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Effect of neurosteroids on a model lipid bilayer including cholesterol: An Atomic Force Microscopy study

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ABSTRACT

Amphiphilic molecules which have a biological effect on specific membrane proteins, could also affect lipid bilayer properties possibly resulting in a modulation of the overall membrane behavior. In light of this consideration, it is important to study the possible effects of amphiphilic molecule of pharmacological interest on model systems which recapitulate some of the main properties of the biological plasma membranes. In this work we studied the effect of a neurosteroid, Allopregnanolone (3α,5α-tetrahydroprogesterone or Allo), on a model bilayer composed by the ternary lipid mixture DOPC/bSM/chol. We chose ternary mixtures which present, at room temperature, a phase coexistence of liquid ordered (L_o) and liquid disordered (L_d) domains and which reside near to a critical point. We found that Allo, which is able to strongly partition in the lipid bilayer, induces a marked increase in the bilayer area and modifies the relative proportion of the two phases favoring the L_d phase. We also found that the neurosteroid shifts the miscibility temperature to higher values in a way similarly to what happens when the cholesterol concentration is decreased. Interestingly, an isoform of Allo, isoAllopregnanolone (3β,5α-tetrahydroprogesterone or isoAllo), known to inhibit the effects of Allo on $GABA_A$ receptors, has an opposite effect on the bilayer properties.

1. Introduction

Many commercially available drugs are directed to membrane proteins. Membrane proteins are strongly coupled to the lipid component of the biological membrane both by specific chemical interactions [1] and by longer range, aspecific physical interactions [2]. The most commonly accepted mechanism of drug action relies on specific and saturable interactions between the exogenous molecules and membrane proteins. This mechanism has been demonstrated in many cases and the binding sites for many ligands and their receptors have been clearly identified [3,4]. A well accepted evidence for the specificity of the interaction between drugs and proteins lies in the different pharmacological properties of the enantiomeric forms of a drug [5]. These differences are typically related to the chiral structure of proteins and to the specific docking of the drugs to the proteins. Nevertheless, the effect of a drug on a membrane protein cannot be studied independently of the presence of the lipid bilayer whereas it is possible to study the effect of drugs on pure lipid systems. The kinetics of the drug/membrane protein interaction is typically described by schemes in which conformational changes of proteins with characteristic rate constants are involved. In many cases, several conformational states are introduced to reproduce theoretically the observed kinetic behavior, even if the introduced states are often difficult to identify experimentally. However, conformational states of membrane proteins can be affected also by the changing properties of the hosting lipid bilayer. Properties of a lipid bilayer could vary both as a consequence of changes in environmental parameters such as pH and temperature, but also, and probably in a more effective way, by modifications of the lipid bilayer components. Drugs, besides interacting specifically with membrane proteins, can also adsorb to the lipid bilayer affecting its properties hence favoring, from an energetic point of view, a specific protein conformation. Drugs/lipid bilayer interaction is considered as an adsorption process when the drug molecules remain confined at the lipid bilayer/water interface, whereas can be considered as an uptake process when the molecules are able to get inside the bilayer. Anyway, the rate constants for conformational changes could depend on the amount of drug interacting with the lipid bilayer. Many investigations demonstrated that lipophilic drugs affect the physical properties of lipid bilayers favoring for example the open or closed conformation of ion channels [6,7]. For example, it has been shown that the efficacy of amphiphilic molecules in activating mechanosensitive channels depends on their lipid solubility and has typically slow kinetics [8].

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Besides these aspects, the up to date view of the biological membrane behavior involves lateral heterogeneity in the organization of lipids and proteins. Specific domains with a lateral extension in the order of tens of nanometers could be present in the membrane [9]. Many proteins could preferentially partition in these domains and the partitioning could affect their interactions and their activation or deactivation of signaling pathways [10,11]. The membrane organization in nanometer-sized lipid aggregates could be related to its thermodynamic conditions [12] considering also the non-equilibrium situation of a biological cell membrane. The interaction of drugs with the membrane could in turn affect its thermodynamics and alter the bilayer organization [13]. These changes could have strong effects on the activity of membrane proteins which preferentially associate with specific domains. Accordingly, it would be very important to study how the insertion of exogenous molecules in a lipid bilayer affects its thermodynamics.

