studies. NMR is one of the very few analytical techniques that allow for quantification of low-affinity binding with great precision.

The best examples of low affinity drug interaction with membrane proteins can be found in the studies of molecular mechanisms of general anesthesia, one of the greatest unsolved mysteries in modern medicine. Two prevailing schools of thought about the modes of the action of anesthetic drugs diverge at the level of involvement of lipids and proteins. One theory believes that anesthetic drugs cause a non-specific perturbation to the membrane lipid matrix, thereby disrupting the neuronal communication due to loss of membrane functionality. The other subscribes to the

notion that proteins, particularly postsynaptic neurotransmitter-gated receptor channels, are the primary targets of the anesthetic drugs. Other than indirect functional studies by mutagenesis (at either *in vivo* or receptor level), however, direct evidence of anesthetic binding to receptors is lacking. Xu *et al.* (Xu *et al.*, 2000b) recently studied the specific interaction between isoflurane, a clinically important volatile anesthetic, and membrane-bound nicotinic acetylcholine receptors (nAChR) from *Torpedo electroplax*, using ^{19}F NMR and gas chromatography of isoflurane. The receptors were reconstituted into DOPC lipid vesicles. The NMR chemical shifts and the T_2 of isoflurane resonance were used to determine the dissociation constant (K_d), the on- and off-rate and the resident time of isoflurane at the bound state. Figure 2 depicts the representative approaches to quantifying the low-affinity binding in the fast-exchange regime. These measurements, combined with gas chromatography, revealed on average 9–10 specifically bound isoflurane molecules per nAChR, with an apparent K_d of ~0.36 mM and characteristic time of the bound state of ~58 μs. Given the pentameric architecture of nAChR from five subunits and the absence of subunit selectivity in anesthetic binding (Eckenhoff, 1996), it follows that for a pentameric nAChR, each subunit on average has at most one specific binding site for two anesthetic molecules or two specific binding sites for one anesthetic molecule each. Although this approach does not resolve the specific sites where the binding occurs, the results have profound implication in that the number of sites for low-affinity drug binding in proteins is limited and that the binding as brief as several microseconds is unlikely to result in changes in the secondary, or even tertiary, structures of the proteins. Indeed, mutagenesis studies have identified as few as two amino acid residues in the transmembrane domains of each glycine or $GABA_A$ receptor subunit that are essential for the receptor's sensitivity to general anesthetics (Mihic *et al.*, 1997). The rapid binding kinetics may suggest that low-affinity drugs may exert their specific action on proteins not by changing protein structures, but by altering protein dynamics (Xu *et al.*, 2000c).

To resolve the submolecular details of low-affinity drug binding to membrane proteins and ion channels, high resolution structural information about the proteins and channels is required. At the present time, this information is generally lacking and limited only to a few functional peptides and small segments from large receptors. Thus, simplified models for channels, consisting of small transmembrane peptides, are widely used. Of note are the studies with gramicidin A channel, the function of which, as measured by Na^+ transport, was found to be modulated differently by volatile anesthetics and structurally similar nonanesthetics (Tang *et al.*, 1996b). Figure 3 depicts the non-destructive NMR measurements of Na^+ transport across gramicidin A channels using the magnetization inversion transfer technique. The intensity of the intra-vesicular $^{23}Na^+$, which is distinguished from the extra-vesicular $^{23}Na^+$ by non-permeable $^{23}Na^+$ shift reagent, is modulated as the selectively inverted extra-vesicular $^{23}Na^+$ recovers to its thermal equilibrium state. This modulation is due to the exchange of $^{23}Na^+$ in the two pools. Tang *et al.*, 1996b elaborated the theory of extracting the apparent unidirectional transport rate constants from the intensity profiles of the intra- and extra-vesicular $^{23}Na^+$ signals and found that anesthetic drugs increased the influx and efflux rate constants by 23 and 28%, respectively. In contrast, the structurally similar nonanesthetics had no effects on

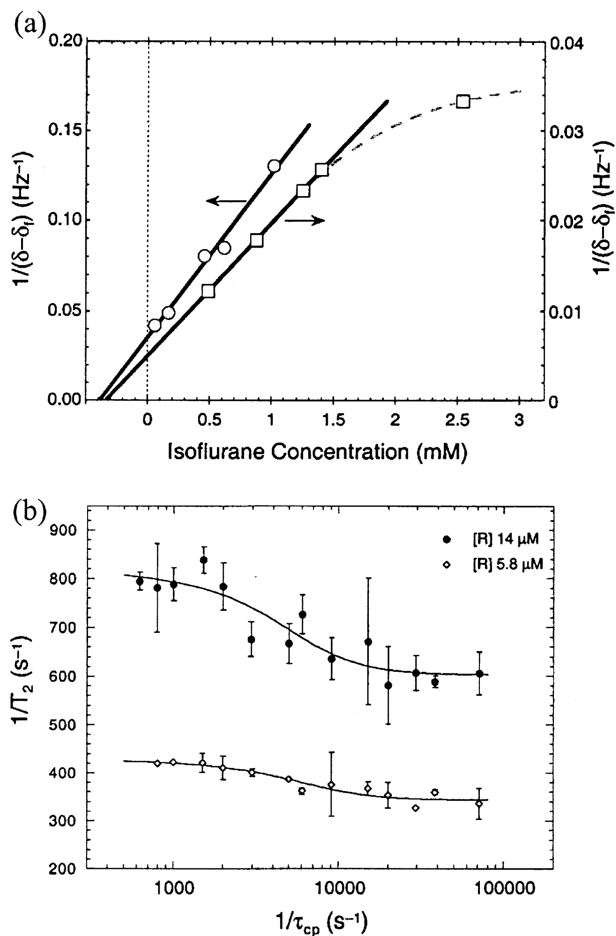
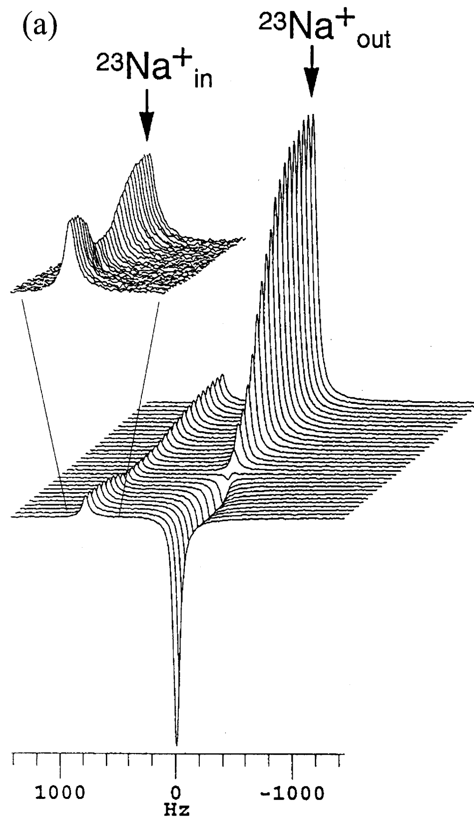


Fig. 2. An example of using NMR to determine low-affinity, yet specific, drug binding to post-synaptic nicotine acetylcholine receptors reconstituted in DOPC vesicles. (a) The reciprocal of changes in resonance frequency of the drug molecule in the bound state relative to the free state is linearly dependent on the drug concentration before saturation of binding sites occurs. The negative value of the intercept on the x-axis yields the dissociation constant, K_d, which equals 0.36 mM in this example. (b) Because the characteristic time of binding and unbinding falls within the time frame of NMR relaxation measurements, the T_2 of the drug molecule depends on how fast the NMR data are sampled. Shown here is the dependence of $1/T_2$ on $1/\tau_{cp}$, the so-called spin echo time. This allows for the mean lifetime of the bound state to be determined. Adapted from Xu *et al.* (2000b) with permission.



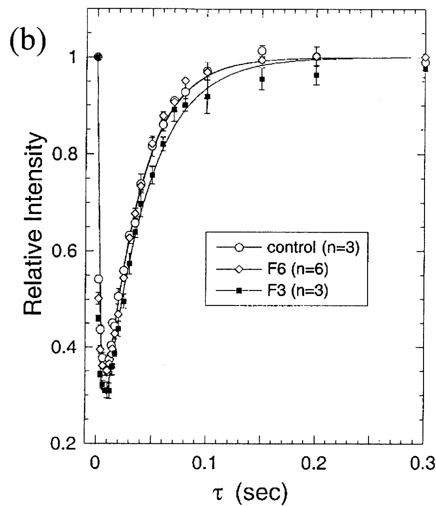


Fig. 3. An example of using NMR to study drug effects on ion transport across transmembrane channels. (a) The stack plot shows that the intensity of the intra-vesicular $^{23}Na^+$ signal (see insert) of the magnetization inversion transfer spectra is modulated by the selective inversion recovery of the extra-vesicular $^{23}Na^+$ in the presence of gramicidin A channels. (b) The signal intensity profiles of $^{23}Na_{in}^+$ are plotted as a function of the $^{23}Na_{out}^+$ inversion recovery time under the control condition and in the presence of anesthetics (labeled here as F3) or structurally similar nonanesthetics (labeled here as F6). The data revealed that anesthetics increased the apparent unidirectional influx and efflux rates by 23% and 28%, respectively, whereas the nonanesthetics had minimal effects on the ion transport. Adapted from Tang *et al.* (1999b) with permission.