Dealing with the possible role of drugs in affecting the functional activities of biological membranes by an aspecific mechanism, one of the most studied effects is the interplay between anesthetics and lipid bilayers [14–18]. The longstanding debate between the specific effects of anesthetics on membrane proteins and their indirect action, mediated by changes they may produce in the lipid bilayer properties, has its roots in the Meyer–Overton rule. This rule states that the activity of anesthetics is strongly related to their partition coefficient in lipid bilayers. Among the mechanisms related to aspecific interactions, the lateral pressure profile change across the lipid bilayer has been proposed as a way to affect efficiently the equilibrium distribution of the membrane proteins in their different conformational states and the rate constants for their transitions [19]. Moreover, recent studies on model bilayers showed that the interaction of anesthetics with lipid bilayers affect their thermodynamic state by changing the relative distribution between different phases [20–22] or by changing the temperature of the bilayer at which a separation in two liquid phases occurs [13]. Another interesting class of molecules whose interactions with lipid bilayers could be relevant for their activity is exemplified by neurosteroids [NSs] [23] which are endogenous molecules able to modulate the activity of ion channels and relevant in the propagation of electrical signals in the nervous system [24]. Their action is typically explained by an allosteric interaction with a membrane protein, i.e., the $GABA_A$ receptor Cl channel, that, affecting the receptor conformational states, produces variation in the channel open time that ultimately results in a changed activity [25,26]. Their activity is usually detected at very low concentrations (in the nM range) and this aspect initially suggested a highly specific interaction with the corresponding membrane proteins [27]. Evidences for a specific NS binding site on $GABA_A$ receptor were provided by several groups. Using site-directed mutagenesis, single residues in the receptor protein that influence NS regulation of GABA receptor have been identified [28–30]. However, NSs are strongly lipophilic and, according to their structure, in some cases they have a partition coefficient which can produce, from a nM concentration in aqueous solution, a μM concentration inside the lipid bilayer [31]. In this case, a highly specific docking mechanism would not be required to explain their effect at a very low solution concentration. Moreover, it has been found that their docking site to the membrane protein could be located in their intramembraneous portion [27]. Accordingly, the effects of NSs could be also related to their partition and diffusion inside the lipid bilayer. It is also well known that, at high concentrations, NSs which modulate the activity of $GABA_A$ receptors can, independently from the GABA presence, activate the receptors [32]. This direct gating effect of the NS has typically slow kinetics which has been connected to its accumulation in the lipid bilayer [33]. The gating kinetics could be attributed both to the required increase of NS concentration inside the bilayer to bind the channels and to the increasing modification of the lipid bilayer properties as the NS concentration inside the bilayer increases.

In this work we studied the effect of two NSs, Allo and one of its isoforms, isoAllo, on a ternary lipid model-membrane containing a natural sphingomyelin extract. We concentrated on the DOPC/bSM/chol mixture with different lipid proportions. bSM is a mixture per se, even if it is mainly composed (~50%) by a 18:0 fatty acid chain. Accordingly, the mixture will be considered as a "pseudo-ternary lipid mixture". This lipid combination is considered a very representative case for the studies of phase behavior of ternary lipid bilayers [34]. One of its main characteristics is related to the fact that the typically used 1:1:1 molar mixture is, at room temperature, very near to a critical point. In fact, the presence of critical points in the thermodynamics of biological membranes is nowadays considered one of the possible explanations for the existence of small and dynamic domains usually called "lipid rafts" [12]. Here, to study the effects of Allo and of its isoform isoAllo we exploited Atomic Force Microscopy (AFM) after forming Supported Lipid Bilayers (SLBs) of the specific lipid composition. While Allo potentiates $GABA_A$ receptor activity in the presence of GABA and, at high concentrations, directly gates the $GABA_A$ receptor channel [25,35], isoAllo is devoid of modulatory activity but acts as a non-competitive antagonist of Allo [36]. In particular, isoAllo decreases the effect of potentiation by Allo and the effect is particularly evident at high GABA concentrations. Herein, we focussed on the modification of the thermodynamic state of the bilayer as the concentration of the NSs in the imaging chamber increased, measuring the relative proportions of different phases which might be present in the bilayer. We also used a temperature-controlled stage to investigate the behavior of the lipid bilayer as a function of temperature.

2. Materials and methods

2.1. Lipid bilayer formation and neurosteroid injection

The lipids 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), Sphingomyelin (Brain, Porcine) (bSM) and cholesterol were purchased from Avanti Polar Lipids (Alabaster, USA) and used without further purification. Allopregnanolone was purchased from Sigma-Aldrich and isoallopregnanolone was a kind gift from Dr A. Guidotti (refer to [25] for details on this molecule). Specific lipid mixtures were prepared by mixing chloroform lipid solutions in the desired molar amount. Chloroform was then evaporated under a flow of nitrogen while being heated in a water bath at 50 °C. Thereafter, the sample was kept under vacuum (10^{-2} mbar) for at least 2 hours in order to remove the remaining chloroform. Then lipids were rehydrated in a buffer solution (150 mM KCl, 8 mM Hepes, pH 7) to obtain a lipid concentration of 0.12/0.06 mg/ml. The sample was sonicated at room temperature for 15 min resulting in a homogenous lipid suspension.

SLBs were prepared by the vesicle fusion technique [37–39]. Briefly, immediately after sonication of the lipid suspension, 70–100 μl of the suspension was deposited onto a freshly cleaved mica sheet (SPI Supplies/Structure Probe, Inc., USA) fixed on a PTFE disc attached to a metal disc. The lipid suspension was incubated for 15 min at a temperature above 40 °C and then the sample subjected to extensive rinsing with the imaging buffer. The sample was then slowly cooled to 25°C.

Small amounts of the NSs (10^{-6} M concentration in the same buffer used for AFM imaging, diluted from a 10^{-2} M DMSO solution) were injected in the imaging chamber in order to reach the desired final concentration. After each injection, we waited for about 10–15 min in order to reach an equilibrium condition for the bilayer before acquiring images. It is important to stress that control experiments on lipid bilayer patches prepared in the same conditions but not exposed to NSs conserved good time stability in their properties. All the reported variations after the insertion of the exogenous molecules are therefore ascribable in large part to their effect on the bilayers.

2.2. Atomic Force Microscopy

Atomic Force Microscopy imaging was performed with a Bioscope I microscope equipped with a Nanoscope IIIA controller (Veeco