the transport. This difference in the functional response is correlated with the high-resolution measurements of drug-channel interaction in a series of thorough investigations (Tang *et al.*, 2000b; Tang *et al.*, 1999b; Tang *et al.*, 1999e; Tang *et al.*, 2000c) using high-resolution ^{1}H NMR, truncated driven intermolecular 19F-{^{1}H} NOE between fluorinated anesthetic-nonanesthetic pairs and different residues in the channel peptide, and large-scale molecular dynamics simulations. Distinctly different patterns of interaction for anesthetics and nonanesthetics emerged from these high-resolution characterizations. Anesthetics specifically targeted the anchoring residues (four tryptophans in the case of gramicidin A) at the channel-membrane-water interface and altered the water exchange with the indole amide hydrogen of the tryptophan side chains. The structurally similar nonanesthetics have no effects in the interfacial region. It is interesting to note that although neither anesthetics nor nonanesthetics altered the gramicidin A channel structure (Tang *et al.*, 1999e), the specific drug interaction with typtophan side chains in gramicidin A channel is not due to tryptophan residue *per se* but requires the channel conformation in a membranous environment. In methanol, in which gramicidin adopts various double-helical conformations, no specific interaction of the drugs with tryptophan residues was detectable by NMR and photoaffinity labeling (Tang *et al.*, 2000b). It was suggested that the spatial arrangements of the tryptophan residues with the amphiphilic regions at the channel-lipid-water interface might create a specific structural motif favorable for anesthetic binding. Other groups have also reported that anesthesia (or, at least, immobilizing action of the anesthetics) requires amphipathic properties of the interacting sites or amphipathic cavities within the target proteins (Harrison, 2000; Sonner, 2000).

Taken together, the results of the studies of general anesthetics in model membranes and membrane proteins with different complexity have led to the hypothesis of a unitary mechanism of general anesthesia based on protein dynamics (Xu *et al.*, 2000c). The essence of the hypothesis is that anesthetic molecules exert their action on all proteins through a common *molecular* mechanism of modulating protein dynamics, not necessarily by fitting into structurally compatible pockets, but by becoming an integral part of amphipathic domains where they can either disrupt the association of the channel with its surrounding or facilitate the formation of structured water clusters within the protein or at deep levels of protein-lipid interface where the water presence is normally scarce and brief . In doing so, they alter the functional characteristics of protein motion and thereby change the protein function.

NEW AND EMERGING APPROACHES

The use of transferred NOE in 1D, 2D, and 3D experiments has become increasingly popular in the studies of interaction of small molecules with proteins, receptors, monoclonal antibodies, ribosomes, and micelles (Barsukov *et al.*, 1996; Bertho *et al.*, 1998; Mayo *et al.*, 1996; Phan-Chan-Du *et al.*, 2001; Polenova *et al.*, 1997). Another novel NMR technique, the transverse relaxation optimized spectroscopy or TROSY, has recently been applied to experiments with membrane proteins (Arora *et al.*, 2001; Fernandez *et al.*, 2001). This method enabled substantial

progress in studying the conformational dynamics of the main protein chain by ^{15}N relaxation at very high magnetic fields.

The multiplet relaxation in proton-coupled ^{13}C NMR spectra has recently been applied to peptides and proteins. It was shown that differential effects between relaxation and the NOE of different transitions of a spin are related to the local anisotropy of rotational motions of the corresponding methyl and methylene groups, and therefore may be used in characterizing their motional dynamics, as reviewed recently (Daragan and Mayo, 1997).

The high-pressure ^{19}F NMR in the presence of molecule oxygen has been proposed to determine immersion depth and topology of membrane proteins (Prosser *et al.*, 2000; Prosser *et al.*, 2001). Paramagnetic and apolar O_2 is known to partition with an increasing concentration gradient toward the hydrophobic interior of the lipid bilayer. At partial pressures above 100 Atm, paramagnetic shifts and T_1 relaxation rates of fluorinated probe (such as labeled lipid or membrane-associated macromolecule) become appreciably depth-dependent due to the interaction with O_2 thus allowing for protein topology to be determined.

Whereas rapidly tumbling aggregates, such as surfactant micelles discussed earlier, enable high-resolution NMR methods to be used to study membrane processes (Henry and Sykes, 1994), solid-state NMR techniques may provide useful information about bilayers and natural membranes. There is no limitation on the molecular mass for solid-state NMR, and novel experiments allow for completely resolved spectra to be obtained from uniformly isotopically labeled membrane proteins in phospholipid bilayers. The resulting constraints may be used to determine the structures of membrane proteins (Howard and Opella, 1996; Opella *et al.*, 2001). The effects of nisin, a positively charged antibacterial peptide, on a phospholipid membrane have been studied by the solid-state ^{2}H and ^{31}P methods (Bonev *et al.*, 2000), and a ^{19}F NMR signal of a fluorinated inhibitor in a pellet of native ghost membranes has been reported (Grage and Ulrich, 1998). Magnetically oriented bicelles are quite suitable for solid-state NMR studies (Glover *et al.*, 2001; Howard and Opella, 1996; Luchette *et al.*, 2001; Sanders and Prosser, 1998). Structure and orientation of a wasp venom mastoparan X in bicelles has been reported (Whiles *et al.*, 2001).

The application of magic angle spinning (MAS) NMR to macroscopically oriented biomembrane samples combines the advantage of MAS with static solid-state NMR on uniformaly aligned membranes (Glaubitz, 2000). This technique allows one to obtain resolved ^{1}H, ^{13}C, and ^{31}P spectra (Glaubitz and Watts, 1998). The method has been applied to several membrane proteins of various sizes, such as M13 coat protein, gramicidin D, bacteriorhodopsin, and rhodopsin (Glaubitz, 2000;Glaubitz and Watts, 1998).

To use MAS on liquid inhomogeneous samples to reduce inhomogeneous line broadening is a novel approach. Its advantage was first suggested for monitoring combinatorial organic synthesis on resins (Anderson *et al.*, 1995; Meissner *et al.*, 1997; Sarkar *et al.*, 1996). It was later used in a variety of NMR experiments, including ^{13}C T_1 relaxation and ^{1}H NOESY measurements on protein solutions, model membranes, cells, and tissue biopsy samples (Gil *et al.*, 1997; Huster *et al.*, 1998; Moka *et al.*, 1998; Wind *et al.*, 2001). Using ^{1}H NOESY-MAS and solid-state ^{2}H

NMR, Yau *et al*., 1998) found that four tryptophan analogues resided in the vicinity of the glycerol group in PC membranes. In combination with Fourier transform infrared spectroscopy, 1D NOE-MAS and 2D NOESY-MAS were applied to monitor the interaction of a number of derivatives of a new class of antineoplastic agents, 1-aryl-3-(2-chloroethyl) ureas, with lipid bilayers (Saint-Laurent *et al*., 1998b). The study showed the incorporation of the aromatic ring of the drugs near the interfacial region of the PC bilayers. The use of high magnetic fields (750 MHz) and spherical rotor inserts enable further improvements in the quality of the ^{1}H and ^{13}C MAS spectra of membranes, as exemplified by the determination of the structure and location of the polypepide AFA-*o*-*t*-butyl in multilamellar membranes by DQF-COSY, intermolecular NOE, and natural abundance ^{13}C T_1 measurements (Pampel and Volke, 1998).

Conclusions on the molecular mechanisms of membrane processes drawn from the studies using synthetic model membranes are subject to further verification on isolated natural membranes and, ideally, on whole tissues and organs. As an example, a comparison if anesthetic and nonanesthetic effects on depolarization-evoked glutamate and GABA release from mouse cerebrocortical slices has been performed (Liachenko *et al*., 1998; Liachenko *et al*., 1999). At clinically relevant concentrations, the anesthetics inhibited both glutamate and GABA release, while structurally similar nonanesethetic suppressed only glutamate release. *In vivo* NMR may be used to measure the ionic fluxes through membrane channels in the isolated intact organs, as exemplified by the ^{87}RB, ^{23}Na, and ^{31}P NMR study of the effects of cardioselective antiischemic channel openers (e.g., pinacidil and cromakalim) on the ionic fluxes through ATP-sensitive K^+ channels in rat hearts (Kupriyanov *et al*., 1998). It was suggested that the fraction of immobilized cations in tissue can be monitored based on their characteristic relaxation pattern by means of the multiple-quantum filtering without the use of toxic shift reagents (Dizon *et al*., 1996). Thus, NMR studies of molecular mechanisms of the drug-membrane interaction using model membrane systems, combined with the studies of natural biological systems, and may ultimately result in more complete understanding of membrane-related processes in living organisms.

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